The homeodomain protein IPF-1/STF-1 is expressed in a subset of islet cells and promotes rat insulin 1 gene expression dependent on an intact E1 helix–loop–helix factor binding site

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The mouse homeodomain protein insulin promoter factor-1 (IPF-1) and the rat homologue somatostatin transactivating factor-1 (STF-1) are involved in early pancreatic development and have been implicated in the cell-specific regulation of insulin- and somatostatin-gene expression in mature islet β- and δ-cells. The cell specificity of IPF-1/STF-1 expression in mature islets is, however, still unclear. Using antisera against recombinant IPF-1 and STF-1 in combination with antisera against islet hormones we find that all β-cells in monolayers of newborn rat islet cells express STF-1, as do a fraction of the δ-cells. In adult rat and mouse pancreas we find a similar distribution. IPF-1/STF-1 expression was not detected in glucagon-producing α-cells. In islet cell tumour models we found that a glucagon/islet amyloid polypeptide (IAPP)-producing pluripotent rat islet cell line (NHI-6F-GLU) expresses STF-1 in all cells prior to insulin gene activation induced by *in vivo* culture. In contrast, a mouse α-cell line (αTC1) exclusively expressed IPF-1 in a small subset of insulin-producing cells while an insulin-negative subclone (αTC1.9) was negative for IPF-1. In transfection experiments using αTC1.9 cells STF-1 activated a rat insulin 1 reporter gene dependent not only on both STF-1-binding sites, but also on the E1-binding site for the helix–loop–helix factor IEF-1. However, the endogenous mouse insulin genes remained inactive in these cells. These results suggest that the insulin promoter acquires its very high, yet cell-specific, activity at least partly through the action of IPF-1/STF-1. This action is dependent on helix–loop–helix factors bound to the E1 element.

INTRODUCTION

The mouse pancreas forms as an outpocketing from the embryonic gut beginning at day 10 of gestation (e10) [1]. Later, the attachment of the pancreatic buds to the gut narrows, forming the pancreatic duct and the cells lining the duct differentiate into exocrine and endocrine tissues [1]. The cells in the dorsal and ventral gut wall that give rise to the pancreatic anlage can be distinguished already at day e8 by expression of the homeodomain protein IPF-1 [2]. This protein was originally cloned as an insulin gene transcription factor (insulin promoter factor-1) but its early expression indicates that it serves an additional function in early pancreatic development. This was recently demonstrated convincingly as mice homozygous for an IPF-1 gene disrupted by homologous recombination completely failed to develop the pancreas [3].

β-Cell-specific expression of the rat insulin 1 gene has been found to be dependent on *cis*-acting elements located in the 5′-flanking sequence [4,5]. A number of discrete *cis*-acting elements have been defined by mutational analysis [6]. Two sites (E1 and E2) (throughout this paper we use the newly adopted nomenclature for insulin gene *cis*-elements [7]), containing eight base pairs of identical sequence (−110 to −103 and −238 to −231, respectively) which include a so-called E-box (CANNTG), are critical for a high expression level. These sites bind the factor, insulin enhancer factor-1 (IEF-1) [8,9]. IEF-1 is a dimer composed of either one of the ubiquitous helix–loop–helix (HLH) proteins, Pan-1 or Pan-2, in conjunction with an islet-specific HLH protein [10–14].

Additionally, two 'TAAT'-motif-containing sites have been implicated in the β-cell-specific activity of the rat insulin 1 promoter [2,10,15]. These two sites, the A3/A4 element (−222 to −208) and the A1 element (−81 to −74) both bind the homeodomain protein IPF-1/STF-1/IDX-1 [2,15,16]; additionally, the A3/A4 element binds insulin enhancer factor-2 (IEF-2) [10]. The cloning of mouse IPF-1 and the rat homologue, named alternatively somatostatin transactivating factor-1 (STF-1) or islet duodenum homeobox factor-1 (IDX-1) (in the remaining part of this paper we will refer to the mouse protein by the name IPF-1 and the rat protein by the name STF-1), showed that they belong to a new class of homeodomain proteins [2,16,17], distinct from Isl-1 and lmx-1, two LIM-homeodomain proteins which in recombinant form are able to bind to the A3/A4 element [18,19]. We and others have recently reported that STF-1 is capable of binding to and transactivating the human insulin gene and, furthermore, appears to be involved in the glucose-regulated transcription of the insulin gene [20,21]. STF-1 is also capable of activating a transfected rat insulin 1 promoter in synergy with Pan-1 when introduced into HeLa cells [15].

The expression pattern of IPF-1 in the adult pancreas is still unresolved. Although found only in the islets within the pancreas, one report describes IPF-1 expression as restricted to the β-cells [2], whereas two other reports describe IPF-1 expression in the majority of islet β-cells but also in approximately 20% of the

Abbreviations used: CAT, chloramphenicol acetyltransferase; FITC, fluorescein isothiocyanate; HLH, helix–loop–helix; IAPP, islet amyloid polypeptide; IDX-1, islet duodenum homeobox factor-1; IEF-1, insulin enhancer factor-1; IPF-1, insulin promoter factor-1; STF-1, somatostatin transactivating factor-1; SV40, simian virus 40.

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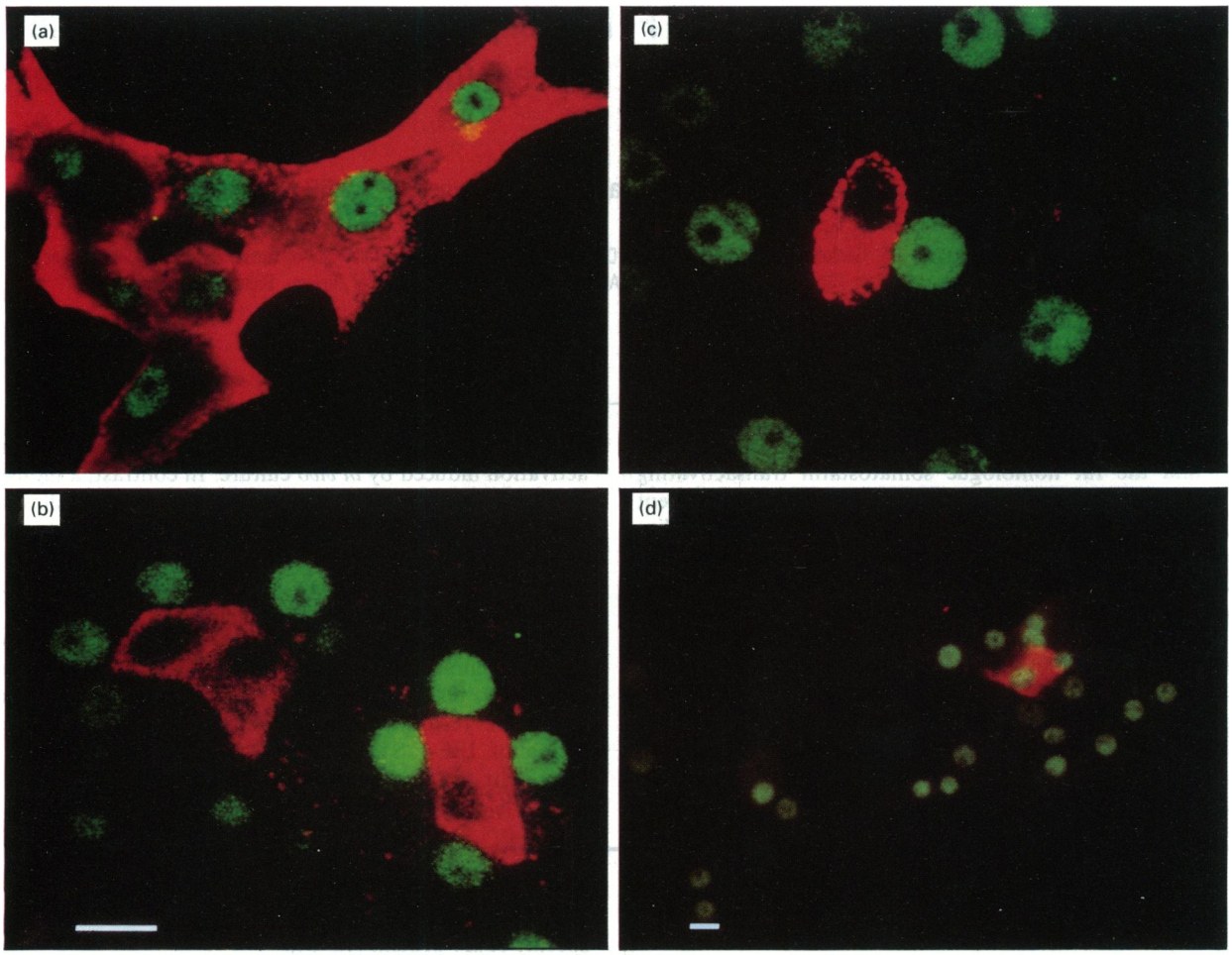

Figure 1 Confocal laser scanning and convential micrograph images of monolayers of newborn rat islet cells

Confocal laser scanning images are shown of monolayers of newborn rat islet cells double stained with antiserum to STF-1 followed by FITC-conjugated secondary antibody (**a–c**), and with monoclonal antibodies to insulin (**a**), somatostatin (**b**), or glucagon (**c**) followed by Texas Red-conjugated secondary antibody. (**d**) A conventional micrograph of the same culture used in (**b**) was stained with antiserum to STF-1 followed by FITC-conjugated secondary antibody and a monoclonal antibody to somatostatin followed by Texas Red-conjugated secondary antibody. Bars, 10 μm.

δ-cells and 1–2 % of the α-cells. Therefore the aim of the present study was (a) to elucidate the cell specificity of IPF-1/STF-1 in both primary and transformed islet cell cultures, and (b) to investigate its mechanism of action on insulin gene transcription.

MATERIALS AND METHODS

Cell lines and nuclear extract preparations

NHI-6F-GLU (previously NHI-6F-Primary), a glucagon-producing rat cell line carrying a silent human insulin gene [22], and NHI-6F-INS (previously NHI-6F-28), an insulin-producing cell line derived by *in vivo* culture of NHI-6F-GLU with high levels of insulin expression in early passages [22,23], were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum (Gibco, BRL), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) (Gibco, BRL). αTC1, a transgenically derived glucagon-producing α-cell line [24], αTC1.9, a subclone of αTC1 [25], and βTC3, a transgenically derived insulin producing β-cell line [26], were grown in Dulbecco's modified Eagle's medium (DMEM) with the same supplements as used for RPMI 1640.

Nuclear extracts were prepared from these cells as described [27].

Plasmids, transfections, and analysis of gene expression

A Rat I–CAT reporter construct (pIns.CAT) containing 346 bp of the rat insulin 1 gene promoter as well as block mutants of this reporter (S7-, S8-, S10-, S20-, and S22-CAT) have all been described previously [6]. The P1M2 mutant of pIns.CAT has been described [2]. Rat II–CAT was constructed by ligating a 745 bp *Bam*HI–*Hin*dIII fragment from RIPTag [28], containing 660 bp of rat insulin 2 promoter sequences, and a 1.6 kb *Hin*dIII–*Bam*HI fragment from pSV2-CAT [29], containing chloramphenicol acetyltransferase (CAT) gene coding sequence and simian virus 40 (SV40) poly(A) and splice site, into *Bam*HI-digested pUC18. Transfections were by the calcium-phosphate co-precipitation method [29], using 15 μg of reporter plasmid and 5 μg of an RSV-luciferase expression plasmid as internal control. Luciferase activities were determined (Luciferase Assay System, Promega). CAT assays were performed as described [29]

after normalization to luciferase activity. Co-transfections were done either with 1 μg of a CMV-STF-1 expression vector [17] or with 1 μg of the control vector pcDNA1-Neo (In Vitrogen, Abingdon, U.K.) containing a cytomegalovirus promoter without insert.

Antisera and immunochemistry

The anti-STF-1 serum has been described [15], as has the anti-IPF-1 serum [2]. Insulin-, glucagon-, and somatostatin-antisera have previously been described [30]. Cells were seeded in 8-well chamber slides (Nunc, Glostrup, Denmark) prior to immunocytochemistry which was performed as described [23].

Newborn rat islets were isolated as described previously [31]. Growth hormone-promoted monolayers of newborn rat islets were prepared and cultured on microscope slides (Nunc, Glostrup, Denmark) as described previously [31]. After 4 weeks of culture the monolayer islet cells were fixed in 1% neutral paraformaldehyde for at least 24 h. Primary antibodies to islet hormones (insulin, glucagon and somatostatin) were combined with an antiserum to STF-1 in double-staining experiments by indirect immunofluorescence with species-specific secondary antibodies labelled with fluorescein isothiocyanate (FITC) or Texas Red [32]. The stainings were examined with a Multiprobe 2001 inverted confocal laser scan microscope system (Molecular Dynamics, Stockholm, Sweden) equipped with an argon–krypton laser and a 100×, n.a. 1.40 Nikon objective.

RESULTS

STF-1 is expressed in all β-cells and a fraction of the δ-cells in newborn rat islet monolayer cells

Isolated islets from newborn rats were dispersed and monolayer cultures were allowed to form prior to immunocytochemistry. Double staining with anti-insulin and anti-STF-1 sera revealed that all insulin-positive cells also showed intense nuclear staining with the STF-1 antiserum (Figure 1a). When double staining was performed with anti-somatostatin antibody only a small fraction of somatostatin-positive cells displayed nuclear staining for STF-1 (Figures 1b and 1d). Double staining for glucagon and STF-1 failed to detect glucagon/STF-1 double-positive cells (Figure 1c). Immunohistochemistry performed on sections of adult rat and mouse pancreas revealed a similar distribution of STF-1 or IPF-1 immunoreactivity with staining of all β-cells and no staining of α-cells while a fraction of the δ-cells did stain for STF-1 or IPF-1, although the staining was less intense than the β-cell staining (results not shown).

STF-1 immunoreactivity is present in both NHI-6F-INS and NHI-6F-GLU cells

To examine further the distribution of STF-1 in NHI-6F-GLU and NHI-6F-INS cells we performed immunocytochemistry with the anti-STF-1 serum. As can be seen in Figure 2, STF-1 is localized to the nucleus. Strikingly, the number of STF-1-positive cells approached 100% in both NHI-6F-GLU (results not shown) and NHI-6F-INS (Figure 2a) even though the number of insulin-positive cells is below 1% in NHI-6F-GLU and approximately 50% in NHI-6F-INS cells [22,23]. Double staining for STF-1 and insulin did not reveal any obvious correlation between intensity of STF-1 staining and insulin expression as intense STF-1 immunoreactivity could be found in cells not expressing insulin (Figure 2). Thus immunocytochemistry confirms that STF-1 is equally abundant in NHI-6F-GLU and NHI-6F-INS cells, as previously demonstrated in band shift analysis [20].

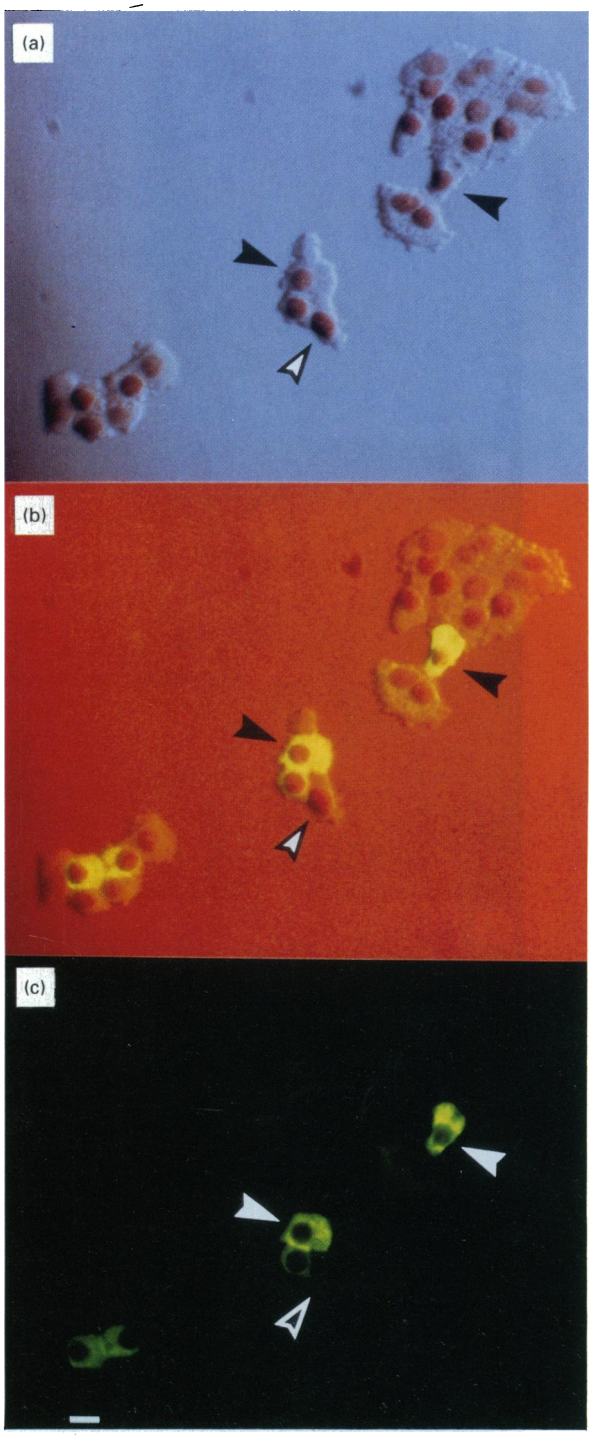

Figure 2 Localization of STF-1 and insulin in NHI-6F-INS cells

Cells grown in chamber slides were stained with antiserum to STF-1 followed by immunoperoxidase staining using a Histostain-SP streptavidin–biotin/enzyme immunostaining kit. Following the peroxidase staining the cells were stained with a monoclonal antibody to insulin followed by FITC-conjugated secondary antibody. Phase contrast exposure (**a**), double phase contrast–FITC exposure (**b**), FITC exposure (**c**). Note insulin/STF-1 double-positive cells (filled arrowheads) and STF-1-positive but insulin-negative cells (open arrowhead). Bar, 10 μm.

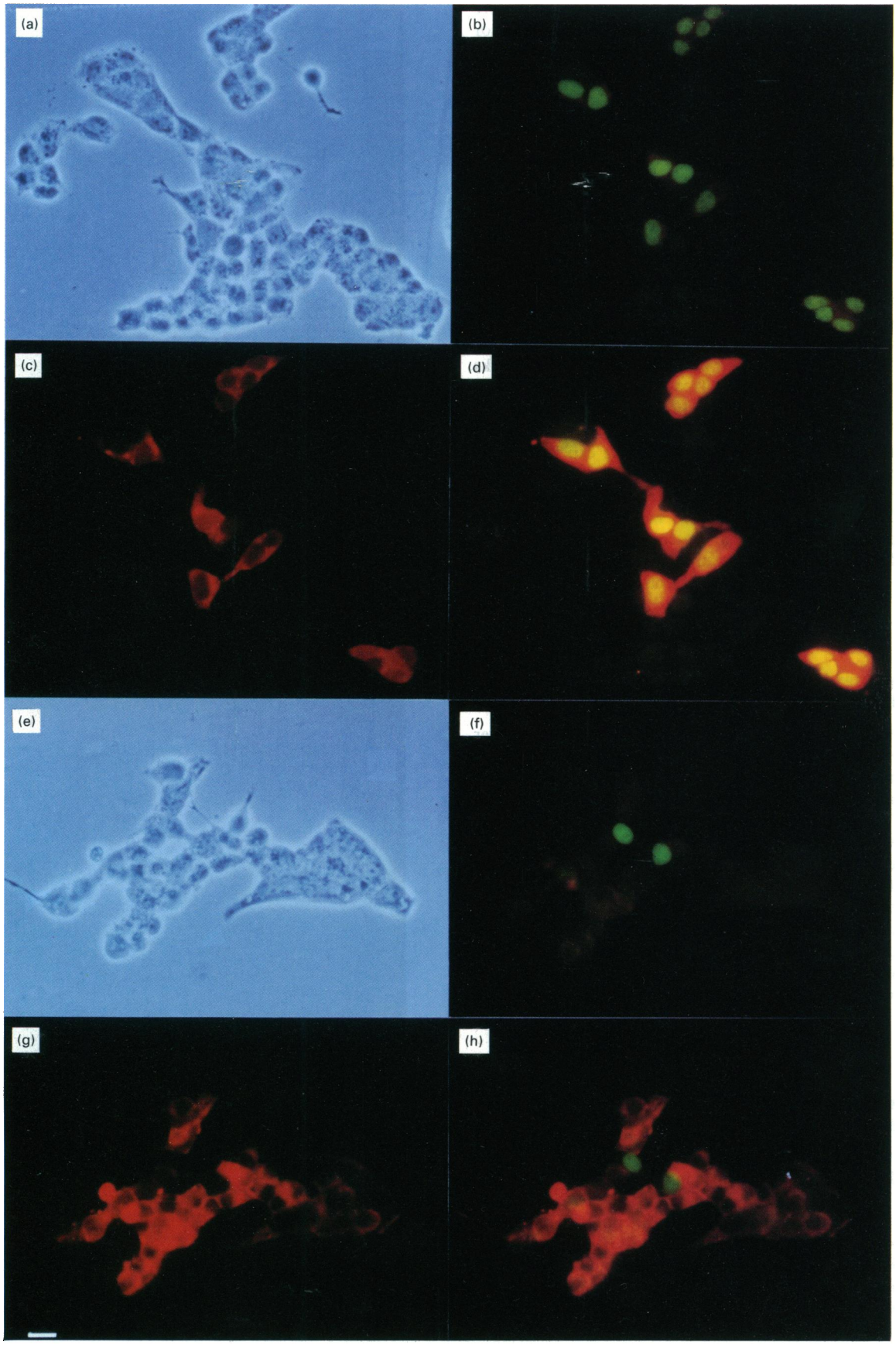

Figure 3 **For legend see facing page.**

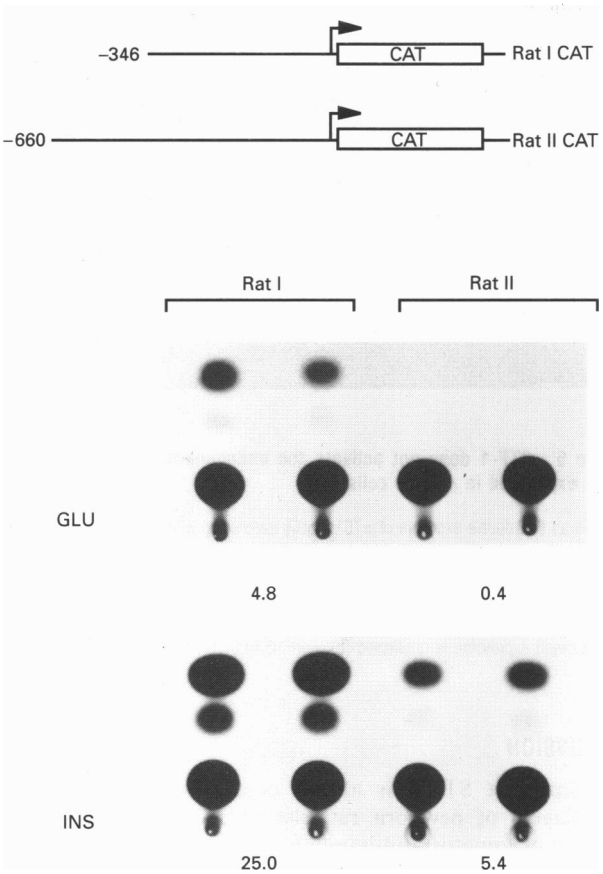

Figure 4 Insulin promoter activity is induced by *in vivo* culture of NHI-6F-GLU cells

Representative transient assay of rat insulin 1 and 2 promoter activity in NHI-6F-GLU (GLU) and NHI-6F-INS (INS) cells. Cells were transfected with 15 μg of the indicated reporter plasmid together with 5 μg of a RSV-luciferase expression vector. Extracts were prepared after 48 h and assayed for CAT activity after normalization against luciferase activity. Numbers below chromatograms indicate percentage conversion of [^{14}C]chloramphenicol. Schematic representation of the insulin enhancer/promoter-CAT reporter constructs showing the extent of 5′-flanking sequences used from either of the two non-allelic rat insulin genes is shown above the autoradiograms.

IPF-1 is specifically localized to an insulin-producing subset of αTC1 cells

Some glucagon-producing αTC1 cultures obtained from glucagon–promoter SV40 large-T transgenic mice have been reported to contain a subset of insulin and islet amyloid polypeptide (IAPP)-positive cells [25,32], the number of which varies between cultures. If IPF-1 is required for insulin expression this insulin-producing subset must have regained IPF-1 expression. To test this directly we performed double immunocytochemical stainings of αTC1 and of the subclone αTC1.9 which does not contain insulin-producing cells [25]. Staining of αTC1.9 confirmed previous findings in that no insulin-producing cells are present in this subclone (results not shown). When analysing for IPF-1 expression we were not able to detect any positive cells (results not shown). Double staining of αTC1 for insulin and IPF-1 did indeed reveal that all the insulin-positive cells contained IPF-1; furthermore, all IPF-1-positive cells produced insulin (Figure 3d). Double staining for glucagon and IPF-1 showed that all glucagon-producing cells were negative for IPF-1 (Figure 3h). Thus, a perfect correlation between insulin production and IPF-1 expression was observed, strengthening the notion of IPF-1 as a transcription factor required for insulin expression.

The transcriptional activity of insulin 5′-flanking DNA is higher in NHI-6F-INS than in NHI-6F-GLU cells

We have previously shown that the endogenous insulin genes are activated upon *in vivo* culture of NHI-6F-GLU cells. To test whether the insulin gene transcription thus induced was caused by a concomitant induction of a diffusible transactivating activity we performed transient transfection assays. Plasmids containing rat insulin 1 (−346 to +1) and 2 (−660 to +8) enhancer/promoters directing expression of CAT showed 5- and 13-fold higher CAT activity, respectively, in NHI-6F-INS cells than in NHI-6F-GLU cells (Figure 4). When comparing the activity of the two promoters in NHI-6F-INS cells we found that the rat insulin 1 enhancer/promoter had about 5-fold higher activity than the insulin 2 enhancer/promoter (Figure 4).

Activation of the rat insulin 1 gene by STF-1 is dependent on an intact E1, but not E2, HLH-factor binding site

The different distribution of STF-1/IPF-1 suggests that introducing STF-1 to αTC1.9 cells should activate co-transfected insulin reporters whereas the same experiment performed in NHI-6F-GLU cells would not be expected to activate insulin reporters to the same degree due to the already high expression of STF-1. To test the effect of expressing STF-1 cDNA in αTC1.9 and NHI-6F-GLU cells upon rat insulin 1 promoter activity we co-transfected pIns.CAT with either an STF-1 expression vector or a control vector. After normalization to a firefly luciferase internal control, CAT activities were determined. As seen in Figure 5, co-transfection of STF-1 leads to only a moderate (1.7-fold) increase in CAT activity when assayed in NHI-6F-GLU cells but to a 3- to 5-fold increase when assayed in αTC1.9 cells. We then asked which *cis*-elements were required for STF-1-mediated transactivation in αTC1.9 cells. In addition to determining the importance of the 'TAAT'-motif-containing A1 and A3/A4 elements we also wanted to examine the involvement of IEF-1, a heterodimeric HLH-protein binding to the E1 and E2 sites in the rat insulin 1 promoter which is present in αTC1 cells and its subclones αTC1.6 and αTC1.9 ([10,33]; P. Serup, unpublished work). We reasoned that αTC1.9 cells would offer a unique system in which to examine the possible cooperation between STF-1 (provided exogenously) and IEF-1 (provided endogenously). By co-transfecting STF-1 with rat insulin 1 reporters containing block mutations in either of the above-mentioned *cis*-elements we found that the A3/A4 binding site was required for activation as only 1.4-fold activation of the S20 mutant construct was seen (Figure 5). The three mutations affecting the A1 site yielded slightly different results. The S7 and

Figure 3 Co-localization of IPF-1 and insulin in αTC1 cells

Cells grown in chamber slides were stained with antiserum raised against IPF-1 followed by FITC-conjugated secondary antibody (**a–h**). The cells were stained with a monoclonal antibody to insulin (**a–d**) or a monoclonal antibody to glucagon (**e–h**) followed by Texas Red-conjugated secondary antibody. Phase-contrast exposure (**a** and **e**), FITC exposure (**b** and **f**), Texas Red exposure (**c** and **g**), double exposure of FITC and Texas Red (**d** and **h**). Note perfect co-localization of IPF-1 and insulin (**d**) and complete absence of IPF-1 in glucagon-positive cells (**h**). Bar, 10 μm.

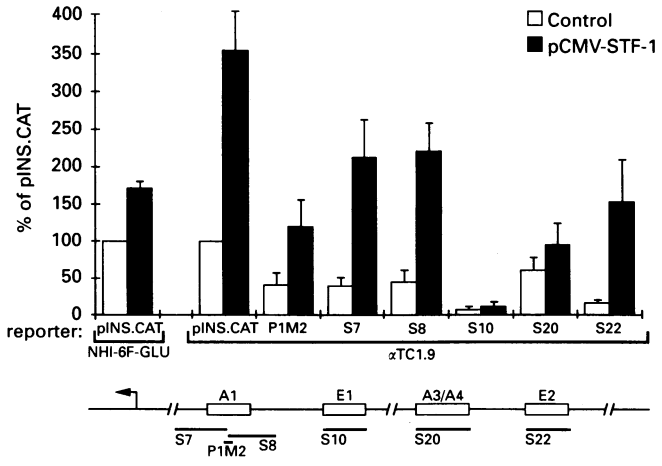

Figure 5 Insulin gene activation by STF-1 requires the A1, A3/A4 and E1 elements

Samples (15 μg) of the indicated reporter plasmids were transfected together with 1 μg of either an STF-1 expression plasmid or a control plasmid and 5 μg of an RSV-luciferase expression vector into the indicated cell line. Transfections, extract preparations, and CAT-assays were performed as described in Figure 4. The location of the mutants in relation to the *cis*-elements are shown in the lower part of the Figure. Each experiment was done in duplicate between two and nine times. Error bars indicate the S.E.M. Note the low activation of the wild-type reporter (pIns.CAT) in NHI-6F-GLU compared with αTC1.9 cells, and the impaired STF-1 mediated activation in the P1M2 and FLAT mutants and the complete absence of both basal and STF-1-mediated activation of the S-10 mutant in αTC1.9 cells.

S8 block mutants were activated to the same degree as the wild-type construct. These mutants, however, coincidentally create new potential binding sites for STF-1 right next to the original site and thus the activation could be mediated by these. To test this we next assayed the P1M2 mutant which only affects the central nucleotides in the 'TAAT' motif. The STF-1-mediated activity of this mutant is clearly reduced, although not to the same extent as seen with the A3/A4 mutant. When examining activation of the E1 and E2 mutants (S10 and S22, respectively) we found that the E1 site was absolutely required for basal activity as well as for activation by STF-1, indicating that binding of the HLH-factor IEF-1 to E1 is required for the activity of STF-1 on the insulin promoter. In contrast, mutation of the E2 site significantly lowered the basal activity but showed approximately 10-fold activation by STF-1.

STF-1 does not activate the endogenous insulin genes in αTC1.9 cells

If STF-1/IPF-1 was the sole determinant for lack of the insulin gene expression in α-cells, as suggested by the co-expression of insulin and IPF-1 in the αTC1 subpopulation, then expression of STF-1 in αTC1.9 cells might activate not only transfected insulin genes but also the endogenous mouse insulin 1 and 2 genes. We tested this by transfecting αTC1.9 cells with the STF-1 cDNA and, after 48 h, we performed double-labelling immunocytochemistry for STF-1 and either insulin or glucagon. STF-1-positive cells were readily detected and often found in pairs where the cells were in contact, indicating that the transfected cells had undergone cell division. As seen in Figure 6(a), none of the STF-1 transfected cells had begun to express insulin. In some instances STF-1/glucagon double-positive cells could be detected, confirming that STF-1 does not inhibit glucagon expression (Figure 6b).

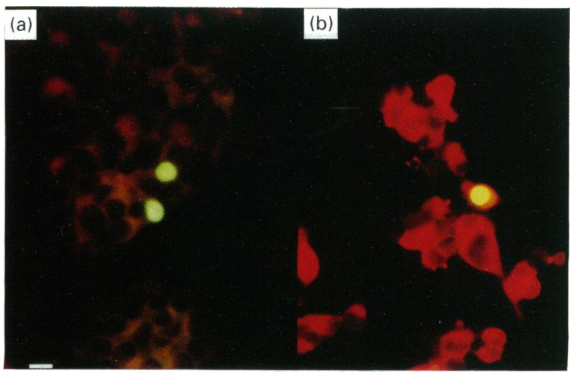

Figure 6 STF-1 does not activate the endogenous mouse insulin genes when expressed in αTC1.9 cells

FITC/Texas Red double exposure of αTC1.9 cells transfected with 10 μg of STF-1 expression plasmid. After 48 h the cells were fixed in 4% paraformaldehyde and stained with antiserum to STF-1 followed by FITC-conjugated secondary antibody (**a** and **b**) and with a monoclonal antibody to either insulin (**a**) or glucagon (**b**) followed by Texas Red-conjugated secondary antibody. Note the complete absence of insulin immunoreactivity in STF-1-positive cells (**a**) and that glucagon expression is unaffected (**b**). Bar, 10 μm.

DISCUSSION

We find that STF-1 is always co-expressed with insulin in monolayers of newborn rat islet cells; furthermore, a small fraction of somatostatin-immunoreactive cells also express STF-1. Using sections of adult rat and mouse pancreas we find STF-1/IPF-1 expressed in all β-cells as well as in a fraction of the δ-cells. The use of monolayers of newborn rat islet cells ensures that co-expression is not due to overlapping cells as might happen using sections. In this system we do not find co-expression with glucagon, nor do we in 3-μm sections of adult rat or mouse pancreas. These results are generally in good agreement with previous results which find strictly β-cell-specific expression [2], or, in addition to β-cell expression, expression also in a small fraction of α- and δ-cells [15,34,35]. The absence of IPF-1/STF-1 in the majority of δ-cells shows that it is not strictly required for somatostatin expression, but does not exclude a possible role in the activation of somatostatin expression during the islet development.

In spite of the very low frequency of insulin-positive cells, most if not all of the pluripotent NHI-6F-GLU cells expressed STF-1 immunoreactivity. This might explain the high frequency of IAPP-positive cells in NHI-6F-GLU [32]. β-Cell-specific expression of the IAPP gene is regulated by factors shared with the insulin gene; in particular, an A3/A4 binding factor (possibly IPF-1/STF-1) has been implicated in cell-specific expression of the IAPP gene [36]. Only the small subset of αTC1 cells which express insulin do also express IPF-1. The perfect correlation between insulin- and IPF-1-expression in αTC1 cells suggests that IPF-1 is required for insulin gene activity. Taken together the IPF-1/STF-1 expression in the two glucagon-producing cell lines, however, suggests that this protein constitutes only one of several factors restricting insulin expression to the β-cell.

We find that the induction of insulin gene expression that occurs during *in vivo* culture [22] is reflected by increased activity of transiently transfected rat insulin 1 and 2 enhancer/promoters. As STF-1 is uniformly expressed in NHI-6F-GLU cells it was not surprising that co-transfection of an STF-1 expression vector into these cells had only a minimal effect upon a rat insulin 1 reporter gene. The *in vivo* induction of insulin gene expression

therefore cannot be explained by induction of either IPF-1/STF-1, IEF-2 or IEF-1, which are all present in NHI-6F-GLU cells.

Using αTC1.9 cells as an IPF-1/STF-1-negative background we find that rat insulin 1 gene expression could be enhanced 3- to 5-fold by co-transfection of STF-1 cDNA. This is in agreement with our previous finding that human insulin gene activity can be stimulated 4-fold in αTC1 cells by co-transfection of STF-1 cDNA [20]. We then used this system to assay which *cis*-elements were required for activation by STF-1. Activation of the A1 mutant (P1M2) was clearly reduced compared with wild type, although some activation was still detectable, while activation of the A3/A4 mutant (S20) was severely reduced. This indicates that both of these elements are required for full activity of STF-1 on the insulin promoter. This is in agreement with results obtained on the rat insulin 2 promoter [34] and the human insulin promoter [20]. Early transfection experiments have indicated that the A3/A4 element by itself cannot stimulate promoter activity in insulin-producing cells when linked to a heterologous promoter but instead it is capable of augmenting expression dependent upon E1 and E2 sites [37]. Interestingly, we found that the E1 but not the E2 element was required for STF-1 activation. This suggests that STF-1-mediated activation of the rat insulin promoter is dependent on the binding of the HLH-factor IEF-1 to the E1 site and presence of an E2 site cannot compensate for the loss of the E1 site. This apparent cooperativity between STF-1 and IEF-1 is in agreement with a recent report which finds that in HeLa cells STF-1 is capable of activating the insulin promoter synergistically with one component of IEF-1 namely the HLH-protein Pan-1 [15]. Previous work by German and co-workers has suggested that the A3/A4 element cooperated with the E2 element in the so-called FF-mini-enhancer [36]. These data, however, were obtained with multimerized FF-mini-enhancers. In contrast, our data are obtained with the complete promoter. In this context it is noteworthy that neither the rat insulin 2 nor the human insulin gene contains an E2 box capable of binding IEF-1 [38].

Introduction of STF-1 cDNA into αTC1.9 cells transiently did not activate the endogenous insulin genes, and glucagon expression remained unaffected. Using immunocytochemistry we readily detected the STF-1 protein properly localized to the nucleus. The failure of STF-1 to activate the mouse insulin genes indicates that one or more factors in addition to IPF-1/STF-1 and IEF-1 are required in order to activate chromosomally located insulin genes.

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