Establishment of Rat Pancreatic Endocrine Cell Lines by Infection with Simian Virus 40

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The feasibility of infection and transformation by SV40 (simian virus 40) of primary cell cultures derived from newborn-rat pancreas was investigated. As judged by the presence of intranuclear SV40 T-antigen, exposure to the virus resulted specifically in infection and transformation of epithelioid (predominantly endocrine) cells. The transformed cells were subcultured (more than 64 passages) and cloned. Culture medium and acid/ethanol extracts of the cells did not contain detectable amounts of immunoreactive insulin after the third subculture. However, inoculation of such SV40-transformed pancreatic cells into immunodeficient rats results in tumours in which insulin production was partially restored through the passage *in vivo*, since the tumour cells contained and synthesized small amounts of immunoreactive insulin which co-migrated with an insulin marker on gel chromatography. Interestingly, the transformed cells maintained under tissue-culture conditions produced a protein immunologically related to insulin, soluble in aqueous buffer but insoluble in acid/ethanol. This 30000-dalton protein is too large to be a translation product of the rat preproinsulin 9S mRNA. SV40-transformed pancreatic cells might prove useful in the investigation of the factors controlling and maintaining insulin biosynthesis.

Pancreatic endocrine cells of mammalian origin can readily be established in primary cultures (Lambert *et al.*, 1972). However, these endocrine cells had a limited potential for growth *in vitro*: they failed to form confluent cell layers and could not be subcultured. Furthermore, no stable insulin-producing cell lines have as yet been derived from pancreatic endocrine tumours (Murray & Bradley, 1935; Yip & Schimmer, 1973; Chick *et al.*, 1973; Adcock *et al.*, 1975), even though transplantable insulinomas of mixed cell types have been reported by Grillo *et al.* (1967) and Chick *et al.* (1977). The availability of pancreatic B-cell lines would be of considerable use to study the control of insulin biosynthesis at the molecular level.

The virus SV40 exhibits a striking mitogenic effect and converts primary cultures and cell strains from different animal species into continuous cell lines (Defendi, 1966), at least some of which retain their specific enzymic (Wells *et al.*, 1966) and hormonal functions (Deftos *et al.*, 1968; Grimley *et al.*, 1969; de Vitry *et al.*, 1974).

In the present paper we report that infection with SV40 of primary cultures of rat pancreatic endocrine cells leads to establishment of stable cell lines that no longer produce detectable amounts of insulin or pro-

insulin *in vitro*, but synthesize a 30000-dalton protein antigenically related to insulin. We also show that inoculation of the transformed pancreatic cells into immunodeficient rats led to outgrowth of tumours that produced small amounts of an insulin-like polypeptide.

Materials and Methods

Primary cultures of pancreatic endocrine cells

These were prepared from newborn Wistar rats as described by Lambert *et al.* (1972). Unless otherwise indicated, the cells were plated in plastic Petri dishes (60 mm diameter; Falcon Plastics, Oxnard, CA, U.S.A.) in Dulbecco's modified Eagle's medium (Dulbecco & Freeman, 1959) containing 16.7 mM-glucose and 10% (v/v) foetal bovine serum (GIBCO, Grand Island, NY, U.S.A.). At 2 days after plating, this medium was replaced with fresh medium containing 5.6 mM-glucose. In some experiments, the fibroblastoid cells were selectively eliminated by incubation in culture medium without cystine and foetal bovine serum for 2 days before infection, i.e. by a modification of the method reported by Chick *et al.* (1975). At 3 or 5 days after plating, the cultures were infected with a suspension of wild-type SV40 twice plaque purified (0.2 ml/dish). Plaque formation used for virus purification and titration was assessed

Abbreviation used: SV40, simian virus 40.

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in cultures of monkey kidney cells, where a single infectious viral particle will cause an area of lysed cells, the plaque. The suspension contained 5×10^8 plaque-forming units/ml, and absorption was for 90 min at 37°C. The cultures were then covered with 5 ml of Dulbecco's modified Eagle's medium containing 25 mM-glucose and 10% foetal bovine serum, subsequently referred to as 'culture medium'. To infect and mock-infect cells in suspension, primary cultures (from which fibroblastoid cells had been removed) were trypsin treated and centrifuged as described below. The low-speed cell pellets were resuspended in 1 ml of SV40 suspension in culture medium, or in culture medium alone (mock infection).

Preparation of subcultures and clonal derivatives

Cultures were trypsin treated at 37°C for 5 min in 5 ml of phosphate-buffered saline (Dulbecco & Vogt, 1954), pH 6.8, containing 0.1% trypsin (Difco Laboratories, Detroit, MI, U.S.A.) and 0.6 mM-EDTA (Merck, Darmstadt, Germany). The resulting cell suspensions were centrifuged at 500g for 5 min, resuspended in fresh culture medium and then replated. In most experiments, the cells from one confluent SV40-transformed culture were suspended in 50 ml of culture medium and distributed into ten dishes.

To prepare cell clones, trypsin-treated cells were resuspended in culture medium to a concentration of about 30 cells/ml; single drops of approx. 30 μ l were then placed in the centres of 50 small plastic Petri dishes (35 mm diameter). After 24 h incubation at 37°C all cells had attached to the plates. To all dishes containing a single cell, 1 ml of culture medium was added and the development of cell colonies was followed. Petri dishes containing more than one cell were discarded. In some experiments cultures were grown for 1–2 weeks in the presence of 1% Tylocine (GIBCO), an antibiotic known to inhibit growth of mycoplasma.

Detection of SV40-induced-tumour (T) and capsid antigens

Cells grown on cover slips (2 cm $\times$ 2 cm) were subjected to immunofluorescence tests by using either anti-T or anti-capsid serum as described previously (May *et al.*, 1971).

Tumour induction in immunodeficient rats

Female Wistar rats (9–10 weeks old, about 200 g body wt.) bred in our laboratory were subjected to whole-body irradiation of 500 rd from a cobalt source. Each animal was inoculated 24 h later with 0.5 ml of culture medium containing 10^7 SV40-transformed cells. Inoculation was performed subcutaneously over the scapular region.

Extraction of insulin and proinsulin

(a) *Acid/ethanol extraction.* Cell cultures were washed twice with ice-cold phosphate-buffered saline and then incubated overnight at 4°C with 2 ml of acid/ethanol (ethanol/water/conc. HCl, 140:57:3, by vol.), which was later tested for immunoreactive insulin (Herbert *et al.*, 1965). Tumours were cut into small fragments, which were resuspended overnight at 4°C in acid/ethanol (for details see Tables 1 and 2), sonicated at 4°C (MSE, 100 W, tapering probe, 4 $\times$ 15 s), centrifuged at 30000g for 30 min (4°C) and the supernatants assayed for immunoreactive insulin. Rat insulin (Novo Research Institute, Bagsvaerd, Denmark) was used as standard with antisera induced in guinea pigs against pig insulin (kindly provided by Dr. P. H. Wright, Indianapolis, IN, U.S.A.).

(b) *Extraction with glycine buffer.* Cultured cells or tumour fragments were suspended in 0.2 M-glycine buffer (adjusted to pH 8.8 with 5 M-NaOH) in the presence of 0.2% human serum albumin; they were sonicated and centrifuged as above. The supernatants were assayed for immunoreactive insulin.

Studies on biosynthesis of insulin and immunologically related polypeptides

(a) *Tumours.* These (2–3 cm diameter, weighing 3–4 g), induced by subcutaneous inoculation of an SV40-transformed cell clone (B7C), were fragmented and cell suspensions were prepared by incubation in a solution containing 0.2% trypsin and 0.02% collagenase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) in phosphate-buffered saline, in this case at pH 7.7 and without Ca^{2+} and Mg^{2+} . The cells were then washed twice with culture medium and the final pellet (500 g for 5 min at room temperature) was resuspended to a concentration of 5×10^7 cells/ml in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 0.25% bovine serum albumin, 25 mM-glucose and 100 μ Ci of L-[4,5- ^3H]leucine (22 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). After incubation at 37°C for 3 h, the cells were centrifuged at 500g for 5 min, washed with 3 $\times$ 5 ml of ice-cold phosphate-buffered saline and resuspended in 1 ml of glycine buffer supplemented with 0.2% human serum albumin and 0.025% Merthiolate (Fluka, Buchs, Switzerland). The cell suspensions were then sonicated as described above.

(b) *Cultured cells.* Cells grown in 10 cm-diameter plastic Petri dishes (about 10^7 cells/dish) were incubated at 37°C for 3 h in 2 ml of Krebs–Ringer bicarbonate buffer containing 100 μ Ci of L-[^3H]leucine, as above, or 100 μ Ci of L-[^3S]methionine (410 Ci/mmol; The Radiochemical Centre)/ml. Then the cultures were washed, scraped off with a rubber blade (rubber 'policeman'), pelleted and sonicated as above.

The 30000g supernatants of the sonicated prepara-

tions from tumours or cultured cells were applied to an affinity-chromatography column (1 ml bed volume) containing CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AG, Zurich, Switzerland) to which guinea-pig anti-insulin γ -globulin had been covalently linked. The binding capacity of the column was 1–2 μ g of pig insulin/ml bed volume. After incubation for 14 h at 4°C, unbound radioactivity was eluted by washing the columns with about 30 ml of glycine buffer, pH 8.8; the radioactive material that remained bound to the columns was then displaced and eluted with 5 ml of 1 M-acetic acid, pH 2.6, containing 0.2% human serum albumin.

In control experiments, the 30000g supernatants of the sonicated preparations were mixed with a large excess of unlabelled pig insulin (100 μ g/ml) and subjected to affinity chromatography as above. Radioactivity that under these conditions was bound to the columns and also eluted with 1 M-acetic acid was considered to be non-specifically bound (see the Results section).

After affinity chromatography, the labelled immunoreactive material obtained from either cultured cells or tumour-cell suspensions was chromatographed on a column (40 cm $\times$ 1.6 cm) of Sephadex G-50 (medium grade; Pharmacia). To avoid non-specific attachment of radioactive insulin or insulin-related polypeptides we generally pre-exposed the Sephadex columns to 1 mg of pig insulin (Novo Research Institute). The columns were calibrated with Dextran Blue 2000 (Pharmacia), 125 I-labelled insulin and 3 H]leucine. A flow rate of 10 ml/h was maintained, with 1 M-acetic acid containing 0.25% human serum albumin as the elution buffer. Fractions (1 ml) were collected, and 100 μ l portions of each fraction were mixed with 5 ml of Instagel (Packard Instrument International SA, Zurich, Switzerland) and counted for radioactivity in a scintillation spectrometer.

Results and Discussion

Establishment of pancreatic cell lines by infection with SV40

Properties of uninfected primary cultures. At 3–4 days after plating, primary cell cultures derived from neonatal-rat pancreas contained numerous clusters of epithelioid cells corresponding to a total of 5×10^4 – 10^5 cells/6 cm-diameter dish. Cultures prepared under standard conditions contained various amounts of fibroblastoid cells; however, these could be selectively removed (see the Materials and Methods section). Judged by cytoplasmic immunofluorescence after exposure to anti-insulin antibodies, about 60–80% of the epithelioid cells were pancreatic B cells. Most of the remaining endocrine cells were glucagon-containing A cells (Orci, 1976).

Despite some residual mitotic activity (Blondel *et al.*, 1972; Chick, 1973), no significant proliferation of epithelioid cells was ever observed, even if the fibroblastoid cells had been eliminated (Braaten *et al.*, 1974; Chick *et al.*, 1975), nor did the size of the clusters of epithelioid cells detectably increase later than 3–4 days after plating.

Early events after infection (1–30 days). The results of 13 independent experiments can be summarized as follows. Cultures were infected 3–5 days after plating; by 24 h after infection, about 1% of the epithelioid cells contained intranuclear SV40 T-antigen, detectable by immunofluorescence. Thereafter this number increased and reached a maximum about 3 days after infection, when 60–80% of the epithelioid cells gave a positive immunofluorescence reaction (Fig. 1). In contrast, T-antigen was never detected in fibroblastoid cells. Since at no time after infection could viral capsid proteins be demonstrated, we conclude that epithelioid cells from rat pancreas are non-permissive for SV40 and underwent an abortive infection leading only to the expression of the early viral gene(s). Virus-induced mitoses could first be observed 3–4 days after infection and led, within 1–2 weeks after infection, to the development of numerous microscopic colonies of T-antigen-positive cells. From the beginning, SV40-induced cell proliferation was accompanied by a relatively high incidence of abnormal (tri- and multi-polar) mitoses, resulting in cell death. Analogous cytological effects of SV40 have been observed in non-permissive cell cultures derived from other animal species (Defendi, 1966). By 3 weeks after infection, most infected cultures contained numerous macroscopic colonies of rapidly growing T-antigen-positive cells. From this time on, the incidence of abnormal mitoses markedly decreased, the rate of cell proliferation increased and the T-antigen-positive cells began to form confluent cultures. The results were essentially the same whether or not fibroblastoid cells had been removed from the primary cultures before infection.

Fig. 1. T-antigen in epithelioid cells after infection with SV40. Intranuclear SV40 T-antigen was detected by immunofluorescence in a primary culture of rat pancreas; cells were fixed 3 days after infection.

Late events after infection (later than 30 days). In more than 10 different experiments, subcultures were prepared around 30–40 days after infection, when cell proliferation had markedly increased. The average doubling time of early SV40-transformed cells was in the order of 36 h, when determined during the second passage, i.e. 6–8 weeks after infection. Thereafter the doubling time decreased, approaching 12 h after 10 or more subcultures (4 months or more after infection). To date, some of the cells have been kept in culture for more than 2 years (64 subcultures). Infection with SV40 thus converted a fraction of the epithelioid cells into rapidly proliferating transformed cells with apparently unlimited potential for growth *in vitro*.

Establishment of clonal derivatives. In one experiment we established 11 clones from an SV40-transformed culture during the third subculture (60 days after infection). They consisted of rapidly growing T-antigen-positive epithelioid cells, which formed confluent cultures. By now, one of these clones (B7C) has been subcultured more than 50 times (2 years). Karyotype analysis (Ford, 1962) of cells from this clone revealed a modal chromosome number of 40 ± 2 (mean $\pm$ S.E.M. of nine observations), which was the same as that found in primary cultures of rat pancreatic cells (40 ± 1). This does not exclude, however, the possible existence in the SV40-transformed

cells of karyotypic abnormalities which could not be detected by the method used.

Tumour formation in immunodeficient rats. SV40-transformed, cloned or uncloned pancreatic cell cultures did not give rise to tumours in normal rats after subcutaneous inoculation of 10^7 cells per animal. By contrast, inoculation of the same number of cells into rats rendered immunodeficient by cobalt irradiation led in most animals to the development of localized, subcutaneous tumours.

In a preliminary experiment, cells from two different transformed cultures (Table 1) were inoculated into three irradiated rats. All three rats developed palpable tumours after about 3 weeks. Tumour formation was then induced in 13 immunodeficient rats by inoculation of cells from clone B7C (Table 1). The injected animals were killed between 1 and 8 weeks after inoculation. In two rats, examined after 1 week, small single tumours (2–3 mm diameter) were found. In the remaining animals, the tumours rapidly increased in size, reaching diameters of 2–3 cm. By 6 weeks some tumours tended to regress, possibly as a result of recovering immunocompetence of the hosts. Cultures were prepared from two additional tumours induced by clone B7C (1 and 4 months after inoculation) either by the use of tumour-derived cell suspensions (enzymic digestion) or by explantation of small tumour fragments. In either case cells rapidly grew

Table 1. *Acid/ethanol-soluble and glycine-buffer-soluble immunoreactive insulin extracted from tumours induced by subcutaneous inoculation into immunodeficient rats of 10^7 cells from SV40-transformed rat pancreatic cells*

For full details see the text. TSV C15 is an SV40-transformed, T-antigen-positive, hamster fibroblast cell line obtained from Dr. R. Cassingena (Paris).

Cell line inoculated	Subculture no.	Duration of tumour growth (weeks)	Tumour weight (g)	Acid/ethanol-soluble immunoreactive insulin (ng/g of tissue)	Glycine-buffer-soluble immunoreactive insulin (ng/g of tissue)
7	29	4	1.38	3.2	Not measured
7	30	4	1.96	6.2	Not measured
B7C	15	4	2.02	4.2	Not measured
B7C	33	1	0.04	0.4*	10.1
B7C	33	1	0.03	0.4*	10.4
B7C	33	2	0.02	8.7	4.3
B7C	33	2	0.16	9.5	0.1*
B7C	33	3	0.55	6.5	0.1*
B7C	33	4	0.56	3.7	7.2
B7C	33	4	0.45	2.4	0.1*
B7C	33	4	1.19	7.3	8.7
B7C	33	6	1.21	3.7	17.5
B7C	33	6	2.05	1.0	13.8
B7C	33	6	0.95	9.0	15.1
B7C	33	8	0.52	7.2	10.2
B7C	33	8	0.42	5.5	2.2
TSV C15		4	5–10†	0.1*†	0.1*†
TSV C15		6	5–10†	0.1*†	0.1*†

* Limits of detection of acid/ethanol- and glycine-buffer-soluble immunoreactive insulin were 0.4 and 0.1 ng/g of tissue respectively.

† 2–3 g of tissue used per assay.

out, contained intranuclear SV40 T-antigen and exhibited essentially the same growth and immunological properties as the original B7C cells. These pancreatic cells therefore demonstrate another property of SV40-transformed cells, the ability to form tumours in immunodeficient animals (Weil, 1978).

Insulin production before and after infection with SV40

Uninfected control cultures. As determined in more than 10 independent experiments, acid/ethanol-soluble cellular immunoreactive insulin (comprising insulin and proinsulin) varied between 200 and 1000 ng of insulin/dish, when measured 3–4 days after plating. At this time, the hormone accumulated in the culture medium during 24 h corresponded to the amount of cellular immunoreactive insulin soluble in acid/ethanol. These results are similar to those reported by Lambert *et al.* (1972).

By 4 weeks after plating, the cellular content of immunoreactive insulin had decreased to 50–400 ng/dish. Attempts to establish secondary cultures 4–20 days after plating failed, since only a small and variable fraction of the trypsin-treated epithelioid cells reattached to the surface of the dishes and only a few of these reattached cells divided (see also below). As expected, the acid/ethanol-soluble immunoreactive insulin content of such secondary cultures was very low, varying between 14 ng and less than 0.4 ng/dish, the latter value corresponding to the limit of detection.

Infected cultures. In two individual experiments, acid/ethanol-soluble cellular immunoreactive insulin and accumulation of immunoreactivity in the culture medium were measured every third day between 3 and 20 days after infection of primary cultures containing both epithelioid and fibroblastoid cells. The same measurements were performed on mock-infected control cultures. During the first week there was no significant difference between infected and mock-infected cultures, although up to 80% of the epithelioid cells contained T-antigen and the virus-induced mitogenic effect was clearly visible. From the second week on, a small increase in acid/ethanol-soluble cellular immunoreactive insulin and immunoreactivity in the culture medium was observed in some dishes containing macroscopic colonies of SV40-transformed cells. However, the increase in total immunoreactive insulin was always less than expected from the increase in the number of SV40-transformed epithelioid cells. This suggested that production of insulin decreased or ceased in a fraction of the cells undergoing SV40-induced mitoses. Essentially the same results were obtained whether or not fibroblastoid cells had been removed from the primary cultures before infection. Under the standard conditions used, the amount of immunoreactive insulin secreted during a 24 h period into the culture

medium was equivalent to acid/ethanol-soluble cellular immunoreactive insulin.

In an attempt to study in more detail the early effects of infection on insulin production, fibroblastoid cells were removed from primary pancreas cell cultures and a suspension of epithelioid cells was prepared by trypsin treatment of five cultures, 5 days after plating. The cells were infected and mock-infected in suspension. Batches of about 10^4 cells were plated into 6 cm-diameter dishes and colonies were allowed to develop for 7 days in culture medium. The results in Table 2 show that the plating efficiency of uninfected cells was very low. However, the infected cells had a moderately higher plating efficiency and, on average, the colonies formed by infected cultures were larger and contained more cells (average cell size was similar in the two groups). Total cellular immunoreactive insulin soluble in acid/ethanol was slightly increased in the dishes containing SV40-infected colonies; however, the hormone content per colony and per cell was decreased. These results support the assumption that, in a fraction of the infected cells, insulin production decreased or ceased soon after the onset of virus-induced mitotic stimulation.

After the massive outgrowth of SV40-transformed cells (30–40 days after infection) acid/ethanol-soluble cellular immunoreactive insulin and immunoreactivity in the culture medium rapidly decreased. Thus in a typical experiment the cellular content of immunoreactive insulin per 10^7 transformed cells, measured after the first, second and third subcultures

Table 2. Number, size and total acid/ethanol-soluble immunoreactive insulin of epithelioid cell colonies derived from SV40-infected and mock-infected primary cultures of rat pancreas

Cells from primary cultures were infected or mock-infected in suspension. Portions containing 10^4 cells were plated in 20 6 cm-diameter Petri dishes, and 7 days later the dishes were extracted with acid/ethanol, then stained with 10% Giemsa solution (Ford, 1962). Size and number of colonies were measured by projecting the stained dishes on a screen at a magnification of 18 $\times$. Abbreviations: *r*, number of dishes (replicates); *n*, number of colonies measured. Significance of difference between infected and mock-infected cultures was estimated by Student's *t* test; *P* < 0.001 for no. of colonies and insulin content per dish.

Type of cultures	Epithelioid cell colonies		Acid/ethanol-soluble immunoreactive insulin (ng/dish)
	No. per dish	Mean diameter of colonies (cm)	
Mock-infected	26 $\pm$ 2 (<i>r</i> = 20)	0.20 $\pm$ 0.04 (<i>n</i> = 214)	0.84 $\pm$ 0.04 (<i>r</i> = 20)
SV40-infected	47 $\pm$ 3 (<i>r</i> = 20)	0.24 $\pm$ 0.07 (<i>n</i> = 189)	1.03 $\pm$ 0.03 (<i>r</i> = 20)

(30, 45 and 65 days after infection), had decreased to 7.8, 5.4 and less than 0.4 ng respectively. In subsequent experiments, SV40-transformed uncloned cultures and 14 individual clones were assayed for acid/ethanol-soluble immunoreactive insulin and immunoreactivity in the culture medium at different times between 2 and 18 months after infection: in all instances immunoreactivity was below the level of detection (<0.4 ng/ 10^7 cells) even when a large number of cells was used (4×10^8 – 6×10^8 cells, 180 mg wet wt.). When examined by electron microscopy 1, 2 and 8 months after infection (first, second and 18th passage), none of the SV40-transformed cells contained storage granules typical for pancreatic B or A cells. Atypical granules were occasionally seen, but could not be identified. Similarly, studies using immunofluorescence failed to provide evidence for the presence of insulin or glucagon in the cytoplasm of the SV40-transformed cells tested at these stages. This contrasts with the continued, though diminished, production of peptide hormones by other SV40-transformed endocrine cell lines (Wells *et al.*, 1966; de Vitry *et al.*, 1974).

Resumption of synthesis of an insulin-like polypeptide in tumours induced by SV40-transformed cells. Buonassisi *et al.* (1961) have reported that established mouse cell lines from adrenal and pituitary tumours tended to lose the ability to produce hormones during prolonged subculture *in vitro*, but that inoculation of such cells into animals gave rise to tumours which resumed hormone production, albeit at a lower rate. Similar results were reported by de Vitry (1977) with an SV40-transformed mouse hypothalamic cell line producing neurophysin. We tested tumours, induced in immunodeficient rats (see the Materials and Methods section) by inoculation of SV40-transformed rat pancreatic cells, for the presence of acid/ethanol-soluble immunoreactive insulin. First we tested two tumours induced by an uncloned parental culture (inoculated at passages 29 and 30) and one tumour induced by clone B7C (passage 15). At 1 month after inoculation, each of the three tumours measured 2–3 cm in diameter and all three contained small amounts of acid/ethanol-soluble immunoreactive insulin (see Table 1, first three lines) determined at three different dilutions. Subsequently, 13 tumours induced by cloned B7C cells were extracted in acid/ethanol at different times after inoculation. The further results shown in Table 1 establish that all tumours tested contained immunoreactive insulin, except for the very small tumours isolated after 1 week. Since the amounts of acid/ethanol-soluble immunoreactive insulin were rather small, we also extracted thyroid (173 mg of tissue extracted/ml), liver (580 mg/ml) and muscle tissue (484 mg/ml) of the tumour-bearing rats, and two hamster tumours induced by an SV40-transformed, T-antigen-positive hamster fibroblast cell line (TSV C15). We were un-

able to find detectable amounts of immunoreactive insulin in any of the five non-pancreatic tissues tested (<0.4 ng/g of tissue). Table 1 also shows that 10 of the 13 tumours induced by transformed pancreatic cells contained immunoreactive insulin measured in glycine-buffer extracts. The two non-pancreatic hamster tumours did not contain detectable amounts of such immunoreactivity.

To study the molecular properties of the insulin-related material extracted from tumours induced by

Fig. 2. Biosynthesis of insulin-related proteins by SV40-transformed cells before and after passage *in vivo*. SV40-transformed rat pancreatic cells were labelled *in vitro* with [3 H]leucine and total proteins extracted by sonication in glycine buffer; the 30000g supernatants were subjected to immunoaffinity chromatography by using Sepharose-bound antibodies to pig insulin; the columns were washed, and the retained radioactive material was eluted with 1 M-acetic acid, then subjected to chromatography on Sephadex G-50 columns. Fractions were collected and assayed for radioactivity. The Sephadex G-50 column was calibrated with Blue Dextran (void volume, V_0), [125 I]-labelled pig insulin or rat insulin and [3 H]leucine (V_i). Two cell types were extracted and the results are shown in this Figure: (a) an SV40-transformed, cloned rat pancreatic cell line (B7C) maintained in culture (29th subculture); (b) cells isolated from a tumour induced by inoculation of cloned (B7C) cells into rats. The tumour was excised 4 months after inoculation.

SV40-transformed pancreatic cells, we prepared B7C tumour-cell suspensions and incubated them with [^3H]leucine for 3 h. The cells were then sonicated in glycine buffer, centrifuged and the 30000g supernatant was subjected to affinity chromatography, by using anti-insulin antibodies covalently linked to Sepharose 4B. The column was then washed with glycine buffer and the bound material then displaced and eluted with 1 M-acetic acid and chromatographed on a Sephadex G-50 column. The results in Fig. 2(b) show that most of the radioactivity was eluted with the void volume (mol.wt. > 12000), and a smaller fraction co-migrated with ^{125}I -labelled insulin. No radioactive peak could be detected in the region of proinsulin. The identically treated B7C parent cell line, which induced the tumour after subcutaneous injection, yielded radioactivity exclusively in the void volume (Fig. 2a).

These results indicate that, *in vivo*, the SV40-transformed pancreatic cells resume the synthesis of a peptide indistinguishable from insulin by the methods used. It is suggested that the environment *in vivo* contains factors that are essential for the maintenance of insulin synthesis.

Presence in SV40-transformed rat pancreatic cells of a protein antigenically related to insulin. The results obtained with the B7C-induced tumours (Fig. 2) pointed to the possibility that SV40-transformed pancreatic cells might produce a protein antigenically related to insulin, but larger than preproinsulin (11 500 daltons) (Chan *et al.*, 1976). Such a protein might be insoluble in acid/ethanol and thus escape detection when acid/ethanol is used to extract insulin, proinsulin and preproinsulin (Chan *et al.*, 1976). SV40-transformed cultures of rat pancreatic cells, both cloned and uncloned, were therefore sonicated in glycine buffer and the 30000g supernatants were assayed for immunoreactive insulin. The results in Table 3 show that five SV40-transformed pancreatic cell lines tested contained small

but significant amounts of immunoreactivity, whereas identically treated non-pancreatic cell cultures used as controls yielded lower contents. As expected, acid/ethanol extracts of the different cell types in Table 3 did not contain immunoreactivity (results not shown). The results of Table 3 were not altered when the cell lines were grown for 2 weeks before extraction in the presence of an antibiotic known to inhibit growth of mycoplasma. This makes it unlikely that immunoreactivity provided by SV40-transformed pancreatic cells is due to mycoplasma infection.

To examine further the molecular properties of the immunoreactive material, four rat pancreatic cell lines, independently transformed with SV40, were labelled with [^3H]leucine or [^{35}S]methionine. One line (B7C) had been cloned, but the others had not. The 30000g supernatant of the glycine-buffer extracts after labelling was subjected to immunoaffinity chromatography. In this small group of different pancreatic cell lines, between 2 and 4% of total trichloroacetic acid-precipitable radioactivity was found to bind to the columns and was eluted with 1 M-acetic acid. To test the immunological specificity of attachment and elution of this substance, unlabelled pig insulin (100 $\mu\text{g}/\text{ml}$) was added to the 30000g supernatant in a separate experiment, and the mixture again subjected to affinity chromatography. The presence of unlabelled pig insulin decreased the percentage of bound and eluted radioactivity to 0.7–0.9% of total acid-precipitable radioactivity. As a further control, a monkey kidney cell line (BSC) was labelled with [^{35}S]methionine, sonicated and the 30000g supernatant subjected to affinity chromatography in the presence or absence of pig insulin (100 $\mu\text{g}/\text{ml}$). In both instances, about 0.9% of total radioactivity was retained by the columns and subsequently eluted with acetic acid, thereby suggesting that the radioactive material from BSC cells that bound to the affinity columns was unrelated to insulin.

Table 3. Glycine-buffer-soluble immunoreactive insulin content of five SV40-transformed rat pancreatic cell lines, and of two cell lines of non-pancreatic origin

Cells from confluent Petri dishes containing about 1.5×10^7 cells were scraped off and their glycine-buffer extracts assayed for immunoreactive insulin (results are mean values $\pm$ S.E.M. for *n* dishes). The limit of detection under these conditions was 0.1–0.4 ng/dish. BSC is a monkey kidney cell line. SV101 is a standard SV40-transformed, T-antigen-positive cell line derived from Swiss 3T3 mouse fibroblasts.

	Cell line	Subculture no.	Glycine-buffer-soluble immunoreactive insulin (ng/Petri dish)	<i>n</i>
SV40-transformed pancreatic cell lines	7	30	9.3 $\pm$ 0.1	4
	B7C	19	4.0 $\pm$ 0.2	3
	24F	4	1.2 $\pm$ 0.2	4
	24	8	6.2 $\pm$ 0.1	4
	25	8	2.1 $\pm$ 0.3	3
Non-pancreatic cell lines	BSC		0.6	2
	SV101		0.5	2

In other experiments the radioactive material extracted from SV40-transformed rat pancreatic cells was eluted from affinity columns and the eluate was subjected to chromatography on Sephadex G-50 columns. As previously indicated in Fig. 2(a), virtually all of the radioactivity appeared in the void volume. In parallel experiments, chromatography on Sephadex G-50 columns was performed under denaturing conditions in the presence of 8M-urea. Again all the radioactivity appeared in the void volume. Finally, unlabelled, SV40-transformed rat pancreatic cells (B7C) were sonicated in glycine buffer in the presence of added pig ^{125}I -labelled insulin; the 30000g supernatant was again applied to an affinity column, eluted with 1M-acetic acid and chromatographed on Sephadex G-50; on parallel columns ^{125}I -labelled pig insulin was chromatographed alone. In both instances ^{125}I -labelled insulin exhibited the elution pattern of unlabelled native insulin. Furthermore, there was no evidence for proteolytic degradation of ^{125}I -labelled insulin.

In two independent experiments, the glycine-buffer extracts (30000g supernatants) obtained from 16 B7C cultures (about 3×10^8 cells) were pooled and applied to affinity-chromatography columns. The acetic acid eluate was dialysed against water, freeze-dried and assayed for immunoreactive insulin at sequential dilutions (1:2 to 1:512) in the standard radioimmunoassay system. The presence of 0.2% serum albumin in the acetic acid eluate did not allow for chemical quantification of the eluted proteins. However, Fig. 3 shows that displacement of ^{125}I -labelled insulin by progressive dilutions of the eluate exhibited a pattern similar to that obtained with unlabelled rat insulin, when assayed at concentrations from 10 to 0.1 ng/ml.

Experiments were also performed with an antiserum (GP8, kindly provided by Dr. R. H. Jones, St. Thomas's Hospital, London, U.K.) directed against the N-terminus of the B chain of ox insulin, and known not to react with rat insulin (R. H. Jones, personal communication). In repeated experiments we observed, however, that antiserum GP8 reacted with rat proinsulin and also with the eluates from affinity chromatography of extracts from the SV40-transformed rat pancreatic cell lines described above. The absence of detectable proteolytic degradation of ^{125}I -labelled insulin in the presence of large concentrations of added protein during purification and immunological assay strongly suggests that a protein in the extracts from SV40-transformed rat cultures did, in fact, compete with ^{125}I -labelled insulin for binding sites on antibodies.

In an attempt to define the nature of the immunoreactive protein(s) present in the extracts of SV40-transformed rat pancreatic cells we performed the following experiments: three different uncloned and one cloned (B7C) cultures were labelled with [^{35}S]-methionine, sonicated in glycine buffer and the

Fig. 3. Comparison by radioimmunoassay of rat insulin and proteins extracted from an SV40-transformed pancreatic cell line (B7C)

Rat insulin was assayed at concentrations from 0.1 to 10 ng/ml. Proteins from B7C cells were extracted in glycine buffer and partially purified by affinity chromatography. The extracts were assayed at dilutions from 1:2 to 1:512. For details see the Materials and Methods and the Results sections.

30000g supernatants applied to the affinity columns. The radioactive material was eluted with 1M-acetic acid, dialysed against water, denatured by boiling for 5 min in 2% (v/v) sodium dodecyl sulphate and 5% (v/v) mercaptoethanol and finally analysed by electrophoresis on sodium dodecyl sulphate/polyacrylamide slab gels (10% polyacrylamide). The gels were fixed with 20% (w/v) trichloroacetic acid, dried and subjected to radioautography. As shown in Fig. 4, there was a distinct band, with an apparent molecular weight of about 30000, which comprised 10–20% of total radioactivity present in the gels, and the remainder of the radioactivity was present in several slower-moving bands (40000–60000 daltons). The presence of 100 μg of pig insulin in the radioactive extracts from B7C cultures prevented the binding of the 30000-dalton protein to the affinity columns. In

Fig. 4. Electrophoretic mobility of proteins extracted from an SV40-transformed pancreatic cell line

Densitometric tracing of [35 S]methionine-labelled proteins, partially purified by affinity chromatography of extracts from an SV40-transformed pancreatic cell line (B7C). Molecular weights are given on the ordinate.

contrast, no evidence for the presence of a radioactive 30000-dalton protein was observed in extracts prepared from [35 S]methionine-labelled cell lines of non-pancreatic origin (BSC, CV-1), whereas radioactive proteins were present in the 40000–60000-dalton range.

These observations suggest that the 30000-dalton protein produced by SV40-transformed rat pancreatic cells is antigenically related to insulin.

Furthermore, this protein (or a related labelled protein) was also secreted into the culture medium.

The 30000-dalton protein differs from rat preproinsulin with respect to molecular size and its insolubility in acid/ethanol. Its nature and biological significance remain unknown. It is too large, however, to be coded by rat preproinsulin mRNA, which comprises about 600 (Duguid *et al.*, 1976) or 450 (Ullrich *et al.*, 1977) nucleotides. Experiments are required to establish whether the 30000-dalton protein shares amino acid sequences with rat insulin.

As a working hypothesis we consider the possibility that the rat insulin genes are considerably larger than expected from the size of preproinsulin mRNA and that in SV40-transformed pancreatic B cells the expression of these genes may be abnormal, perhaps as the result of altered processing (splicing?) (Berget *et al.*, 1977) of a primary gene transcript. Alternatively, SV40 may activate in rat pancreatic cells (cf. Weil, 1978) a host-cell gene or genes that code for a larger protein from which insulin evolved (Steiner *et al.*, 1969; de Haen *et al.*, 1976).

In conclusion, infection of primary cultures of the endocrine pancreatic cells allows the establishment of cell lines which lose the ability to synthesize insulin *in vitro*, but partially recover this ability after passage *in vivo*. The transformed cells maintained *in vitro* produce a 30000-dalton protein immunologically related to insulin.

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References

- Adcock, K., Austin, M., Duckworth, W. C., Solomon, S. S. & Murrell, L. R. (1975) *Diabetologia* **11**, 527–534
- Berget, S. M., Moore, C. & Sharp, P. A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3171–3195
- Blondel, B., Orci, L., Like, A., Amherdt, M., Marliss, E. B. & Wollheim, C. B. (1972) *Diabetes* **21**, Suppl. 1, 346
- Braaten, J. T., Lee, M. J., Shenk, A. & Mintz, D. H. (1974) *Biochem. Biophys. Res. Commun.* **61**, 476–482
- Buonassisi, V., Sato, G. & Cohen, A. I. (1961) *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1184–1190
- Chan, S. J., Keim, P. & Steiner, D. F. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1964–1968
- Chick, W. L. (1973) *Diabetes* **22**, 687–693
- Chick, W. L., Lauris, V., Soeldner, J. S., Tan, M. H. & Grindbergs, M. (1973) *Metab. Clin. Exp.* **22**, 1217–1224
- Chick, W. L., Like, A. A. & Lauris, V. (1975) *Endocrinology* **96**, 637–643
- Chick, W. L., Warrens, S., Chute, R., Like, A. A., Lauris, V. & Kitchen, K. C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 628–632
- Defendi, V. (1966) *Prog. Exp. Tumor Res.* **8**, 125–188
- Deftos, L. J., Rabson, A. S., Buckle, R. M., Aurbach, G. D. & Potts, J. T. (1968) *Science* **159**, 435–436
- de Haen, C., Swanson, E. & Teller, D. C. (1976) *J. Mol. Biol.* **106**, 639–661
- de Vitry, F. (1977) *Nature (London)* **267**, 48–50
- de Vitry, F., Camier, M., Czernichow, P., Benda, P. H., Cohen, P. & Tixier-Vidal, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3575–3579
- Duguid, J. R., Steiner, D. F. & Chick, W. L. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3539–3543
- Dulbecco, R. & Freeman, G. (1959) *Virology* **8**, 396–397
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182
- Ford, C. E. (1962) in *Methodology in Human Genetics* (Burdette, W. F., ed.), pp. 227–259, Holden-Day, San Francisco

- Grillo, T. A. I., Whitty, A. J., Kirkman, H., Foa, P. P. & Kobernick, S. D. (1967) *Diabetes* **16**, 409-414
- Grimley, P. M., Deftos, L. J., Weeks, J. R. & Rabson, A. S. (1969) *J. Natl. Cancer Inst.* **42**, 663-680
- Herbert, V., Lau, K. S., Gottlieb, C. W. & Bleicher, S. J. (1965) *J. Clin. Endocrinol. Metab.* **25**, 1375-1384
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33-66
- Lambert, A. E., Blondel, B., Kanazawa, Y., Orci, L. & Renold, A. E. (1972) *Endocrinology* **90**, 239-248
- May, E., May, P. & Weil, R. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1208-1211
- Murray, M. R. & Bradley, C. F. (1935) *Am. J. Cancer* **25**, 98-107
- Orci, L. (1976) *Polypeptide Hormones: Molecular and Cellular Aspects: Ciba Found. Symp.* **41**, 313-323
- Steiner, D. F., Clark, J. L., Nolan, C., Rubenstein, A. H., Margoliash, E., Aten, B. & Oyer, P. E. (1969) *Recent Prog. Horm. Res.* **25**, 207-282
- Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischler, E., Rutter, W. J. & Goodman, H. M. (1977) *Science* **196**, 1313-1319
- Weil, R. (1978) *Biochim. Biophys. Acta* **516**, 301-388
- Wells, S. A., Wurtman, R. J. & Rabson, A. S. (1966) *Science* **154**, 278-279
- Yip, C. C. & Schimmer, B. P. (1973) *Diabetologia* **9**, 251-254