Two HNF-1 binding sites govern the glucose repression of the human sucrase-isomaltase promoter

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We have previously shown, using the Caco-2 clone PF11, that glucose represses transcription of the human sucrase-isomaltase (SI) gene and that the $-370/+30$ fragment of the SI gene conferred glucose-regulated expression on a heterologous gene. Different fragments beginning at the already characterized SI footprint (SIF) 1 ($-53/-37$), SIFR ($-153/-129$) or SIF3 ($-176/-156$) elements [Wu, Chen, Forslund and Traber (1994) *J. Biol. Chem.* **269**, 17080–17085] were tested, in comparison with the $-370/+30$ fragment, for their capacity to inhibit reporter gene expression under high-glucose (25 mM) conditions. Unlike SIF1 and SIFR, the addition of the HNF (hepatocyte nuclear factor)-1-binding element SIF3 to the promoter fragment was required for repression under high-glucose conditions. This effect was enhanced when the SI promoter was extended to

position -370 , indicating that the $-370/-176$ region contains elements that may co-operate with SIF3 to increase the metabolic control of the SI promoter. We have characterized an additional HNF-1-binding site near to and upstream from SIF3; SIF4. By mutagenesis of the three HNF-1-binding elements we show that the two distal HNF-1-recognition sites are the most important for the glucose regulation of the SI gene. Moreover, this glucose regulation was abolished in PF11 cells overexpressing vHNF-1C (variant HNF, an isoform of the HNF-1 family). We thus propose that the differential binding of HNF-1-family proteins to their DNA targets on the SI promoter constitutes the molecular mechanism that controls the glucose regulation of the SI gene transcription.

INTRODUCTION

The presence, at a precise moment and in a precise site, of different positive and negative transcription factors dictates, in multicellular organisms, the subset of genes that are specifically expressed within a cell and thus determines its phenotype. Conversely the expression of particular genes is currently used to specify the lineage of a given cell or to determine its differentiation status. The sucrase-isomaltase (SI) gene belongs to this category. Its expression is restricted to a subtype of epithelial intestinal cells, tightly controlled during development and is widely used as a characteristic functional marker of the fully differentiated enterocytes. In the human colon, SI is transiently expressed at readily detectable levels between the 12th and 25th weeks of gestation [1–4], but may be re-expressed in some colon carcinomas [5–7]. In the small intestine the SI gene expression starts at the 8th week of gestation and is maintained throughout life [8,9]. The constant renewal of the intestinal epithelium is ensured by the highly dividing stem cells located in the crypts. These precursors migrate along the crypt–villus axis and simultaneously stop their divisions and differentiate into the four epithelial subtypes: mucus-secreting goblet cells, enteroendocrine cells, Paneth cells or enterocytes [10]. The latter migrate toward the apices of the villi and are the only ones able to express the SI gene [11–13].

The 5' portion of the SI gene has been sequenced [11,14] and efforts have been made to identify the DNA elements and the different transcription factors that could be responsible for the intestine-specific expression of the gene. These studies have shown that, in transgenic mice, a 3.4 kb fragment from the 5' region of the gene can confer intestine-specific expression on a human-growth-hormone reporter gene, but was not sufficient to restrict this expression to the enterocytes [15]. Besides these studies *in vivo*, cellular models have also been used and have

contributed to the understanding of the molecular mechanisms governing the SI gene regulation.

Most studies on the biosynthesis, intracellular transport and regulation of the intestinal brush-border hydrolases have utilized two carcinoma-derived cell lines which can differentiate *in vitro* into enterocyte-like cells: HT-29 and Caco-2 [16,17]. The HT-29 cells constitute a heterogeneous population that does not express the SI gene under standard culture conditions (25 mM glucose); the gene is de-repressed when these cells adapt to growing in glucose-free medium [18]. The Caco-2 cell line originates from a human adenocarcinoma of the colon and differentiates spontaneously after confluency into enterocytes expressing the SI gene [19]. Previous studies from this laboratory have established that, in these cells, all the experimental conditions that led to an increased glucose uptake also led to a decrease of the SI enzymic activity provoked by a diminution of the transcription rate of the gene [14,20]. The glucose consumption of the Caco-2 cells decreases with the number of passages. We have cloned these cells from early or late passages and obtained metabolic variants. Their analysis confirmed the inverse relationship between the level of glucose consumption and that of SI gene expression [21]. Of these, PF11 was found to be the highest-glucose-consuming clone and expressed the lowest SI mRNA level in the presence of high amounts of glucose (25 mM) in the culture medium. Culture of PF11 cells in a low-glucose medium (1 mM) has been shown to increase the expression of SI at late confluency [21]. Using this model we have previously shown that a 400 bp fragment that includes the promoter of the SI gene was sufficient to confer glucose-dependent expression on a luciferase reporter gene [22]. The results presented here define some DNA elements that are implicated and show that proteins of the HNF (hepatocyte nuclear factor)-1 family govern the glucose-dependent repression of the SI gene.

Abbreviations used: SI, sucrase-isomaltase; PSI, promoter SI; SIF, SI footprint; SIFR, SI footprint repressor; HNF, hepatocyte nuclear factor; vHNF, variant HNF; ma, mutated antisense; ms, mutated sense; EMSA, electrophoretic mobility-shift assay; DTT, dithiothreitol.

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MATERIALS AND METHODS

Construction of luciferase plasmids

p205Luc [22] was constructed by insertion of a polylinker and the promoterless luciferase gene into the Epstein–Barr-derived vector p205-GTI [23]. In this plasmid, a hygromycin-resistance gene, utilized for the selection of stably transformed cells, is controlled by the HSV1 thymidine-kinase promoter. The SI promoter fragments used in this study were generated from a plasmid containing the $-3600/+30$ SI genomic fragment [22]. The $-370/+30$ fragment was obtained by digestion with *AlwNI* and *KpnI* and the other subfragments were generated by PCR with a single antisense oligonucleotide, SIKa: 5'-CCTCTAGGTACCAGACTTG-3' (where the *KpnI* recognition site is underlined), and the following sense primers:

SIF1s,

5'-GGCTGGTGAGGGTGCAATAAACTTTATGAGTA-3'

SIFRs, 5'-TCCCAGAGAGAAACATTTATGTAACTA-3'

SIF3s, 5'-CAGTACAATTACTAATTAAGTTAGAT-3'

where SIF is SI footprint and SIFR is SIF repressor.

The p205Luc vector was linearized with *HindIII*, blunt-ended by Klenow treatment and then digested with *KpnI* to insert the fragments upstream from the luciferase gene. All recombinant plasmids were sequenced with the dideoxynucleotide technology and unmodified T7 DNA polymerase (Pharmacia, Orsay, France) and then purified by two successive isopycnic centrifugations in CsCl gradients [24].

Mutagenesis

A recombinant pTZ18R (Pharmacia) plasmid containing a *AlwNI/KpnI* subfragment from the SI gene promoter was used as the starting material [22]. Site-specific mutations were introduced in the three HNF-1-binding sites using a PCR-based overlap-extension method [25]. The following sense and antisense primers bracketing the multi-cloning site of pTZ18R were used as outside oligonucleotides for PCR: 5'-GGGTTTCCCAGT-CACGACG-3' and 5'-GCGGATAACAATTTACACAGG-3'. For screening facilities the internal oligonucleotide pairs were designed to introduce an additional restriction site into the vector (the mutated positions, for each HNF-1-binding site, are indicated in bold and the new restriction site is underlined; ms and ma stand for mutated sense and mutated antisense respectively):

SIF2ms, 5'-GAAGATTATT**GTGCACT**GATAGGC-3'

SIF2ma, 5'-GCCTATCAGT**GCAACA**ATAATCTTC-3'

SIF3ms, 5'-GTACAATT**ACTCGAGA**ACTTAGA-3'

SIF3ma, 5'-TCTAAGTT**CTCGAGT**AATTGTAC-3'

SIF4ms, 5'-CTTAAGAT**AGATCT**AGTTCAC-3'

SIF4ma, 5'-GTGA**ACTAGATCT**ATCTTAAG-3'

The three restriction sites are respectively for *ApaLI*, *XhoI* and *BglII*.

A first series of PCR was done by combining, in one tube, the pTZ sense primer with an antisense internal primer and, in another tube, the complementary sense internal primer and the antisense pTZ primer. The PCR products were gel-purified and combined as overlapping pairs to generate the single or double mutants with a second PCR using the two outside pTZ primers. The triple mutant was generated in the same way but starting with a recombinant plasmid containing two HNF-1-mutated sites (at SIF2 and SIF3) and the third pair of mutating primers (SIF4ms and SIF4ma). The final PCR products were cloned into

pTZ18R; the recombinant plasmids were digested with the appropriate restriction enzyme and the putative candidates were sequenced. The modified fragments of the SI gene promoter were gel-purified after *HindIII/KpnI* digestion and introduced into the p205Luc vector as described above.

Cell culture and cellular transformation

For standard conditions PF11 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM, Eurobio, Paris, France) containing 25 mM glucose, 20 % (v/v) fetal bovine serum (Boehringer Mannheim, Mannheim, Germany), 1 % (v/v) non-essential amino acids (Gibco, Glasgow, U.K.) and in a 10 % CO₂ atmosphere. For growth in 1 mM glucose the same conditions were used except that glucose-free DMEM was used, the final glucose concentration being supplied by the serum. For transfection experiments, 25 cm² Petri dishes (Corning Glasswork Co, Corning, NY, U.S.A.) were seeded with 0.45×10^6 cells. After attachment (48 h), the medium was changed and transfections were performed 4–5 h later with the CaPO₄ procedure [26] using 20 µg of total DNA/dish. The precipitate was left overnight on the cells and the culture medium was then changed daily. Hygromycin B was added at confluency at a final concentration of 210 µg/ml, which was determined to be the minimal lethal concentration for the untransformed cells. Hygromycin-resistant populations were not cloned and were subcultured under the following conditions: the cells were allowed to attach during 48 h without the drug and hygromycin B was then added at a concentration increasing progressively from 40 to 210 µg/ml, depending on cell density. After selection, all experiments were performed with cells cultured in 25 mM or 1 mM glucose-containing medium without hygromycin [22].

For transient expression, PF11 cells were transfected on day 3 with 20 µg of pRSV-vHNF-1C or with 20 µg of pRSV for the control. When appropriate the cells were switched two days later to 1 mM glucose medium.

RNA extraction and Northern blot analysis

Total RNA was extracted by the guanidinium isothiocyanate method [27]. After glyoxal denaturation, 20 µg were electrophoresed on 1 % agarose and transferred to nylon membranes (Appligene, Illkirch, France) with $20 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate). Prehybridization and hybridization with the vHNF-1C or with the SI probe were as described previously [22]. The filter was stained with Methylene Blue [28] to control the uniformity of the loadings.

The vHNF-1C-specific probe was isolated as an *AvrII/BalI* fragment from pRSV-vHNF-1C plasmid [29] and the SI probe was the 2 kb *EcoR I* fragment excised from pSI2 [30].

Plasmid extraction and DNA analysis of transformed cells

Extra-chromosomal and genomic DNAs were purified by a small-scale SDS lysis procedure adapted from the method of Birnboim and Doly [31]. The total DNA from each cellular population was analysed by slot-blot and hybridization with a luciferase probe (a 759 bp *EcoRI/KpnI* fragment from pTZLuc [32]) to estimate the plasmid copy number and with the SI2 probe [30] for the endogenous SI gene. Probes were ³²P-labelled using a megaprime DNA labelling kit (Amersham Pharmacia Biotech, Les Ulis, France). After 18 h of hybridization in the presence of 10 % dextran sulphate (Pharmacia), the blots were washed twice in $2 \times$ SSC/0.1 % SDS, at 65 °C for 30 min and then for 15 min in $0.1 \times$ SSC at 65 °C. Quantification was achieved by densitometric scanning (Mark III CS, Joyce, Loeb and Co, Gateshead,

Figure 1 Effect of glucose on luciferase activities in relation to the size of the transfected human SI gene promoter fragments

The stably transformed PF11 cells were cultured in 1 mM (open bars) or 25 mM glucose (hatched bars). The pooled hygromycin B-resistant populations were used and the drug was omitted during the course of each experiment (14 days). Results are presented as means of 3 independent experiments $\pm$ S.D. and expressed as light units/s per μ g of protein per plasmid content.

U.K.) of different film exposures to achieve conditions of linearity.

Luciferase assays

The cell monolayers were rinsed twice with calcium- and magnesium-free PBS and frozen at -20°C . After thawing on ice, the cells were scraped in lysis buffer (15% glycerol/25 mM Tris/8 mM MgCl_2 /1 mM EDTA/1% Triton X100/1 mM dithiothreitol (DTT)/0.4 mM PMSF). The extracts were again frozen at -20°C , thawed and then centrifuged for 15 min at 4°C and 10000 g (Microfuge 2, Beckman). Supernatant (20 μ l) was used for the enzymic determination with a luciferase assay kit (Promega France, Charbonnières, France) in a Lumat LB9501 luminometer (Berthold, Elancourt, France). Protein concentrations were estimated with the bicinchoninic acid (BCA) protein-assay reagent (Pierce, Rockford, IL, U.S.A.). Luciferase activities are expressed as light units/s per mg of protein per plasmid copy.

Preparation of nuclear extracts

All of the nuclear extracts were prepared from confluent cultures, i.e. 16 days after plating, by the method of Shapiro et al. [33]. Standard undifferentiated HT-29 cells and HepG2 cells were maintained in 25 mM glucose medium, PF11 cells were cultured either in 1 or 25 mM glucose medium. All manipulations were performed at $0-4^{\circ}\text{C}$ in the presence of protease inhibitors. Aliquots were frozen and stored in liquid nitrogen for subsequent analysis of DNA-binding proteins.

Electrophoretic mobility-shift assays (EMSAs)

The double-stranded oligonucleotides were labelled with Klenow DNA polymerase and [α - ^{32}P]dCTP (Amersham). Nuclear extracts (5–10 μ g) were first incubated during 15 min at 20°C with 1 μ g of poly(dI-dC) in a buffer containing 15 mM Hepes (pH 7.9), 0.1 mM EGTA, 0.1 mM EDTA, 40 mM KCl, 0.5 mM DTT and 5% glycerol; 0.2 ng of ^{32}P -labelled double-stranded oligodeoxynucleotide was added to the reaction mixture and incubated for additional 15 min at 20°C . For competition experiments a 100 $\times$ molar excess of the unlabelled oligonucleotide was added during the first incubation. For supershift experiments, 0.25–1 μ l of the anti-HNF-1 polyclonal antiserum Ac 284 (a kind gift from M. Yaniv, Institut Pasteur, Paris, France) or 1 μ l of the preimmune rabbit serum was preincubated at 4°C during 4 h with nuclear extracts before addition of the radioactive probe.

Free DNA and DNA-protein complexes were fractionated by electrophoresis through a 5% (19:1, acrylamide/bis-acrylamide) non-denaturing polyacrylamide gel run in $1 \times$ TBE buffer (90 mM Tris/90 mM boric acid/1 mM EDTA) at 10 V/cm for 2–3 h at room temperature. The gels were then dried and subjected to autoradiography.

The following oligonucleotides were used for this study (where PSI is promoter SI):

PSI 1 sense,
5'-ATAGAAAGAACTACTTTTATGTTCTTAA-3'

PSI 1 antisense,
5'-TATCTTAAGAACATAAAAGTAGTTCTTT-3'

PSI 2 sense,
5'-TAAGATATTAATAGTTCACAGCTTTGAGAAAT-3'

PSI 2 antisense,
5'-TTTGATTTCTCAAAGCTGTGAAGTATTAATAT-3'

PSI 3 sense,
5'-TTTGAGAAATCAAAGAGTATCTGACAGTA-3'

PSI 3 antisense,
5'-ATTGTACTGTCAGATACTCTTTGATTCT-3'

SIF3 sense, 5'-CAGTACAATTACTAATTAAGTATAGAT-3'

SIF3 antisense,
5'-GGGAATCTAAGTTAATTAGTAATTGT-3'

Dnase I footprinting

The $-370/+30$ fragment of the human SI gene, cloned in both orientations in the unique *Xba*I site of pTZ18R (Pharmacia), was digested by *Hind*III and *Sac*I, and each strand was end-labelled at the *Hind*III restriction site with [α - ^{32}P]dCTP and Klenow DNA polymerase. The nuclear extracts (15 μ g) were incubated in a buffer containing 15 mM Hepes (pH 7.9), 0.1 mM EDTA, 0.1 mM EGTA, 40 mM KCl, 0.5 mM DTT, 5% glycerol and 100 ng of poly(dI-dC) for 15 min at room temperature. Labelled DNA (60000 c.p.m.) was then added and incubated for 20 min at room temperature. CaCl_2 was added to a 2.5 mM final concentration and then 4 μ g of DNase I/ml (0.5 μ g/ml for naked DNA) were added for 1 min at 20°C . Reactions were stopped with 0.2% (v/v) SDS/15 mM EDTA/450 μ g of proteinase K/ml. Samples were then incubated at 42°C for 30 min. Nucleic acids were extracted with phenol/chloroform (1:1, v/v), precipitated with ethanol and dissolved in loading buffer (99% formamide/

1 μ g/ml xylene cyanol/1 μ g/ml Bromophenol Blue/10 mM EDTA, pH 8). Samples were heat-denatured and loaded on to a 6% polyacrylamide sequencing gel. After electrophoresis at 1200 V for 3 h, the gel was fixed, dried and autoradiographed.

RESULTS

Functional analysis of the glucose-regulated SI gene promoter

In the present study, different subfragments from the $-370/+30$

region were tested in order to identify the *cis*-acting sequence(s) involved in the glucose regulation. As shown in Figure 1, luciferase activities were low in PF11 cells transformed with the $-56/+30$ (beginning at SIF1) or with the $-154/+30$ (beginning at SIFR) construct and similar under the two sets of culture conditions. Sequences located upstream from the SIF1, SIF2 and SIFR elements were thus necessary for glucose repression. The addition of the SIF3 element in the $-180/+30$ construct had two effects: (i) a net increase of the luciferase activities in both

Figure 2 DNA-protein interactions, in the $-370/+30$ region, are modulated by the glucose concentration in the culture medium

(A) Nuclear extracts were purified from PF11 cells maintained in 1 mM (lanes 1 and 6) or in 25 mM glucose (lanes 2 and 7), from HepG2 cells (lanes 3 and 8) and from undifferentiated standard HT-29 cells (lanes 4 and 9); nuclear extracts were omitted in the control reactions loaded in lanes 5 and 10. The footprints were done with the 3'-labelled lower strand in lanes 1–5 and with the 3'-labelled upper strand in lanes 6–10. A precise localization of the protected regions was achieved with a sequencing reaction of the pTZ18R vector, initiated with a 20-mer forward primer, which was loaded on the GATC lanes. The square brackets indicate the regions whose protection varies with the glucose status of the PF11 cells and the black bars cover the already described SIF elements. (B) EMSA experiment showing the differences in the complexes formed between the SIF3 probe and the nuclear extracts (NE) from 25 mM (lanes 1 and 2) or from 1 mM (lanes 3 and 4) PF11 cells. A 100-fold excess of the unlabelled oligonucleotide was preincubated with the extracts in lanes 2 and 4. ○, HNF-1 monomer; ●, vHNF-1 monomer; comp., competitor.

Figure 3 The DNA–protein complexes formed in the $-228/-193$ region differ in the two glucose conditions

The PSI 1 ($-253/-222$), PSI 2 ($-228/-193$) or PSI 3 ($-206/-173$) oligonucleotides were incubated with nuclear extracts (NE) purified from PF11 cells cultured in 1 mM (1) or in 25 mM (25) glucose. The probes were incubated without nuclear proteins in lanes 1, 4 and 7 (–). ○, HNF-1 monomer; ●, vHNF-1 monomer.

glucose conditions; and (ii) the appearance of the glucose effect on the expression of the reporter gene, the luciferase activities being 1.4 times higher in 1 mM than in 25 mM glucose. The repressive effect of glucose was enhanced when the promoter fragment was extended in the 5' direction ($-370/+30$). The luciferase activity was then three times higher in 1 mM than in 25 mM glucose.

Glucose-dependent variations of the footprints within the SI promoter

DNase I footprinting experiments were undertaken to compare the protections obtained with nuclear extracts purified from PF11 cells cultured in 1 or 25 mM glucose. In the fragment extending from -370 to $+30$ (Figure 2A), the four protected elements already described by Traber's group [34,35] were detected in the two culture conditions. As expected, the intestine-specific transcription factor Cdx2/3, which binds to SIF1, was absent in the nuclear extract prepared from the liver-derived HepG2 cells (Figure 2A, lane 8). This site was equally well protected with extracts from the PF11 cells maintained in 1 or 25 mM glucose as well as the SIFR element, which was always protected by the ubiquitous E4BP4 transcription factor. The previously identified HNF-1-binding sites SIF2 and SIF3 were more protected with the nuclear extracts from the control HepG2 cells than from the intestinal cells. The SIF3 site was well protected with the nuclear extracts purified from the undifferentiated HT-29 cells, which do not express the SI gene (Figure 2A, lanes 4 and 9). Two strong bands appearing at the SIF1 upper limit in lane 9 were also present with the PF11 nuclear extracts (lanes 6 and 7) but, immediately above, the three strong ones and the fainter one were absent with HT-29 extracts (lane 9). Different DNA–protein complexes were thus formed at the

Figure 4 Specificity of the DNA–protein complexes detected with the PSI 2 oligonucleotide

The nuclear extracts purified from PF11 cells cultured in 1 mM (lanes 2–5) or in 25 mM (lanes 6–9) glucose medium were incubated with a 100-fold molar excess of the oligonucleotide indicated above the corresponding lanes before adding the radioactive PSI 2 probe. The asterisk on the left indicates the non-specific complex which, as discussed in the text, is never detected under high-glucose conditions. ○, HNF-1 monomer; ●, vHNF-1 monomer.

SIF2/SIF1 junction in these two cell types. Glucose-dependent variations in the protection were observed for SIF2, SIF3 and, more distally from the transcription start, in the two regions indicated by the brackets in Figure 2A (at $-222/-205$ and $-280/-240$). Within these two regions a hypersensitive site appeared with nuclear extracts from the 25 mM glucose-cultured cells but not from the 1 mM glucose-cultured cells. Although this region was not studied here (see below), the strong band at position -260 present in lanes 2 and 7 was also observed with HT-29 cells (Figure 2A, lanes 4 and 9). Different members of the HNF-1 family can, at least *in vitro*, bind to the same sequence. The slight differences observed in the SIF2 and SIF3 regions could thus result from cellular variations in the members of the HNF-1 family between the two culture conditions. This was confirmed by a gel-mobility-shift experiment using a SIF3 oligonucleotide (Figure 2B). The characteristic specific triplet [36] of the HNF-1-bound complexes was observed, but the relative intensity of the intermediate band was reproducibly higher in 25 mM than in 1 mM glucose.

Characterization of the glucose-dependent DNA–protein interactions in the SI promoter

EMSA were used to further characterize nuclear proteins that bind to the $-253/-180$ fragment in PF11 cells cultured in 1 or 25 mM glucose. The $-370/-253$ region has an AT-rich com-

Figure 5 Supershift experiments identify HNF-1 as one of the nuclear proteins bound to the PSI 2 oligonucleotide

In lanes 3–5 and 8–10 increasing amounts (i.e. 0.25, 0.5 or 1 μ l) of the anti-HNF-1 antiserum were preincubated with the nuclear extracts prior to the addition of the PSI 2- (A) or SIF3- (B) labelled oligonucleotide. Lane 1: free probe; lanes 2 and 7, no antiserum; pre (lanes 6 and 11), extracts preincubated with 1 μ l of a non-immune serum. The same amounts of the two probes were used for each experiment but the autoradiography shown on the left of (A) (25 mM) results from a 6 $\times$ longer exposure than the right (1 mM) or than (B). $\circ$, HNF-1 monomer; $\bullet$, vHNF-1 monomer.

position, which caused practical hybridization problems for PCR or for EMSA, and was not studied further.

The –253/–183 region was covered with three partially overlapping double-stranded oligonucleotides, PSI 1 (–253/–222), PSI 2 (–228/–193) and PSI 3 (–206/–173), which were used for mobility-shift assays. Incubation of PSI 1 with nuclear extracts from 1 mM glucose cells (Figure 3, lane 2) gave rise to a major complex C1a, which was also present, but to a lesser extent, with 25 mM glucose extracts (lane 3), and to two less abundant complexes C1b and C1c. All the nuclear preparations that we have tested gave these three DNA–protein complexes. However, with most of them we did not observe any qualitative or quantitative differences in the two culture conditions (results not shown). No differences could be detected in the two DNA–protein complexes C3a and C3b formed with PSI 3 and extracts from PF11 cells maintained under the two sets of culture conditions (Figure 3, lanes 8 and 9).

A more interesting situation emerged with the use of the PSI 2 oligonucleotide. A band, here at the level of C1b, was present under the two sets of glucose conditions (Figure 3, lanes 5 and 6). Other very faint bands were also observed but their intensities were not reproducibly correlated with the carbohydrate status of the culture. A group of three slowly migrating complexes was formed with this same probe. The relative positions and the mobilities of the two upper bands (referred to as C2) were strikingly similar to those observed with the SIF3 probe (for

example, see Figure 2B). A comparison of lanes 5 and 6 revealed that the same complexes were formed in the two conditions but that the lower band in C2 was reproducibly more intense with nuclear extracts from cells cultured in 25 mM glucose. It must be noticed that the C2 complex contains three bands, like those observed with SIF3, but that the lower band (indicated by two black dots in Figure 2B) is usually very faint and not systematically detected (compare Figures 3, 4 and 5).

The specificity of the formation of these complexes was tested in competition experiments using PSI 2 as a probe and either the homologous PSI 2, or the heterologous PSI 1 or PSI 3 as unlabelled competitors (Figure 4). Only the more slowly migrating complexes were suppressed with the homologous oligonucleotide (Figure 4, lanes 2 and 3) and were thus specific. The band indicated by the asterisk in Figure 4 was obtained only with 1 mM extracts (lanes 2–5) but, unfortunately, it was not constantly observed (see Figure 5B) and was not eliminated with any competitor.

HNF-1 binds to the PSI 2 oligonucleotide *in vitro*

The three upper bands formed with PSI 2 were reminiscent of those obtained with the SIF3 oligonucleotide (Figure 2B) or with an HNF-1-specific oligonucleotide (results not shown). An analysis of the sequence revealed a strong similarity, 10 nucleotides identical out of 13, with the HNF-1-consensus-binding site

Table 1 *In vitro* mutagenesis of the three HNF-1-binding sites differently affects the activity of the SI gene promoter in cells cultured in 1 or 25 mM glucose

The luciferase activities were measured in the stably transformed populations cultured in 1 or 25 mM glucose (see legend of Figure 1). Each number expresses the ratio of the normalized luciferase activity measured in 1 mM glucose versus that measured in 25 mM glucose.

Construct	SIF 2 ⁺ 3 ⁺ 4 ⁺	SIF 2 ⁻ 3 ⁺ 4 ⁺	SIF 2 ⁺ 3 ⁻ 4 ⁺	SIF 2 ⁺ 3 ⁺ 4 ⁻	SIF 2 ⁻ 3 ⁻ 4 ⁺	SIF 2 ⁻ 3 ⁺ 4 ⁻	SIF 2 ⁺ 3 ⁻ 4 ⁻	SIF 2 ⁻ 3 ⁻ 4 ⁻
Luciferase 1 mM/25 mM	2.8	2.1	0.8	1.4	0.8	1.2	0.8	1.2

[37]. Supershift experiments were undertaken to determine if an HNF-1 peptide was present within one of the three upper complexes formed with PSI 2. Aliquots of the two nuclear extracts were pre-incubated with increasing amounts of a rabbit polyclonal anti-HNF-1 antibody or with non-immune serum as a control. For comparison the gel-mobility-shift assays were done with the PSI 2 or with the SIF3 oligonucleotide (Figures 5A and 5B respectively). Since the experimental conditions were identical in the two series, it was clear that the amount of complexed probe was higher with SIF3 than with PSI 2. The greater affinity of the nuclear factor(s) for the SIF3 element was confirmed by reciprocal competition experiments (results not shown). Within C2, the more slowly migrating band was the first to disappear with the Ac 284 antiserum and the other one was only supershifted with a higher amount of the antibody (see Figures 5A and 5B, lanes 3–5 for 1 mM glucose and lanes 8–10 for 25 mM). The non-immune serum did not alter the intensities or the positions of the bands corresponding to the different DNA–protein complexes (Figures 5A and 5B, lanes 6 and 11).

The two distal HNF-1-binding sites are necessary for glucose regulation

Individual or combined mutations were introduced in the three HNF-1-binding sites and the modified promoters were tested in the PF11 cells. The stably transformed populations were cultured in low- or high-glucose conditions to estimate the contribution of each site to the SI gene regulation. The results of these experiments, shown in Table 1 as the ratio between the luciferase activities measured under the two sets of conditions, revealed that SIF3 and SIF4 are more important for glucose-dependent regulation. Altering these sites completely abolished or strongly diminished the response of the promoter to glucose. Adding a mutation in the SIF2 site to these constructions had no effect and the SIF2 single mutant was only marginally affected in the glucose repression (a ratio of 2.1 compared with 2.8 for the wild-type).

Effect of vHNF-1C on the SI gene expression

Of the the six HNF-1 and vHNF-1 isoforms described, vHNF-1C alone behaved as a transcriptional inhibitor [29]. This isoform is thus a candidate for a transcriptional repression of the SI gene under the high-glucose culture conditions. The possible role of vHNF-1C was tested by transfection of the PF11 cells with a pRSV-vHNF-1C construction. In 25 mM glucose the level of SI mRNA was not affected by the overexpression of vHNF-1C (Figure 6, compare lanes 1 and 3). The situation was strikingly different in 1 mM glucose where the level of SI mRNA was dramatically decreased (Figure 6, lanes 2 and 4). The overexpression of vHNF-1C abolished the glucose regulation of the SI gene (Figure 6, compare lanes 3 and 4 with lanes 1 and 2).

Figure 6 PF11 cells were transiently transfected on day 3 with pRSV/vHNF-1C (lanes 3 and 4) or with the empty vector, pRSV, as a control (lanes 1 and 2)

Total RNA was isolated at day 14 from cells cultivated in 1 mM (lanes 2 and 4) or 25 mM glucose (lanes 1 and 3). The membrane was hybridized with an SI- or vHNF-1C-specific probe (upper panels) and then stained with Methylene Blue (lower panel).

DISCUSSION

The intestine-specific transcription of the SI gene is controlled *in vitro* by a 183 bp region located immediately upstream from the transcriptional start site. Within this fragment four interacting sites for nuclear-binding proteins have previously been identified and were named SIF1–3 [34] and SIFR [35]. SIFR binds a protein related to the transcriptional repressor E4BP4 but its functional characteristics in intestine are still undetermined. The SIF1 element binds Cdx-2, a caudal-related protein that positively controls the intestine-specific expression of SI [38], whereas SIF2 and SIF3 bind homoeoproteins of the HNF-1 family [39]. Besides these studies on the tissue-specific expression of the gene, the molecular mechanisms that govern the repressive effect of glucose on the transcription of the human SI gene have not yet been deciphered. We have previously shown that, in Caco-2 cells, the –370/+30 region of the SI gene promoter was sufficient to confer this glucose effect on a heterologous gene [22]. It was therefore important to test whether the SIF elements characterized by Traber's group were involved in the glucose regulation.

Stable transfection experiments indicate that the addition of SIF3 upstream from the –154/+30 fragment enhances the luciferase activity of both 1 and 25 mM glucose-cultured cells.

We also show here that SIF3 is a necessary component of the glucose regulation of the SI gene. As mentioned above, SIF3 binds the homeoproteins of the HNF-1 family [also called HNF-1 α or liver factor (LF)-B1 encoded by the gene transcription factor 1, TCF1] and the related vHNF-1 (HNF-1 β or LF-B3 encoded by the gene TCF2) (for review, see [40]). HNF-1 and vHNF-1 proteins bind as homo- or heterodimers to the same motif and are co-expressed at different levels not only in liver but also in intestine, kidney, pancreas and thymus. In all these organs, HNF-1 and vHNF-1 expression is limited to specialized epithelial cells. Gel-mobility-shift assays using the SIF3 element and nuclear extracts from the PF11 clone of Caco-2 cells cultured in the two glucose conditions gave the characteristic triplet of HNF-1/DNA complexes. Each band of the triplet was previously identified as one of the three combinations of the HNF-1 and vHNF-1 dimers [36], the upper band corresponding to the HNF-1 homodimer, the intermediate band to the HNF-1/vHNF-1 heterodimer and the lower band to the vHNF-1 homodimer. In supershift experiments (Figure 5) anti-HNF-1 antiserum shifts, at low concentration, the upper band of the triplet, then the intermediate one and has no effect on the homodimeric vHNF-1 complex. Interestingly, the relative proportions of each dimeric species vary with the glucose concentration in the culture medium. More particularly, that of the HNF-1/vHNF-1 heterodimer is always greater with nuclear extracts from PF11 cells maintained in 25 mM glucose than from PF11 cells cultured in 1 mM glucose (as illustrated in Figure 5). From these data, we conclude that, in addition to its positive role on the SI gene transcription, the SIF3 element participates through differential binding of the HNF-1 proteins in the glucose-dependent regulation of SI in Caco-2 cells. However, the existence of additional regulatory elements, that may co-operate with SIF3 to mediate the glucose effect on the SI gene transcription, was strongly suggested by the transfection experiments showing a greater difference of luciferase expression between the two glucose conditions with the $-370/+30$ construct when compared with the $-180/+30$ fragment (containing SIF1, SIF2, SIFR and SIF3).

The footprinting analysis of the $-370/+30$ fragment of the SI promoter using nuclear extracts from PF11 maintained in 1 or 25 mM glucose shows protected sites that may be implicated in the glucose regulation. In addition to SIF3, two protected regions located to $-222/-205$ and $-280/-240$ were differently protected in the two glucose conditions. Given that the PF11 cells cultured in 25 mM glucose express the SI gene, albeit at a low level, the transcriptional factors required for the SI expression are thus present in both culture conditions. It is therefore not surprising that the differences observed in the footprints were quantitative rather than qualitative. The analysis of the $-253/-183$ region by EMSA shows that proteins of the HNF-1 family bind to $-222/-209$, which constitutes the newly characterized SIF4 site. The comparison of EMSA using the PSI 2 fragment ($-228/-193$) with those using SIF3 clearly indicates that it binds HNF-1 and vHNF-1 dimers but with a lower affinity than SIF3. The SIF4 site is, however, functional in PF11 cells and its suppression, by *in vitro* mutagenesis, alters the glucose regulation to a similar extent as that of SIF3, whereas that of SIF2 has only a marginal effect (Table 1).

As observed for SIF3, the heterodimeric HNF-1/vHNF-1 complex formed on PSI 2 is relatively more abundant in 25 mM than in 1 mM glucose-cultured cells. This raises the possibility that this site may, together with SIF3, be involved in the glucose regulation of the SI gene through the differential binding of the HNF-1 proteins. In the Caco-2 cell line, a functional study of the HNF-1 family demonstrated that HNF-1 isoforms could not increase the transcription from a minimal intestine-specific SI

gene promoter [35]. It has previously been shown that there are three isoforms of HNF-1 (A, B and C), and three isoforms of vHNF-1 (A, B and C) [29]. The vHNF-1C isoform lacks the C-terminal activation domain and behaves as a transdominant repressor when co-expressed with HNF-1. The proportion of the different HNF-1 or vHNF-1 mRNAs have been estimated by RT-PCR in several fetal or adult human tissues and in human cell lines [29]. In all cases, the HNF-1A and the vHNF-1A/B isoforms were the predominant species and among intestinal cells the higher levels for vHNF-1C mRNA were found in fetal intestine and in the Caco-2 cell line. From these data, we propose that in Caco-2 cells the glucose regulation of SI might be related to the relative amounts of the vHNF-1C/HNF-1A and HNF-1A/HNF-1A dimers. The results of EMSAs are consistent with this proposition in so far as the intensity of the band that corresponds to vHNF-1/HNF-1 heterodimer is always stronger in the high glucose (25 mM glucose) than in the low glucose (1 mM glucose) culture condition either with PSI 2 or with SIF3. vHNF-1C might therefore inhibit the transactivator effect of HNF-1A by heterodimerization with HNF-1A in high-glucose conditions. This hypothesis is supported by transient co-transfection experiments in COS7 cells where we have observed that vHNF-1C inhibits the transactivating effect of HNF-1A on the SI gene promoter (results not shown). Also in support of such a mechanism, we have shown that in 1 mM glucose the transient overexpression of vHNF-1C is sufficient to lower the level of the SI mRNA to that observed in repressing conditions (Figure 6).

Many genes that participate in the carbohydrate metabolism are positively or negatively regulated by glucose and/or insulin. Among them, the L-pyruvate kinase gene has been particularly well studied and its promoter has been shown to contain a glucose response element constituted by two degenerate E-boxes (CANNTG) that bind proteins related to upstream stimulatory factor [41]. We did not detect any canonical glucose response element in the proximal upstream region of the SI gene. An HNF-1 binding site has nevertheless been reported in the L-pyruvate kinase promoter [42], and upstream that of the glucose transporter 2 [43]; it is noticeable that these two genes are regulated in the same way as SI in PF11 cells cultured in 1 or 25 mM glucose [44].

Several studies reported a major role of HNF-1 in the regulation of genes involved in carbohydrate metabolism and expressed in kidney. In a proximal tubule cell line, PKSV-PCT, HNF-1 α (HNF-1) has been reported to behave as a transactivator of the 190 bp promoter of the aldolase B gene, whereas HNF-1 β (vHNF-1) antagonized this action [45]. The HNF-1-null mice have a massive urinary loss of glucose caused by a proximal-tubular dysfunction which suggests a critical role for HNF-1 in the renal expression of the glucose transporters [46]. Furthermore, in mouse kidneys the phosphoenolpyruvate carboxykinase gene, which is normally repressed by glucose, is no longer responsive to a carbohydrate diet when its HNF-1-binding site is mutated [47].

We have shown in this study that the HNF-1 proteins are essential for the glucose regulation of the SI gene. As glucose does not affect the vHNF-1C mRNA level (results not shown) it remains to be determined how it influences the association of this protein with the other members of the family to modulate the expression of the SI gene.

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