Induction of cell surface insulin antigenicity in fibroblasts transfected with islet genomic DNA

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Ltk⁻ cells were transfected with total genomic DNA obtained from rat islets or from insulinoma cells mixed with DNA tagged with fluorescent dye. Flyorescence-activated cell sorter (FACS) was used for initial selection of successful transfectants, monitoring fluorescent DNA incorporated into the cell. For subsequent selections the cells were treated with anti-insulin antiserum labeled with fluorescent dye and selected by FACS. B cell DNA, but not DNA from murine leukemic cells, induced the appearance of cell surface insulin antigenicity in fibroblasts. This phenotypic expression was transient. Together with our previous demonstration of the induction of insulin secreation in Ltk⁻ cells [(1986) Biochem. Biophys. Res. Commun. 136, 638–644], these results indicate that B cell specific characteristics can be transfected to non-endocrine cells by genomic DNA transfection.

Fluorescent marker *Cell surface* *Insulin* *Transient expression* *(L-cell)* *genomic DNA*

1. INTRODUCTION

Employing serum from insulin-dependent diabetic patients, it is possible to show that pancreatic β-cells express cell-specific surface antigens [1]. In addition, recent studies demonstrated that the β-cell plasma membrane contains insulin-like antigenicity [2]. Thus, both antigens may be implicated in the autoimmune pathogenesis of type 1 diabetes mellitus [3]. The induction of surface insulin or other β-cell-specific antigens in heterologous non-endocrine cells could be useful for stimulating autoimmune reactions that mimic the mechanism of autoimmune insulitis. It could also develop into a powerful experimental tool for the identification of the putative enhancer sequence binding factor, which is specific for the activation of the insulin gene enhancer sequence [4]. This study was undertaken to test the feasibility of inducing surface insulin expression in non-endocrine cells by transfection with DNA from insulin-producing cells.

In previous studies from this laboratory, transient secretion of insulin was induced in Ltk⁻ cells transfected with total genomic rat islet DNA [5], using thymidine kinase gene for selection. However, this technique is inappropriate if a large number of successful transfectants are desired, due to the excessive selection of the HAT medium and the interference of dying cells in the FACS. Therefore, to sort the entire transfected population for successful introduction of extraneous DNA, a new method using a trace of fluorescent DNA marker was used.

2. MATERIALS AND METHODS

2.1. *Cells and cultures*

Thymidine kinase-deficient Ltk⁻ fibroblasts and the rat insulinoma line RINm5F were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Biolab, Jerusalem). Islets of Langerhans from the pancreas of male albino Sabra rats were

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isolated by collagenase digestion [6]. Murine leukemic tumor cells (YAC) were grown in an ascitic form in the peritoneal cavity of A/sn mice [7].

2.2. *Immunological reagents*

Guinea-pig anti-pork insulin was purchased from Linco Research Inc. (Eureca, MO), fluorescein isothiocyanate (FITC) conjugate of rabbit anti-guinea-pig IgG and FITC conjugate of rabbit anti-mouse IgG were from Sigma (St. Louis, MO). Anti-leukemic antibodies, shown to bind 50–80% of YAC cells on fluorescence-activated cell sorter (FACS) analysis were kindly provided by Dr R. Sharon, Hebrew University School of Medicine, Jerusalem. All sera were treated at 56°C for 30 min for complement inactivation, absorbed on non-transfected Ltk$^-$ cells at 4°C for 30 min and then spun for 5 min in an Eppendorf minicentrifuge (FRG).

2.3. *DNA extraction*

Cells or islets were suspended in 10 mM Tris, pH 7.5, containing 10 mM EDTA and incubated for 2 h in 2% SDS and 100 μg/ml proteinase (Sigma) at 37°C under gentle swirling. Two rounds of phenol extractions and 2 rounds of chloroform extractions were then applied to remove phenol traces, followed by dialysis against 3000 vols distilled water. The extract was then run on 1% agarose gel electrophoresis [8] to confirm the presence of large DNA fragments (>20 kb). For fluorescence-labelled DNA, 100 μg high-M_r DNA were mixed with 50 μg ethidium bromide in 10 mM Tris, pH 7.5, or with FITC in 10 mM Tris, pH 9.5, incubated at room temperature for 30 min and dialyzed twice against 3000 vols distilled water.

2.4. *Transfection of Ltk$^-$ cells*

Ltk$^-$ fibroblasts (1×10^6), seeded 24–48 h earlier in 100 mm petri dishes (NUNC, Roskildas, Denmark), were cotransfected with 0.1 μg fluorescence-labelled DNA mixed with 10 μg freshly extracted genomic DNA per dish, using the calcium precipitation method [9]. After 6 h the cells were rinsed with DMEM and further cultured in DMEM containing 10% FCS. On the following day, the cells were removed by gentle trypsinization, washed 3 times in cold DMEM containing 10% FCS, and resuspended in the same medium at a concentration of 10^6 cells/ml for cell sorting. During the entire procedure, exposure to light was restricted to a minimum.

2.5. *Cell sorting*

Cells were sorted under sterile conditions on an FACS IV-400 (Becton & Dickinson, Los Angeles, CA). Cells transfected with ethidium bromide were excited at 360 nm while the emission wavelength was 560 nm. For FITC selection 360 nm was used for excitation and 490 nm for the emission wavelength. Rating for high fluorescence intensity was set arbitrarily above the broken cells background. Cells demonstrating high-intensity fluorescence were collected in a sterile tube containing 1 ml growth medium, seeded in 50 mm petri dishes, and grown to a density of $2–5 \times 10^5$ cells per dish. Selection for cell-surface insulin-like antigenicity was carried out 1–6 weeks post-transfection. The transfectants were rinsed with cold Krebs-Ringer (KRB) solution containing 0.5% BSA and incubated for 30 min at 37°C in KRB containing either 3.3 or 16.7 mM glucose. The cells were then incubated for an additional 30 min at 4°C with guinea-pig anti-insulin serum, diluted 1:100 in KRB, rinsed with KRB, and reacted with a 1:200 dilution of FITC conjugate of anti-guinea-pig IgG for 30 min at 4°C. After the excess fluorescent antibodies were removed by washing, the cells were gently trypsinized and washed 3 times more in cold DMEM containing 10% FCS and resuspended at 10^6 cells/ml for FACS analysis.

3. RESULTS

DNA extracted from RINm5F insulinoma cells was marked with ethidium bromide and used to transfect Ltk$^-$ fibroblasts as described in section 2. For selection by the FACS, the vertical gate for positive fluorescent cells was set at 10/255 screen divisions above the upper border of the bulk of fluorescent cells (in accord with the autofluorescence of non-transfected Ltk$^-$ cells). The horizontal gate was set between 60/255 and 255/255 screen divisions. A typical profile of FACS sorting is shown in fig.1A; 2% of the cells were collected (to the right of the arrow) and reseeded. 1 week later the cells were labelled with guinea-pig anti-insulin serum and FITC conjugate

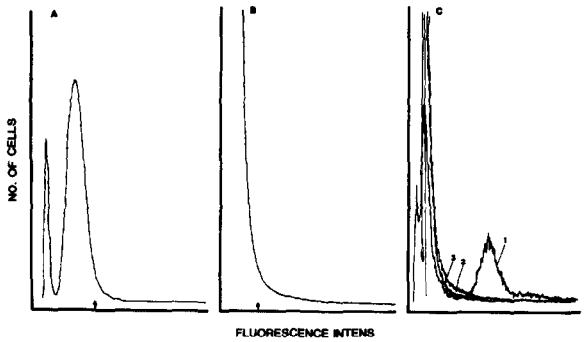

Fig.1. Sorting and analysis for cell-surface insulin expression in fibroblasts transfected with DNA from RINm5F cells, using ethidium bromide as a marker for successful transfections. (A) Ltk⁻ cells 24 h after transfection with RINm5F DNA mixed with 1% of the same DNA tagged with ethidium bromide; excitation wavelength, 360 nm; emission wavelength, 560 nm. All cells to the right of the arrow were collected and cultured for a second sorting. (B) Second sorting using guinea-pig anti-insulin serum and FITC-conjugated rabbit anti-guinea-pig IgG. Emission wavelength, 488 nm. All cells to the right of the arrow were collected and cultured for later analysis. (C) Analysis of surface insulin-expressing cells 2 weeks post transfection. Cells treated with: (1) guinea-pig anti-insulin serum (1:100) as first antibody, (2) normal guinea-pig serum (1:100), (3) KRB buffer alone.

of anti-guinea-pig IgG. A wide range of fluorescence intensities was observed among the positive fluorescent cells of the 2nd FACS analysis (to the right of the arrow in fig.1B). High-intensity fluorescing cells were collected and further grown for 2 weeks, at which time the sorting with anti-insulin antibodies was repeated. A homogeneous population of highly fluorescent cells was obtained in the 3rd FACS analysis (peak in fig.1C) corresponding to the fraction of cells retaining transient insulin antigenicity for long periods.

FITC has not been reported to bind significantly double-stranded DNA. We compared FITC with ethidium bromide as a fluorescent marker for DNA transfection because of its low toxicity [10]. Ltk⁻ cells transfected with FITC, or ethidium bromide-treated DNA mixed with native rat islet DNA, underwent initial sorting on the FACS. The positive fluorescent cells were propagated for 1–3 weeks and then either analyzed and discarded or sorted for further propagation. To obtain a curve

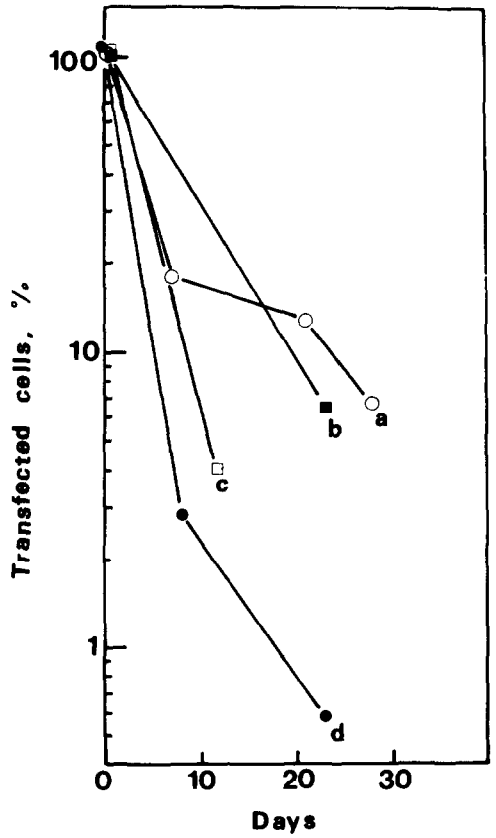

Fig.2. Loss of phenotypic expression of surface antigenicity in transfected fibroblasts. The initial point of total (100%) transfected cells was determined on day 1 post-transfection with either ethidium bromide-treated DNA (a,b) or FITC-treated DNA (c,d). Subsequent points on each curve represent analysis of the phenotype by its respective antiserum and an FITC-conjugated second antibody. (A) Ltk⁻ cells transfected with rat islet DNA mixed with ethidium bromide-tagged rat islet DNA. (B) Ltk⁻ cells transfected with YAC murine leukemic DNA mixed with the same DNA tagged with ethidium bromide. The phenotype was determined by an anti-YAC serum from syngeneic mice. [The anti-leukemic serum was a pool of sera from 10 mice injected with a 170 kDa section of leukemic cell homogenate. These mice became resistant to increasing challenges of live tumor. Their serum pool bound up to 80% of these leukemic cells and stained a 105 kDa protein (not shown).] (C) LTK⁻ cells transfected with YAC murine leukemic DNA mixed with the same DNA tagged with FITC. The phenotype was determined with anti-YAC serum. (D) Ltk⁻ cells transfected with rat islet DNA mixed with the same DNA tagged with FITC. Surface phenotype expression was determined using anti-insulin serum.

for the rate by which the surface insulin expression was lost, the fractional yield of high fluorescence cells was multiplied by that figure of the previous sorting (fig.2). The curves that describe the phenotypic loss in transient transfectants initially selected by FITC-treated DNA had a steeper slope than the ethidium bromide-selected transfectants. This suggests that selection by FITC DNA is less efficient in defining the total transfected population on day 1 by one order of magnitude as compared with the ethidium bromide-labeled DNA method (see differences between curves a and b vs c and d in fig.2). However, since ethidium bromide is highly mutagenic, FITC was preferred for cell sorting. Fig.3 presents FACS analysis of cells initially sorted on the basis of incorporation of FITC-treated islet DNA. Curve C in fig.3 represents the first sorting of cells, monitoring binding of fluorescently labelled anti-insulin antibodies. Curve C2 is the third such sorting. The anti-insulin antibody binding to the cells was inhibited by rat insulin added at the incubation step, confirming the specificity of this antigen-antibody reaction (C2 + insulin).

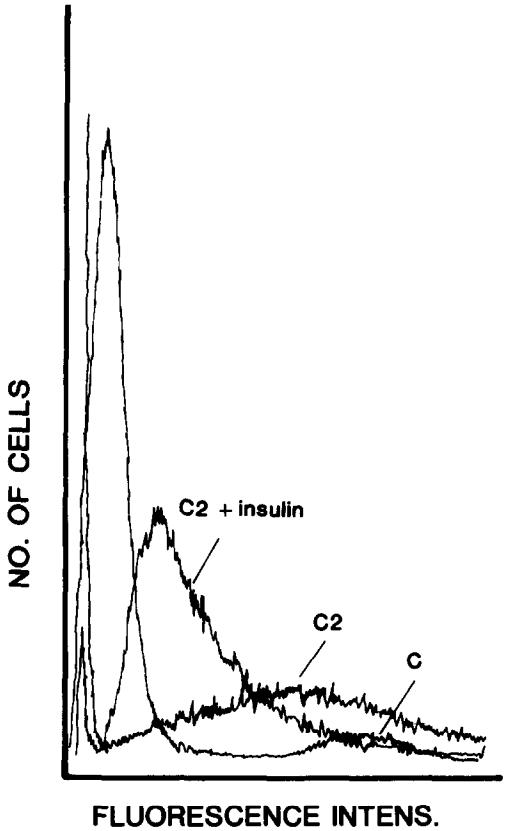

Fig.3. Inhibition of surface insulin antibody binding by insulin. Transfectants were sorted on day 1 by the fluorescence of FITC-treated DNA mixed with native rate islet DNA. (C) First isolation of total transfectants; (C2) third FACS run with anti-insulin antibodies; (C2 + insulin) cell sorted according to anti-insulin binding after excess insulin (10 mU/ml) was added to the incubation medium.

The specific requirement for DNA of B cell origin [11] was examined in our system using DNA extracted from mouse leukemia cells and anti-leukemic serum. Fibroblasts were transfected either with islet DNA (curve 1) (fig.4A) or leukemia DNA (curve 2) and sorted initially on the basis of trace FITC-labeled DNA fluorescence. 3 weeks later, only fibroblasts transfected with islet DNA bound anti-insulin antibodies (curve 1 in fig.4B); transfection with leukemia DNA did not induce expression of insulin (curve 3, fig.4B), nor

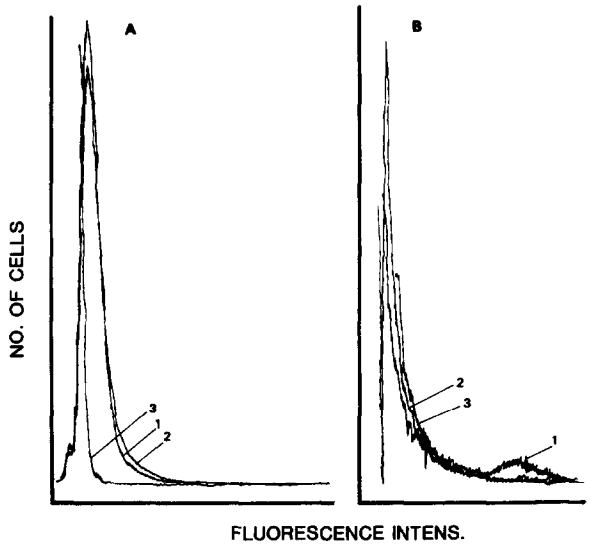

Fig.4. DNA specificity of surface insulin expression in transfected fibroblasts. (A) Ltk$^-$ cells transfected with FITC-treated DNA mixed with (1) islet DNA, (2) leukemic cell DNA, (3) autofluorescence of non-transfected Ltk$^-$ cells. (B) 3 weeks post-transfection of fibroblasts with rat islet DNA. (1) Islet DNA transfectants incubated with anti-insulin antibodies, (2) islet DNA transfectants incubated with anti-leukemic serum, (3) leukemic DNA transfectants incubated with anti-insulin serum. [20% of leukemic DNA transfectants bound the anti-leukemic serum above the background of rat islet DNA transfectants (not shown).]

did islet DNA-transfected cells react with anti-leukemic serum (curve 2, fig.4B); in contrast, fibroblasts transfected with leukemic cell DNA strongly bound anti-leukemic antibodies (not shown).

4. DISCUSSION

Regulated insulin secretion, namely the glucose-sensitive gene(s) transcription, processing and translation of product(s) RNA, processing of the product preproinsulin and controlled secretion of the final product, is believed to be an exclusive characteristic of pancreatic B cells. Attempts to obtain secretion of intact insulin from non-endocrine cells, regulated or unregulated, were mostly unsuccessful. Thus transfection of Ltk^- cells with cloned insulin gene produced no product transcription [12] or low-grade transcription [13]. On the other hand, insulin secretion was reported in an ACTH-secreting cell line transfected with cloned insulin gene [13]. Thus, it seems that transfection of the insulin gene into cells sharing common embryonic origin with B cells, i.e. neural crest APUD cells, may lead to transcription of the complete hormone, a finding compatible with the concept of tissue-specific gene expression.

Recent studies have demonstrated that the immunoglobulin gene enhancer sequence may be introduced in a stable form into Ltk^- cell and activated by specific protein factors [16,17]. Using total genomic DNA from B cells for transcription, we have succeeded in obtaining transient expression of regulated insulin secretion in L cells [5]. In these studies, we have used large DNA fragments (20–100 kb) for transfection. Since the DNA was obtained from insulin-producing cells, it is conceivable that in addition to the preproinsulin gene, genomic regions responsible for the expression of the factors activating the enhancer sequencer [4] have been cotransfected with regions responsible for hormonal processing and release.

Transfection with large fragments of B cell total genomic DNA was also used in this study to demonstrate transient surface insulin-like antigenic expression. Cell-surface insulin-like antigenicity has been previously reported in pancreatic B cells [2], and this study demonstrates that such an expression can only be obtained if the DNA used was of B cell origin (fig.4). This finding is compatible with the cell-specific gene expression concept.

The incorporation of extraneous DNA into host L cells was followed in this study by monitoring the fluorescence of ethidium bromide-tagged DNA or of FITC-tagged DNA. In combination with FITC-labelled anti-insulin antibody binding, this method was also used to obtain an estimate of the rate of disappearance of the phenotypic expression of the donor DNA as described by Scangos et al. [18]. Although cell sorting by the ethidium bromide method was more efficient than by the FITC-labelled DNA procedure (fig.2), due to the relative mutagenicity of ethidium bromide as compared to FITC, the latter was preferred. Attempts to use anti-insulin antibodies for the entire period of sorting were not successful.

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