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Tissue-specific expression of transfected human insulin genes in pluripotent clonal rat insulinoma lines induced during passage *in vivo*

(differentiation/clonal analyses/gene activation)

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ABSTRACT **The pluripotent rat islet tumor cell line MSL-G2 expresses primarily glucagon or cholecystokinin and not insulin *in vitro* but changes phenotype completely after prolonged *in vivo* cultivation to yield small-sized hypoglycemic tumors composed almost entirely of insulin-producing beta cells. When a genomic DNA fragment containing the coding and upstream regulatory regions of the human insulin gene was stably transfected into MSL-G2 cells no measurable amounts of insulin or insulin mRNA were detected *in vitro*. However, successive transplantation of two transfected clones resulted in hypoglycemic tumors that efficiently coexpressed human and rat insulin as determined by human C-peptide-specific immunoreagents. These results demonstrate that cis-acting tissue-specific insulin gene enhancer elements are conserved between rat and human insulin genes. We propose that the *in vivo* differentiation of MSL-G2 cells and transfected subclones into insulin-producing cells reflects processes of natural beta-cell ontogeny leading to insulin gene expression.**

Each of the four distinctive cell types that occurs in the pancreatic islets is highly differentiated and produces its distinctive hormonal product(s) (1) arising in each case from a single precursor molecule—i.e., for beta cells, preproinsulin (2–4); for alpha cells, preproglucagon (5); for delta cells, preprosomatostatin (6); and for PP cells, prepropancreatic polypeptide (7).

The developmental derivation of the islet cells from the embryonic ductal epithelium (8) presupposes the existence of a pluripotent islet progenitor or stem cell to give rise to the four known mature islet cell types. We have established (9) a series of transformed islet cell lines (MSL cells) derived from a liver metastasis of an x-ray-induced islet cell tumor (10). All MSL clones tested so far remain heterogeneous and secrete multiple hormones [i.e., insulin, glucagon, somatostatin, and cholecystokinin (CCK)], even upon repeated cellular cloning (9). Immunocytochemical analysis indicates that the proportion of cells expressing a particular hormone in any given clone is fairly stable over time. In contrast, however, the hormone profile (i.e., the proportion of cells producing insulin, glucagon, somatostatin, and CCK) varies widely among the various clones (9). These results strongly suggest that the MSL cells represent transformed pluripotent islet stem cells with differing, but related, differentiation potentials. Further support for this hypothesis is the independent isolation of two RINm clones (11) and one RINr clone (12) producing insulin, somatostatin, and glucagon, respectively, from the original x-ray-induced tumor.

This study used clone MSL-G2, in which glucagon and CCK are produced at high levels *in vitro* but insulin is secreted at a virtually undetectable level (9). We present data showing that cells from this clone gradually shift toward the expression of the beta-cell phenotype when successively transplanted *in vivo*, resulting in insulin-producing tumors that cause severe hypoglycemia. To determine whether this change in phenotypic expression involved the generation of tissue-specific regulatory factors, we have studied MSL-G2 clones containing a stably transfected single-copy human insulin gene as the reporter gene. Successive *in vivo* transplantations have then been carried out to assess the extent of expression of the human insulin gene during the phenotypic change from CCK/glucagon-producing to predominantly insulin-producing MSL-G2 cells.

MATERIALS AND METHODS

Cell Culture. MSL-G2 cells (9) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO). Transfected cultures were selected for stable transformants with G418 (260 μg/ml) (GIBCO) and expanded in the presence of G418 (300 μg/ml).

Plasmids and DNA Transfection. A plasmid (pBR325) containing a 15-kilobase (kb) *Eco*RI genomic fragment (Fig. 1) carrying the human insulin gene (13) was linearized with *Eco*RI and cotransfected with pSV2-neo (16) by the calcium phosphate precipitation technique (17, 18) by using a fixed ratio of the human insulin gene/pSV2-neo of 5:1 (wt/wt). MSL-G2 cells (1.4×10^6 cells per 12-cm^2 flask) were washed once in 5 ml of HeBS (137 mM NaCl/5 mM KCl/5.5 mM glucose/0.8 mM Na_2HPO_4/21 mM Hepes, pH 7.4) and 1.5 ml of precipitated DNA was added. After 20 min, 10 ml of Dulbecco's modified Eagle's medium (GIBCO) containing 2% fetal calf serum was added and the incubation was continued overnight. The amounts of human insulin DNA tested were: 125 μg, 250 μg, 375 μg, and 500 μg. On day 1 after the transfection the cells were exposed to a glycerol shock (19). At day 2 the medium was changed to RPMI 1640 and the selection was initiated at day 5 by the addition of G418 (260 μg/ml). Proliferating clones were propagated as described (9).

Detection of Human Insulin Gene Expression. Culture supernatants were analyzed for insulin by using a radioimmunoassay that does not discriminate between human and

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Abbreviations: CCK, cholecystokinin; neo, neomycin resistance.

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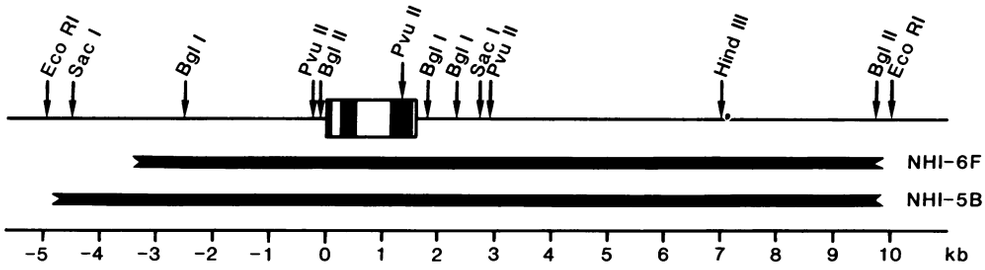

FIG. 1. Restriction map of the 5′ and 3′ flanking regions of the human insulin gene (13, 14). The box indicates the size of the preproinsulin gene transcript and the solid interior areas are the exons. Restriction analyses with combinations of the endonucleases shown revealed integration of a single copy in both NHI-5B and NHI-6F and the size of the fragments of NHI-5B and NHI-6F is indicated (15).

rat insulins (9). Tumors dissected (average wet weight, ≈1 g) from hypoglycemic animals were gently minced in 10 ml of complete tissue culture medium and then incubated for 10 min, and human proinsulin and C-peptide were measured in the supernatants (20, 21). Tumors from hypoglycemic animals were fixed by cardiac infusion of 4% (wt/vol) paraformaldehyde (22). The dissected tumor was prepared for immunocytochemistry (22) and stained for human C-peptide (23) or insulin (rat or human) (24) by the peroxidase-anti-peroxidase technique (25).

RESULTS

Induction of Insulin Gene Expression by *in Vivo* Passage. When MSL-G2 cells were inoculated into rats, large tumors formed that eventually caused hypoglycemia. Successive transplantation gave rise to a phenotype manifested by small-sized insulin-producing tumors (average wet weight, 250 mg representing a reduction in size from primary tumors by a factor of 100) that caused severe hypoglycemia. This tumor line, termed MSL-G2-IN, has remained remarkably stable for 3 years, or >30 successive transplantations, with insulin as the only major hormone released into perifusates of isolated tumor cells (unpublished results). The tumor cells have the capacity to metastasize to the lungs at low frequency and the metastases always express the hypoglycemic phenotype.

Isolation of MSL-G2 Cells Cotransfected with Human Insulin Gene and pSV2-neo. Transfection frequency measured as proliferating G418-resistant clones was 10^{-6}–10^{-7} and the fraction of successful cotransfectants was 15% (3 of 20 clones). All 20 clones were expanded and each was analyzed on Southern blots (16) to verify integration of pSV2-neo DNA sequences and to detect integration of the human insulin gene. Two clones, NHI-5B and NHI-6F, had integrated only a single human insulin gene copy of ≈15 kb and 13 kb, respectively (15) (Fig. 1). Clone NHI-6C integrated only part of the 5′ region of the human insulin gene (data not shown).

Expression of Insulin *in Vitro* and *in Vivo*. Radioimmunoassay for insulin in the culture medium from all 20 G418-resistant clones revealed no detectable insulin, as was found with the mother cell line MSL-G2. The human insulin gene-positive transfected clones, NHI-5B and NHI-6F, and a negative control clone, NHI-5H, which only had integrated pSV2-neo, were all inoculated subcutaneously into NEDH rats. Tumors subsequently formed in all three cases and these were removed and serially subtransplanted for several generations. The first passage of tumors from all three clones resulted in hypoglycemic tumors after various periods (only shown for NHI-5B, Fig. 2). Of these only NHI-5H caused hypoglycemia at the time (56 days) when the first successive transplantation was carried out. The NHI-5B primary tumor was surgically removed and the surviving rat developed

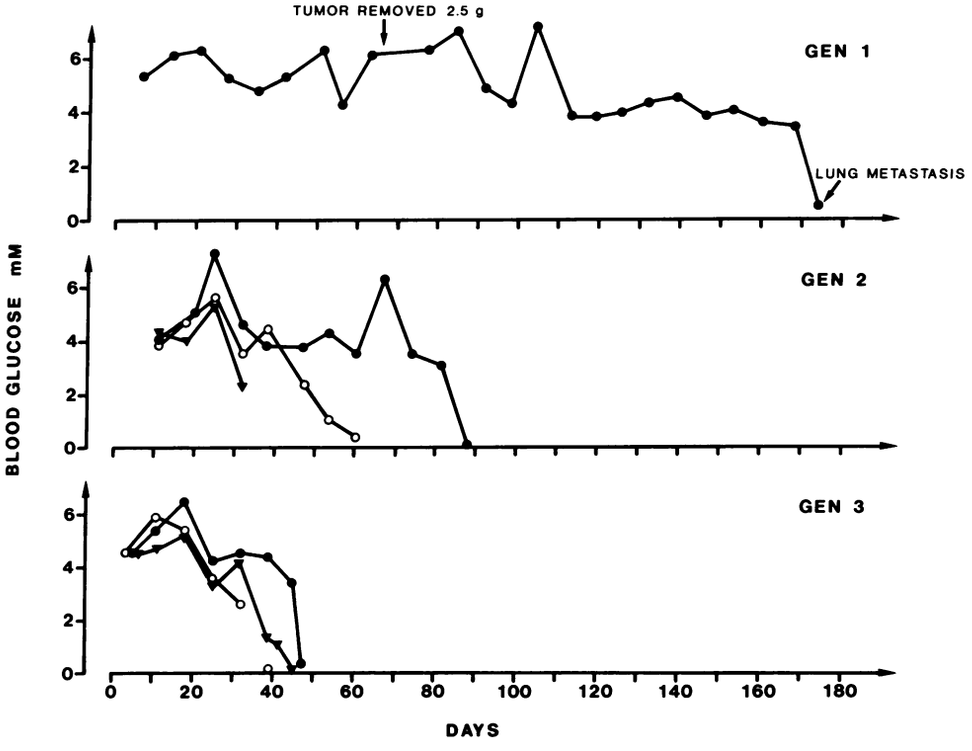

FIG. 2. The transfected clone NHI-5B forms hypoglycemic tumors. The pSV2-neo and human insulin gene cotransfected clone NHI-5B was injected (10^6 cells or 1 mg of freshly excised tumor tissue) subcutaneously between the shoulder blades in NEDH rats (Møllegård, Lille Skensved, Denmark) to form solid tumors. Blood sugar levels in time (Hypocount, Hypoguard Ltd., Woodbridge, U.K.) are shown for several animals (Gen 1–3) in successive tumor generations formed by serial transplantation. Note that the primary NHI-5B tumor metastasized and formed a hypoglycemic tumor in the lung.

hypoglycemia after 180 days (Fig. 2). In this animal a small metastasis was identified in the lung.

On subsequent passages the hypoglycemic activity of the tumor became progressively more dominant resulting in smaller tumors that caused earlier onset of severe hypoglycemia (Fig. 2), similar to the pattern observed with the mother MSL-G2 cell line.

Southern blot analyses demonstrated preservation of the intact *Bgl* I sites of the integrated human insulin gene and the gene for neomycin resistance (neo) during subtransplantation of the NHI-5B and the NHI-6F cells (Fig. 3) as well as of the NHI-5B lung metastasis (Fig. 4*A*). Analysis of restriction fragment length polymorphisms with the neo probe showed a highly heterogeneous pattern indicating a unique integration site in individual clones. Both transfected genes are useful genetic markers to identify the various transfected sister clones (Fig. 3). RNA gel blot analyses by using the MSL-G2-IN tumor and the stable noninsulin-producing MSL tumor MSL-G-AN (O.D.M., unpublished results) as controls clearly indicated the induction of insulin mRNA in NHI-6F after *in vivo* passage (Fig. 4*B*). Insulin mRNA was not detected in the control cells.

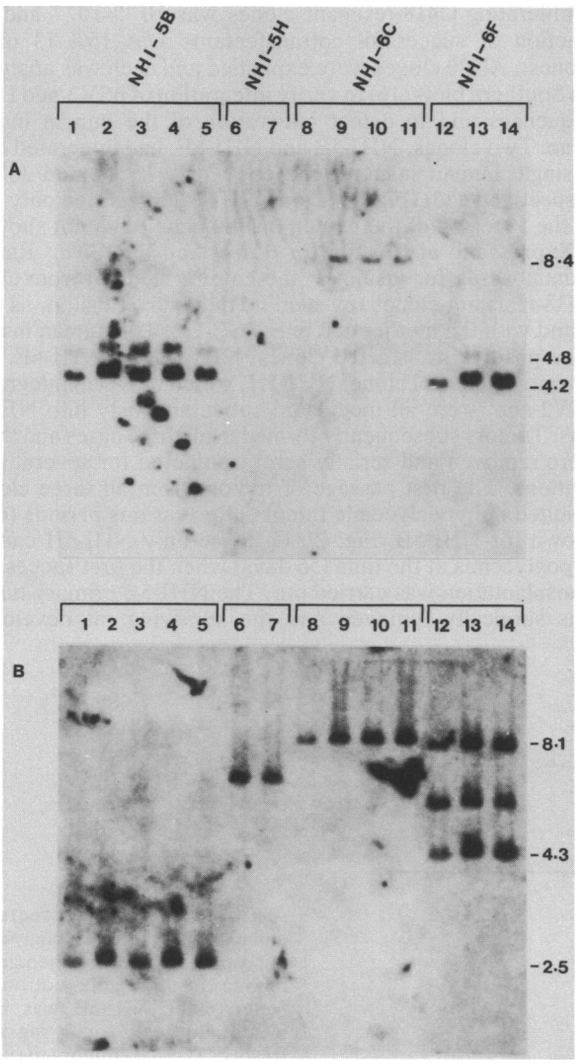

FIG. 3. Restriction fragment length polymorphism studies of transfected sister clones. Southern blot analyses (26) of four transfected clones where DNA from *in vitro* cultures (lanes 1, 6, 8, and 12) and from subsequent *in vivo* hypoglycemic tumors (lanes 2–5, 7, 9–11, 13, and 14) was digested with *Bgl* I (Boehringer Mannheim) and hybridized with a human insulin gene probe (*A*) and a neo probe (*B*). The entire structural neo gene (16) or a 1.6-kb *Pvu* II fragment of the human insulin gene was ^{32}P-labeled by nick-translation (27) or by oligonucleotide labeling (28). The expected 4.2-kb *Bgl* I fragment of human origin (4.8 kb is due to incomplete digestion, see Fig. 1) remained stably integrated in NHI-5B and NHI-6F (*A*). The integrated neo gene is a useful genetic marker to identify each of the various transfected sister clones (*B*).

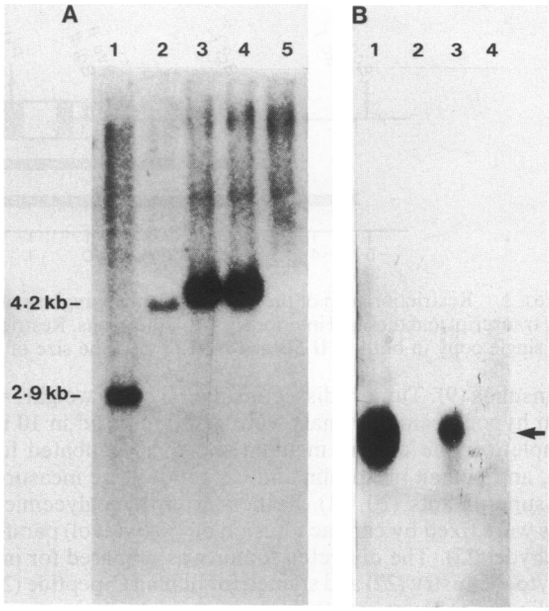

FIG. 4. Southern blot analysis (*A*) showing the monoclonal nature of the NHI-5B *in vitro* cells (lane 2), the primary nonhypoglycemic tumor (lane 3), and the hypoglycemic lung metastasis (lane 4). Lane 5 is DNA from nontransfected control cells MSL-G2, and lane 1 is control DNA human class 1 allele (14). The integrated human insulin gene was derived from a homozygous class-3 individual (13). RNA gel blot analysis (*B*) showing induction of insulin mRNA (arrow) when NHI-6F primary clone (lane 4) is passaged *in vivo* (lane 3). RNAs isolated from MSL-G2-IN (lane 1) and a noninsulin-producing (lane 2) tumor line (O.D.M., unpublished results) were included as controls. The size of the insulin mRNA is 550–650 bases as reported (29, 30). RNA was isolated as described (31), separated (20 μg per lane) on a denaturating 1% formaldehyde gel, and transferred to GeneScreen (New England Nuclear). Hybridization was carried out (31) by using the above insulin gene probe (Fig. 3).

Expression of the Transfected Human Insulin Gene. The levels of human plus rat insulin, human C-peptide, and human proinsulin were measured in extracts of freshly isolated tumors (Table 1). NHI-5B and NHI-6F clones that contained the intact human insulin gene *Bgl* I sites were positive for human C-peptide and proinsulin. In contrast, the NHI-6C clone lacking the complete human insulin gene showed no detectable human C-peptide levels (data not shown). The high level of insulin expression found in the control NHI-5H cells (Table 1) demonstrates the complete

Table 1. Hormone release from freshly isolated tumors

Cell line	Rat no.	Insulin, pmol/ml	Human C-peptide, pmol/ml	Human proinsulin, pmol/ml
NHI-5H	846	3930 (100)	0 (0)	0 (0)
NHI-5B	854	1500 (100)	90 (6)	45 (3.0)
NHI-5B	855	1360 (100)	90 (6.6)	52 (3.8)
NHI-6F	798	1770 (100)	180 (10.2)	121 (6.8)
NHI-6F	818	2430 (100)	320 (13.2)	182 (7.5)

Values are from experiments where a single tumor was dissected in 10 ml of medium and left for 10 min. Insulin values are normalized to 100% and the relative percentage of human C-peptide and proinsulin is shown in parentheses.

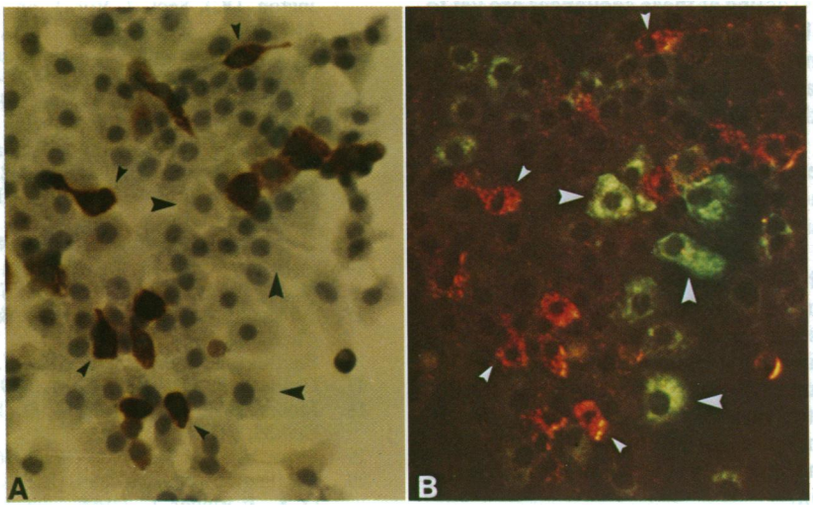

FIG. 5. Insulin and human C-peptide immunocytochemistry of the human insulin gene-transfected MSL-G2 subclone, NHI-6F. Cells isolated from freshly excised tumors were reestablished *in vitro* by using procedures to avoid fibroblast contamination (9). (*A*) Indirect immunoperoxidase staining (9) for human C-peptide with monoclonal rat antibody GN-ID4 (23), which does not crossreact with the rat C-peptides. The indirect immunoperoxidase staining for human C-peptide, where extensive substrate precipitation (aminoethyl carbazole) quenches all other immunoreactivity in that particular cell, allows for subsequent insulin staining with monoclonal mouse antibody AE9D6 (24) and indirect immunofluorescence (32). (*B*) Same field as in *A* is viewed in semidark field illumination combined with epifluorescence, which renders the red human C-peptide cells visible (small arrowheads) and allows simultaneous detection of fluorescent cells only expressing rat insulins (large arrowheads).

lack of crossreaction of the specific human antibodies to rat C-peptide or rat proinsulin.

Immunocytochemical detection of human C-peptide expression was performed on tumor sections (data not shown) or on fixed NHI-5B and NHI-6F tumor cells reintroduced into culture after six successive transplantations. Cell populations from NHI-6F-Tu28 (Fig. 5*A*) and not the control cells MSL-G2-Tu6 showed extensive immunocytochemical staining with the rat monoclonal antibody GN-ID4, specific for human C-peptide/proinsulin (23). Both cell cultures showed strong insulin immunoreactivity (shown only for NHI-6F-Tu28 in Fig. 5*B*).

DISCUSSION

The insulinoma-derived MSL-G2 clonal cell line used in this study exhibits properties suggesting that it may be a derivative of a pluripotent pancreatic stem cell (fur a further discussion of stem cell theory see ref. 33). Thus it gives rise to a variety of neuroendocrine hormone-producing cells when grown under standard culture conditions. Cells expressing insulin occur at a very low frequency ($10^{-3}-10^{-4}$), and cells producing other peptide hormones—such as CCK, glucagon, and somatostatin—are produced at a much higher level. The MSL-G2 cells also share certain immunological epitopes with the pancreatic ductal epithelium, a cell type that is believed to be capable of differentiation into the various cells that make up the islet (34).

The shift in the pattern of differentiation from a predominantly alpha (glucagon producing)-cell phenotype toward an almost exclusively beta (insulin producing)-cell phenotype during prolonged passage of these cells *in vivo* prompted the present study to determine whether this effect would also be observed in a stably transfected clonal derivative of this cell line containing an intact human insulin gene(s). We show that this phenotypic shift indeed occurs within single clones, either those bearing pSV2-neo alone (NHI-5H) or both neo and human insulin genes (e.g., NHI-6F and NHI-5B). Thus, successive passages *in vivo* led to the establishment of lines similar to MSL-G2-IN that expressed a highly stable hypoglycemic phenotype associated with small-sized insulin-producing tumors consisting mainly of beta cells. Perfusion studies of the tumors showed that processed insulin is the only hormone secreted in major amounts. The insulin gene-transfected subclones NHI-6F and NHI-5B behaved almost identically to the parental clone in that essentially no insulin expression was detectable *in vitro* prior to passage, but after passage both the transfected human and the endogenous rat insulin genes were expressed at high levels[‖], as assessed by radioimmunoassay and immunocytochemical staining as well as by RNA gel blot analyses. These results thus indicate that tissue-specific promoter/enhancer segments of the human insulin gene are functional in rat islet tumor cells and that the conditions of passage *in vivo* may somehow reproduce environmental influences that operate in the final stages of differentiation of beta cells in the normal islet.

The beta-cell-specific expression of the insulin gene appears to be mediated by DNA sequences making up the first 300 base pairs of the upstream region of the insulin gene (38–40). Subregions within this portion of the genomic sequence have been reported to be specifically protected in DNase degradation experiments in insulin-producing cells, presumably due to binding of beta-cell-specific nuclear factors (41, 42). In accordance with these experimental results, it has been shown that the 5′ upstream enhancer sequence from the rat insulin gene II efficiently confers beta-cell-specific expression to a foreign gene in transgenic mice (43). Tissue-specific expression of the unmodified human insulin gene in the islets of transgenic mice (in which the entire beta-cell mass expresses human C-peptide) has been reported (44, 45), indicating that the tissue-specific signals are also evolutionarily conserved between mouse and man.

The studies in transgenic mice reenforce the earlier conclusions of Hanahan (43) that the 5′ flanking sequences alone are responsible for the regulation of tissue-specific insulin

[‖]A single copy of the human insulin gene was integrated into the genome in NHI-5B and NHI-6F (15). Assuming that this copy integrated into a diploid cell line containing four endogenous insulin genes [rats have two insulin genes (35–37)], it can be deduced from the data in Table 1 that the human insulin gene in both clones is expressed at levels comparable to the rat genes since human C-peptide/proinsulin accounts for ≈20% of the total insulin immunoreactivity found in the tumor extracts.

expression. The factor(s) acting at these sequences are yet to be isolated (41, 42), but evidence suggests that several proteins able to recognize specific sequences are involved (46). MSL-G2 cells may, therefore, be regarded as transformed islet stem cells that *in vitro* predominantly express an alpha-like phenotype, but, after exposure to the *in vivo* environment, cells differentiate to become insulin producing and thus give rise to the hypoglycemic tumor phenotype.

When the isolated tumor cells (NHI-5B or NHI-6F) from hypoglycemic tumors are reintroduced into culture, the signal for maintaining the beta-cell phenotype is lost and the cultures gradually revert back to noninsulin-producing phenotypes. In both clones the alpha-like phenotype eventually reappears as the dominating cell type (data not shown). Preliminary analyses indicate that the endogenous insulin genes and the transfected human gene are extinguished at different rates. This phenomenon is illustrated in Fig. 5*B*, where only a part of the insulin-producing monolayer cells coexpress human insulin.

The stimulus for the reversible phenotypic shift in the MSL-G2 line is likely to be complex, consisting of combinations of humoral factors and/or extracellular matrix components provided by the tumor host, as suggested by the results of Muschel *et al.* (47) with the insulin-producing RINm-5F line. However, in their experiments increased insulin expression *in vitro* was attributed to mRNA stabilization after growth arrest brought about by added matrix material rather than to any observable change in the proportion of insulin-expressing cells. The results presented here suggest that the cellular environment may play a major role in the functional differentiation of pre-beta cells and further efforts will now be needed to identify the responsible extracellular factors. Equally important will be a better understanding of the intracellular changes responsible for the induction of insulin gene expression. Our findings suggest that the site of integration of the transfected gene is unlikely to be important and thus favor the induction of a trans-activating factor(s), but they do not rule out the possibility that alterations in chromatin structure such as demethylation and altered DNase I sensitivity within the 5′ flanking sequence of the insulin gene may also be involved. A comparison of the proteins expressed in these two phenotypes should make it possible to identify specific trans-acting factors involved in the induction of insulin gene expression.

We dedicate this paper to the memory of the late Ms. Lis Lyngsie. We are indebted to Tove Funder and Heidi I. Jensen for skillful technical assistance. We thank Jane Falk and Susanne Kjellberg for human C-peptide and proinsulin measurements. The expert secretarial assistance of Ms. Bente Völer is gratefully acknowledged. This work was supported in part by the Nordisk Insulin Foundation and National Institutes of Health Grants DK 13914 and AM 20595 to D.F.S.

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