The human hexokinase II gene promoter: functional characterization and detection of variants among patients with NIDDM

M. Malkki^{1,2} · M. Laakso² · S.S. Deeb¹

¹ Departments of Medicine and Genetics, University of Washington, Seattle, Washington, USA

² Department of Medicine, Kuopio University Hospital, Kuopio, Finland

Summary Hexokinase II (HKII) plays an important role in facilitating glucose uptake by skeletal muscle, heart, and adipose tissue in response to insulin. We have cloned and sequenced the proximal promoter region of the human HKII gene, determined the transcription start sites and screened the 2.0 kb of the proximal 5' flanking region for variants in non-insulin-dependent diabetic patients and control subjects. We found three variants in this region, one in the 5' untranslated region (G→C at +217) and two in the promoter region (T→G at -1043 and G→A at -1159). The allele frequencies of these variants did not differ between the diabetic and control subjects and these variants are not associated with insulin resistance. Various segments of the human HKII promoter were tested for driving expression of the luciferase reporter gene. The proximal 500 bp and 400

bp of the promoter were sufficient to drive maximal activity in adipocyte (3T3F442A) and myocyte (C2C12F3) cell lines, respectively. This region of the promoter is GC-rich and contains eight consensus binding sites for the transcription factor Sp-1, five for AP-2, two putative response elements for each of insulin and cyclic AMP. The proximal 175 bases of the promoter retained only 7–15 % of maximal activity. Sequence elements located between positions -304 and -215 accounted for approximately 80 % of the basal HKII promoter. In addition, the region between -215 and -184 contains a negative regulatory element for expression in 3T3F442A but not in C2C12F3 cells. [Diabetologia (1997) 40: 1461–1469]

Keywords Hexokinase II, promoter, NIDDM, insulin resistance, diabetes mellitus.

The isoenzymes of hexokinase (I–IV) (ATP: D-hexose 6-phosphotransferase EC 2.7.1.1) (reviewed in reference [1]) catalyse the first step in intracellular glucose metabolism, the phosphorylation of glucose to glucose 6-phosphate. HK isozymes I–III have high affinity for glucose and are feedback inhibited by glucose 6-phosphate, whereas HK IV (glucokinase) is

not. The glucose phosphorylation step may be rate limiting and, therefore, hexokinases may play a pivotal role in the regulation of glycolysis.

Hexokinase II (HKII) is primarily expressed in insulin-sensitive tissues such as skeletal and cardiac muscle and adipose [2–5]. The cDNA sequence and the structure of the rat [5, 6] and human [7–9] HKII genes have been determined. The human HKII gene spans at least 52 kb and is comprised of 18 exons which encode a protein of 917 amino acids. The human gene has been mapped to 2p13.1 [10].

Insulin was shown to increase HKII mRNA levels by 2 to 3-fold and by 6-fold in differentiated skeletal muscle (C2C12, L6) and adipocyte (3T3F442A, BFC-1B) cell lines [5], respectively. This increase was shown to result from transcriptional activation of the gene in L6 cells. In addition, catecholamines and cyclic AMP were shown to induce expression of

Received: 8 May 1997 and in revised form: 22 July 1997

Corresponding author: Dr. S. Deeb, Genetics Department Box 357360, University of Washington, Seattle WA 98195, USA

Abbreviations: HKII, Hexokinase II; NIDDM, non-insulin-dependent diabetes mellitus; kb, kilobases; SSCP, single strand conformation polymorphism; IRE, insulin responsive element; CRE, cyclic AMP responsive element; PEPCK, phosphoenolpyruvate carboxykinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Fig. 1. Partial restriction map of HKII promoter indicating 5' ends of nested deletions. Shown are genomic clone HKII-L19 and the 2.2 kb *Sst*I subclone derived from this clone (asterisks). Nested deletions have the same 3' end at +106 and various 5' ends (vertical lines). The location of PCR primers indicated by horizontal arrows. Sequence variants indicated by vertical arrows. Open and solid rectangles represent untranslated region of exon 1 and coding region of HKII, respectively. Transcription start site is indicated with +1

the HKII gene in L6 and BCF-1B cells which led to an increase in glucose phosphorylation [11]. Hyperinsulinaemia was observed to be associated with elevated HKII mRNA levels in rat skeletal muscle and adipose tissue [12] and in human skeletal muscle [13]. On the other hand, hyperglycaemia was not found to influence HKII expression in these tissues in the rat [12].

In the KKA^Y mouse, an animal model of non-insulin-dependent diabetes mellitus (NIDDM), HKII mRNA and protein levels are significantly reduced in adipose tissue and skeletal muscle [14]. Furthermore, reductions in basal levels of glucose 6-phosphate [15], in insulin-stimulated glucose uptake (reviewed in reference [16]), and in the levels of HKII mRNA and enzyme production were observed [17] in skeletal muscle of NIDDM patients, suggesting a defect in glucose phosphorylation.

The human HKII gene has been screened for mutations in the coding region, and a total of 7 amino acid substitutions were found [18–21]. Although some of these substitutions may be associated with risk for NIDDM or insulin resistance, they are too infrequent to account for potentially more than 1–2 % of NIDDM cases. The regulatory sequences of the human HKII gene have not been screened. Genetic variability in response of the HKII gene to insulin may underlie insulin resistance in skeletal muscle and adipose tissue. Therefore, we screened the proximal promoter of the human HKII gene for variants that may potentially play an important role in its expression in these tissues and in response to various regulatory agents.

The activity of the proximal rat tumour-type HKII promoter has been studied by transient transfection experiments in rat hepatocytes and hepatoma cells [22, 23]. The HKII gene is not normally expressed in the liver, however, it is expressed at high levels in a variety of rapidly growing tumour cells, including hepatoma cells, which have a high glucose catabolic

rate [24]. In attempts to define the molecular basis of this marked elevation in transcription of this gene in hepatoma cells, Ichihara et al. [22] performed transient transfection of hepatoma cells with plasmids containing various lengths (0.1, 0.6, and 1.3 kb) of the rat HKII promoter. They found that the plasmids containing the 1.3 and 0.6 kb had similar activities, whereas the 0.1 kb construct was almost completely inactive, indicating the presence of important cis-regulatory elements within the proximal 600 bp of the transcription start site. In a second study, Mathupala et al. [23] transfected hepatoma cells with a plasmid containing 4.3 kb of the rat proximal HKII promoter (tumour-type) and showed that promoter activity was enhanced upon treatment with glucose, insulin, glucagon, phorbol 12-myristate 13-acetate (PMA) and cyclic AMP. Incubation in the presence of insulin and glucose together resulted in maximal activation. However, these compounds had no effect on transcription of the endogenous HKII gene in primary hepatocytes and did not activate the 4.3 kb proximal promoter upon transient transfection of primary hepatocytes.

We have initiated characterization of the activity of the human HKII promoter in cell types that express the endogenous gene, namely skeletal muscle and adipose tissue. We have cloned and sequenced the human proximal promoter, determined the transcription start sites of the gene, screened for variants in NIDDM patients and control subjects, and performed analysis of promoter activity in skeletal muscle and adipocyte cell lines. We have identified certain regions of the proximal promoter that play major roles in its activity in these two types of cells.

Subjects, materials and methods

Isolation, sequencing and characterization of genomic clones that span the human HKII promoter. Isolation of human HKII genomic clones that contain exon 1 has been described [8]. Six of these genomic clones which hybridized to a 112 bp cDNA probe (–52 to +60 with respect to the translation start site) were tested for the presence of promoter sequences. Exon 1 contains a unique *Sst*I site (Fig. 1), therefore, these clones were digested with *Sst*I and subjected to Southern blot analysis using the same probe. Two of these clones (HKII-L19 and L-29) were chosen because they yielded the longest *Sst*I fragments (Fig. 1). The hybridizing *Sst*I fragments (0.9 kb from clone HKII-L29 and 2.2 kb from clone HKII-L19) were subcloned into the vector pGEM-3Zi(+) (Promega, Madison, Wis., USA) and sequenced (Sequenase kit; US Biochemicals, Cleveland, Ohio, USA). The 0.9 kb *Sst*I subclone extended

Table 1. Sequence and location of primers used for PCR amplification

Primer	Sequence (5'-3')	Position
HKII 1.1	CCGAGGCGGAGACGCGAGAG	+416/+397 ^a
HKII 1.10	CCAGCCTTTAGCCACGGAC	-254/-236
HKII 1.37	GGCTACCGGCGAGGGTGC	+310/+297
HKII 1.38	CTGTGAATCGGAGAGGTC	+92/+109
HKII 1.28	GGCCTTGCCACAATTTCTCT	-606/-587 ^b
HKII 1.33	GTCCGTGGCTAAAGGCTGG	-236/-254
HKII 1.22	CCTTCATCTTCCTTCCCTG	-1325/-1307 ^c
HKII 1.15	GAGGAAATTGTGGCAAGGCC	-587/-606
HKII 1.34	TTTGGAGTCTGGTGTCTACC	-1194/-1175
HKII 1.35	TGCTGCCTCTCTAAGAGCC	-990/-1008
HKII 1.6	CCCACCCATAGCCGAGCCT	-304/-286
HKII 1.11	GGGCAGTGGGACCTCTCCGATTCA	+118/+95
HKII 1.23	AGATTTCAAGAGACATGAC	-1244/-1262
LUC MC5'	TCTGGATCCAAGCTCAGATC	
LUC MC3'	TACCAACAGTACCGGAATGCC	

^a PCR product generated from this pair of primers was digested with Eco019I and BglI to generate fragments of 148, 212, 182 and 128 bp prior to SSCP analysis;

^b PCR product amplified from this pair of primers was digested with BanI to generate fragments of 192 and 179 bp before SSCP analysis;

^c PCR product generated from this pair of primers was digested with PstI to generate fragments of 286, 220, and 233 bp prior to SSCP analysis. Primers labelled LUC MC5' and 3' correspond to sequences of the multiple cloning site of the luciferase plasmid vector

from the multiple cloning site of the vector λFIX (Stratagene, La Jolla, Calif., USA) to the *Sst* I site in exon 1.

In order to determine whether genomic clone HKII-L19 had additional sequences 5' upstream of the distal *Sst* I site (Fig. 1), this clone was used as template for PCR amplification using vector primer T7 and HKII primer 1.23 (Table 1, Fig. 1) using the Expand Long Template PCR System (Boehringer Mannheim, Indianapolis, Ind., USA) according to the manufacturer's protocol. An amplification product of approximately 2.7 kb was obtained.

Determination of the transcription initiation site. The transcription initiation site of the human HKII gene was determined by primer extension analysis [25]. Total cellular RNA from human skeletal muscle and adipose tissue samples was isolated using the guanidinium isothiocyanate-phenol-chloroform method [26]. The antisense oligonucleotide primer 5'GCGGATTTTCTTAGCTGGGTG-3', position -376 to -396 with respect to the translation start site (underlined in Fig. 2) was used in primer extension analysis as previously

Fig. 2. Sequence of 5'-flanking region of human HKII gene. Proximal 540 bp of the rat HKII promoter aligned with human promoter sequence (conserved nucleotides marked with vertical lines). The transcription initiation site marked with + 1. Putative TATA-like box and CAAT box are in bold; the two *Alu* repeats in lower case. Putative transcription factor binding sites (underlined) were identified by comparison with sequences in the "Transcription Element Search Database" (<http://agave.humgen.upenn.edu/cgi-bin/tess>). The oligonucleotide primer used for primer extension analysis is double underlined

- 2040 Human	GCATGCATAT GATATAACAG TCATGGGCTA GAAGTTAGTT TCTCTCAGG GAGTTTGAAA
- 1980	CTGTAAC TAG TTCAGAGAAG AGGGTGGAGG GCAGCCCCGA TACCCACGCA TTTACCAATA
- 1920	GAGCAACAG GGA CT CAGGA GCCTGGGGAG TGAGGTTAGC CGGAACCCCT CAGAGTGGAG
- 1860	CAC TGTGTCT CTTACTGAGA GAGGAAGGTG TGTCCAGATG <u>GAGGATGTGA</u> TTAAGTGTCC
- 1800	TCAACATCCC TGAGAGAAGG AGTAAGACAA GGGCAGGGGA <u>GAGAGAGGAA</u> TGCAAGATTT
- 1740	GGCAACATGT <u>AGGTCATTAT</u> GATGACTATG ACAAAAGCAG TTTGAGCTCA ATTCTGTGTG
- 1680	GAGTATAGGG AAGGAGGGTT GAGGACGTGC ATTTAGAAGG GTACATAGTT CTCAAGAAGT
- 1620	TTTGTGAGC <u>acatctgttaa tcccagctat ttgggacgct gaagtgggag gactgcttga</u>
- 1560	<u>gccaggaggt tcaagaccag cctggggcaac atatcgagtc cctgctttaa aaaaaaaaaa</u>
- 1500	<u>agGAAGTTT GCTGAGAGGC TAGATGGATT ATGATTTTGT TTTATTTTTC CTGTTTATCC</u>
- 1440	<u>ATATATATT TTTCAACAAT GAGTATTGAT TACTTATATA ATATTTTAA GGCTGTACAC</u>
- 1380	ATTGCGAGCA GCACCCCACT GTTTGAAAA CTCTTCCTCA <u>GTGAACAATG</u> GCAGACCTTC
- 1320	ATCTTCCTTC CTTGAACCTT TTCCAACCTT AGGCTTGCCA TTCTCCACCA GTGCTAATGT
- 1260	CATGTCTCTT GAAATCTGTA TTGAAGTCAG TATTTTCATT TTGCCAGTTT CCACCTGTGTG
- 1200	TTTAAATTTG GAGTCTGTGT TCTAGCATTG GCTGGGGTTG GGGCTTCCAC TCCTCTCAGC
- 1140	ATTGGTAAGC CTCTCATCCC <u>ACCCATCCCC</u> ATGTCCAAGA TCACCCAGTT ACACACTTAC
- 1080	CATCTACCCA GTTCATTAC ATCATCAGTC CCAGAGCTGC AGAGATGCTC TTTTCTTACC
- 1020	TCCTACTTCT CTGGCTCTTA GAGAGGCAGC ATGGGATAAT <u>GGGCAAGCG</u> AATAGGGCCT
- 960	TAAAGTAGAG GGACAAGGGT TCTCTTCCCT ATCTGCCACT TATTAGCTAT <u>GTGACCTCGT</u>
- 900	<u>GTAAGTctct tttctttttg agacagggtc tccctctgtc acctaggctg gactacagtg</u>
- 840	<u>ctatgctcat agctcactgc agcctctgaac tccctgggtc aagctatcct tccacottag</u>
- 780	<u>cctctgagc agcagggact acagggcacat gccacattgt ccgctgatt tatttatatt</u>
- 720	<u>tatttgggaa gatgggggtc tcaactatgc gccaggctg gtcattgaact cctggtctca</u>
- 660	<u>agcaaccctc caacottgga ctcccaagt gctgggatta caggctatgag ccCTGCCTT</u>
- 600	<u>c/cbbp GGCACAAATT CCTCATCTGT AAAACGGGGT TAGTGAACAT CACATCTTAT CAGTGTGTTT</u>
- 540 Human	GAGGATGGGC CGACTCTTGT ATTGCCTGCT CTAGTACAAT CAGCAGCTAA GCGCGCTCAC
- 541 Rat	ATAGGTGTGA CGATTACGAA GGGCTGACTT TTGTAATGCG TTTGCTTCAG GGATCTGCAG
- 480 Human	TTTCCGGCCG TGCTACAATA GGTAAGAACT AGGATGCTTT AGACGTGTGA <u>CTGGGCACTG</u>
- 481 Rat	ACTCGTTGAG CCACAATTAG GATGAGAACT AAGGTGCTTC AGACTGTGTA CAGGGCACTG
- 420 Human	GGAGCCCCCTC ACATGATCCC GAGATGCCAG ACAGTGTCTC TCCGCACAGG <u>CGCTGTGCTG</u>
- 421 Rat	GCGGCCCTTC ACATGATCCT CAGATACCAG ATTGTG GCGTGTGCTG
- 360 Human	GTCCAGAGGC CCGTTTCTTC <u>AGTCGCCCA</u> CACCCCGGG TCCGCGATCAC <u>GCTCCCCCA</u>
- 375 Rat	CTAGGATCAC TTGTCTTTC AGTCTCCCAACATCTCTTGGGTCGTGATCAC GCGCCCCCA
- 300 Human	<u>CCCATAGCCG AGCCTGACGC GCGGTGGCT</u> CATGCGCCTT TCCGTCCAG CTTTATAGCA
- 313 Rat	CCCAAGGCC AGCCTGACGC GCGGTGGCT CATGCGCCTT GAGTCCCGG GCTCTAGCA
- 240 Human	CGGACCACAC GTCCCATCTC AGGCGCCCG <u>CCCTTCCCG</u> CCGGCGCGC
- 253 Rat	CGGAACACAC GTCCCAACTC TGGCGCCCGG CTCC GCCCTAGCG TCGGCGCGT
- 180 Human	<u>CTCCCCAGGC</u> TGCCGGCTCC GGTGTCTGAG CGGCGCGCGC CGCGAGCCGT GAGCGATGAT
- 199 Rat	CTCTCCCGCC GCTGCTTG GGTGTGAG CAGCGCGGCC CGCGGCTCT GGGCGTGT
- 120 Human	<u>TGGCTGCGCC</u> ACGGCGCGCG GCGTCCCTG GCGGCACACA <u>CCCTCCCGC</u> GAGCCAAAT
- 140 Rat	TGGCTGTGGA CTGCGGCGCG GCAGCCGGAG AGCGCACACA CCCTCTTCCC GCAGCCAAAT
- 60 Human	<u>GGCGTGCACA</u> <u>CGTCACTGAT</u> CCGGAGCCCG <u>CGGCGCGCA</u> <u>GCCCTCAAT</u> <u>AAGCCCAAT</u>
- 80 Rat	AGCGCGCCCA CGTCACTGTCTTGGGCGGCCCA ₃ GAGCCGCA GCCCTCAAT AAGCCCAAT
+ 1 Human	transcription TGTGCATGAA ACTCCGGCGC AGGAGTCCCG GGCTGCCGCT GGCAACATCG TGTCAACCG
+ 17 Rat	GTTGCACC A ACTCCAGTGC TAGAGTCTCA GAGACACACA GGCTACACGG AGTATATCCG
+ 61 Human	<u>CTAAGAAAT</u> CCGCGGGGCC GAGGCACGCG CTTGTGAATC GGAGAGTCC CACTGCCCGA
+ 121	GTGGAGCCGG GCTGAGATTC TTCTCAAGTT GAGCTCAGT GATCCTGTGG CCAAGTTAG
+ 181	CGCCTTGAGC TGGGACAACC GGACAGAGCG CCAGGAGAGA ACTGAGGCGC CTTCTAGCAG
+ 241	TTGTGAGGCC AAAATCAGCT CTCGCGAGAG CTCGCGCCCT CCGCAGCGCG CGCACCCCTC
+ 301	GCGGTGAGCC TTCTTTGTGC GCGGTGCGGA CTCGAGCTCT CCGGCGCGCG AGGCCAGGCC
+ 361	CAGCACAAG CAGTGGAGCC GCGCGCGCGC CTCTCCCTCT CGCGTCTCCG CTTGCTTCTC
+ 421 Human	transcription CCAACTCTGC GCGTGGGGC CGCGGCAGGA <u>TGATGTCCTC</u> GCATCTGCTT GCCTACTTCT

described [5]. The procedure was modified as follows: 25 µg of RNA was annealed at 62°C for 16 h, extension was done at 42°C for 1 h using 400 U of SuperScriptII reverse transcriptase (BRL, Grand Island, N.Y., USA) and the reaction products were electrophoresed on an 8 % polyacrylamide-7 Murea-sequencing gel. A sequencing ladder was generated using the 0.9 kb *Sst* I fragment derived from genomic clone L29 as template and the same labelled oligonucleotide as primer.

Screening for variants and association of variants with insulin sensitivity. The screening for variants in the promoter region and in the 5' untranslated region of the HKII gene was performed by single strand conformation polymorphism (SSCP) analysis and sequencing of variants as previously described [20]. The primers and restriction enzymes used in SSCP analysis are shown in Table 1.

The association of variants with insulin sensitivity was determined in 112 patients with NIDDM (56 men, 56 women, age 63 ± 1 years, body mass index 30 ± 1 kg/m², duration of diabetes 8 ± 1 years, 49 % treated with diet, 29 % with oral drugs and 22 % with insulin) and in 97 control subjects with normal glucose tolerance (84 men, 13 women, age 55 ± 1 years, body mass index 27 ± 0 kg/m²). Insulin sensitivity was determined by the euglycaemic hyperinsulinaemic clamp technique (insulin infusion of $80 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ($480 \text{ pmol} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) in control subjects and $120 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ($720 \text{ pmol} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) in patients with NIDDM) as previously described in detail [27].

Preparation of nested deletions of the HKII promoter. Starting with the 0.9 and 2.2 kb *Sst* I-subclones of genomic clones L29 and L19, respectively, nested deletions of the HKII promoter were generated either by cutting with the restriction enzymes shown in Figure 1 (with the 3' end of all segments generated by cleavage with *Eco*0109I) or by PCR amplification of the desired segment. The sequence of oligonucleotide primers used for PCR amplification is given in Table 1. Digested samples were blunt-ended and purified by electrophoresis on a 1 % low-melting-point agarose gel followed by Qiaex gel extraction purification (Qiagen Inc., Chatsworth, Calif., USA) and cloned into the *Sma*I site of the luciferase reporter vector PXP1 (ATCC #37576, [28,29]).

The -4.0 kb HKII construct was prepared in two steps. First, the 2.3 kb *Sal* I/(blunt-ended)/*Sst* I- fragment from genomic clone L19 was cloned into the *Bam*HI (blunt-ended)-*Sst* I site of PXP1 vector. This subclone was designated luc -4.0/-1.7. Next, a 1.8 kb *Sst* I/*Eco*0109I fragment (Fig. 1, -1692 to +106) was isolated, the *Eco*O109I-end bluntended and cloned into the *Sal* I/(blunt-ended)-*Sst* I site of subclone luc -4.0/-1.7 resulting in the -4.0 kb HKII construct.

The sequence of all nested deletions was determined using vector primers LUC-MC5' and LUC-MC3' (Table 1) and several HKII promoter primers. The 3' ends of the nested deletion clones extended to nucleotide +106. Plasmid DNA for transfection experiments was prepared using the Qiagen plasmid kit (Qiagen Inc) according to the manufacturer's protocol.

Internal deletions of various lengths of the GC-rich region located between positions -215 and -180 were frequently observed when we attempted to amplify, using genomic clones as templates, segments encompassing this region. One of these deletions (between -215 and -184) was also used in transfection assays.

Cell culture and transient transfection. The mouse myoblast cell line C2C12F3 [30], kindly provided by Dr. H. Weintraub's laboratory (Fred Hutchinson Cancer Research Center, Seattle, Wash., USA), was maintained in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 2 mmol/l L-glutamine and 20 % fetal bovine serum in a 5 % CO₂/air environment at 37°C. Cells were induced to differentiate into myotubes in DMEM medium supplemented with 2 % horse serum. Approximately 90 % of cells were differentiated into multinucleate myotubes after 3 days of culture in differentiation medium.

The mouse preadipocyte cell line 3T3F442A [31], kindly provided by Dr. M. Reina (University of Barcelona, Spain), was grown in DMEM supplemented with 2 mmol/l L-glutamine, 8 mg/l biotin, 15 mmol/l Hepes and 10 % fetal bovine serum. Cells were differentiated into adipocytes in growth medium further supplemented with 2 nmol/l T₃ and 17 nmol/l bovine insulin. Differentiation was followed by staining lipid droplets with Red Oil O.

Both cell lines were transfected in 24-well plates using the lipofectin reagent (BRL, Grand Island, N.Y., USA) basically following the manufacturer's protocol. Before harvesting, C2C12F3 and 3T3F442A cells were kept in differentiation medium for 1 and 4 days respectively.

For insulin induction studies, C2C12F3 and 3T3F442A cells were transfected and then maintained in differentiation medium for 3 to 5 days, after which they were incubated for 8 to 48 h in serum-, insulin- and glucose-free or glucose-containing growth medium. Subsequently, 10–100 nmol/l insulin, 25 mmol/l glucose or both together were added into the medium and the cells incubated for an additional 4 to 48 h. Cells were harvested, washed once with phosphate buffered saline and lysed with 100 µl of lysis reagent (Promega, Madison, Wis., USA). We used 10 µl of the cell lysate to determine protein concentration [32]. Luciferase and β-galactosidase activities were determined using 20 µl of the cell extract as previously described [33]. Each experiment included two independent control transfections. A promoterless vector, PXP1, was used to correct for background activity and a luciferase reporter gene driven by Rous sarcoma virus promoter, pRSVL 5' [28], was used as an external standard to which HKII promoter activities were compared. The plasmid pncMVlacF, a lacZ reporter gene driven by the cytomegalovirus promoter/enhancer, was used to correct for differences in transfection efficiency.

Results and discussion

Sequence and functional properties of the human HKII gene promoter. We have isolated the proximal promoter of the human HKII gene and sequenced 2.0 kb immediately upstream of the transcription initiation site (Fig. 2). Alignment of the human and rat promoter sequences revealed high conservation up to 450 bp upstream of the transcription initiation site (Fig. 2). The promoter sequence was analysed for transcription factor binding sites using the "Transcription Element Search" database (<http://agave.humgen.upenn.edu/cgi-bin/tess>). The human HKII promoter has a TATA-like box (AATAA) at position -13, a CCAAT-box at -66, and an inverted CCAAT-box (also referred to as a Y-box) at position -118 (Fig. 2). The proximal 550 bp region of the promoter is GC-rich and contains eight putative consensus binding sites for the Sp-1 family of transcription factors [34, 35]. The human HKII promoter also contains five putative binding sites for AP-2 [36,37], four for GCF [38], two for CTF-NF-Y ([39–42] for review see

[43]) and one for ODC-NF-1 [44]. In addition, there are two putative cyclic AMP (CRE) and two insulin response elements (IRE) within this proximal region.

The ubiquitously expressed heteromeric transcription factor NF-Y binds to the CCAAT and Y-boxes and activates transcription of a variety of genes [39–42]. GCF and ODC-NF1 were shown to interact with GC-rich regions and repress transcription from promoters of the epidermal growth factor receptor, β -actin, calcium-dependent protease and ornithine decarboxylase genes [38, 44]. A protein kinase A-dependent signal transduction pathway was proposed to mediate regulation of transcription of the PEPCK and tyrosine aminotransferase gene promoters by insulin via a cAMP response element (CRE) [45–47].

Osawa et al. [48], recently observed that cAMP induces activity of a reporter gene driven by rat HKII promoter constructs. Furthermore, they showed that the CCAAT box and an adjacent CRE cumulatively mediate the inductive effect of cAMP. Both of these elements, including the spacing in-between, are completely conserved in the human HKII promoter (at –44 for CRE and –62 for CCAAT in Fig. 2). The potential role of these putative transcription factors on the regulation of human HKII gene remains to be established.

The human (but not the rat) HKII promoter was found to contain one complete and one truncated *Alu* repeat elements (lower case letters in Fig. 2) which belong to the relatively old J-subfamily of *Alus* [49, 50]. The upstream *Alu* is truncated at its 3' end by approximately 195 bp and is flanked by 14 bp direct repeats and is oriented in the opposite direction of the complete downstream *Alu* element.

The transcription initiation sites of the human HKII gene. Primer extension analysis was used to determine the transcription start site(s) of the human HKII gene. An end-labelled HKII antisense oligonucleotide primer, from –376 to –396 with respect to the translation start site (underlined in Fig. 2), was hybridized to total RNA prepared from human skeletal muscle or adipose tissue and extended by reverse transcription. The length of the resulting fragments indicated a major transcription initiation site of 449 bp upstream of the translation initiation codon (Fig. 3). In skeletal muscle there were minor sites at –12, –20, –39, –40 and –41, which were not observed in adipose tissue. These results differ from those reported for the rat HKII [5] in which the transcription initiation site in rat adipose tissue was mapped to position + 19 with respect to the major start site in human tissues (Fig. 2). Additional start sites were found at positions + 18, + 16, + 15, –4 and –5 (with respect to the start site in human HKII) in the rat AH130 hepatoma cell line [22]. The potential role of these different initiation sites remains to be established.

Fig. 3. Primer extension analysis with human adipose tissue and skeletal muscle RNA. A ^{32}P -labelled oligonucleotide primer (Fig. 2) was hybridized with 25 μg of total RNA extracted from human adipose tissue (lane 1) and skeletal muscle (lane 2). The extended products were run on an 8% sequencing gel adjacent to the DNA sequencing ladder generated by the same labelled oligonucleotide with a subclone of the human hexokinase gene as template. The major band is marked with a thick arrow and minor sites with thin arrows

Sequence variants of the promoter region. SSCP analysis revealed the previously reported variant G→C at position + 217 [21] in the 5' untranslated region and two new variants in the promoter region of the HKII gene (T→G at position –1043 and G→A at position –1159) (Fig. 1). The frequency of the C allele at position + 217 was 0.183 in patients with NIDDM and 0.268 in control subjects ($p = \text{NS}$ between the groups). The allele frequency of the T→G substitution at position –1043 (Fig. 1) was 0.03 in patients with NIDDM and 0.06 in control subjects and the allele frequency of the substitution G→A at position –1159 (Fig. 1) was 0.473 in patients and 0.464 in control subjects ($p = \text{NS}$ between groups). None of these variants was associated with high fasting, 1-h or 2-h insulin levels (data not shown). The association of the + 217 and –1159 variants with insulin resistance was evaluated by the euglycaemic clamp. Neither of these variants was associated with low rates of whole body glucose uptake in NIDDM or control subjects (Table 2). In addition, the –1043 variant (T→A) was not associated with reduced rates of whole body glucose uptake (data not shown). Thus, it seems unlikely that any of these variants contributes to insulin resistance.

Functional analysis of the HKII promoter. A series of nested 5' deletions of the HKII promoter driving

Table 2. Rate of whole body glucose uptake as a function of genotype at the promoter region

	Variant at +217		Variant at -1159	
	GG	GC/CC	GG	GA/AA
Control subjects	58.7 ± 2.2 (41)	55.1 ± 2.1 (29)	58.3 ± 3.8 (17)	56.8 ± 1.7 (53)
NIDDM patients	27.5 ± 1.9 (16)	30.0 ± 5.0 (7)	30.6 ± 2.7 (11)	26.2 ± 2.9 (12)

Rates of whole body glucose uptake ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), as evaluated by the euglycaemic clamp technique, of control and NIDDM patients with different genotypes at the G → C (posi-

tion +217) and the G → A (at position -1159) polymorphism in the 5' untranslated and promoter regions, respectively. Number of subjects in parenthesis

Fig. 4. Functional analysis of the human HKII promoter. C2C12F3 myoblasts and 3T3F442A preadipocytes were transiently transfected with different HKII 5' deletion-luciferase fusion constructs. C2C12F3 cells were harvested 40 h after transfection and 3T3F442A cells were harvested 4 days after differentiation into adipocytes. Cell lysates were assayed for luciferase and β -galactosidase activities. Luciferase activity was first corrected for background activity of the PXP1-vector and then normalized with β -galactosidase activity to correct for difference in transfection efficiency. The data represent mean $\pm$ SEM of six independent experiments. Duplicate transfections were performed in each experiment with two to three different plasmid preparations. The activity of the -1692 HKII construct was set at 100 %. * $p < 0.01$, ** $p < 0.001$ (Student's t -test) indicate statistically significant losses in activity resulting from the deletion of any one segment of the promoter

expression of the luciferase reporter gene were tested for activity by transient transfection of the murine C2C12F3 skeletal muscle and 3T3F442A adipocyte cell lines. Both cell lines have been shown to express endogenous HKII upon differentiation [5]. Relative HKII promoter activities of the nested deletions, shown in Figure 4, were found to be similar in both cell lines. In C2C12F3 cells, deleting the promoter region from -4000 bp to -304 bp resulted in no statistically significant decrease in activity. Therefore, the activity of the -1692 construct was set as 100 % (Fig. 4). Deletion from -304 bp to -254 bp resulted in loss of approximately 60 % of activity, deletion to -174 bp further loss of 25 % of activity, and deletion to -97 bp resulted in additional loss of 5 % of activity. This indicates that the CCAAT and Y-boxes together with the two Sp-1 elements located within this 174 bp region are not sufficient to support maximal basal promoter activity. The activity of the HKII promoter in 3T3F442A cells differed from that in C2C12F3 in that deletion of the region between -505 bp and -406 bp resulted in approximately 35 % decrease in

activity. It appears that the core promoter elements responsible for basal promoter activity in skeletal muscle and adipose cells are located within the proximal 400 bp and 500 bp, respectively.

The sequence variants at positions -1159 and -1043 of the human HKII promoter, which we observed to be equally represented among NIDDM and normal subjects, are unlikely to have any functional impact since deletion of the region between -1692 and -964 did not alter activity of the proximal promoter.

An internal deletion extending from -184 to -215 was prepared from the -254 promoter construct. This deletion encompassed a highly GC-rich region that contains three consensus binding sites for Sp1, 2 for each of AP-2 and GCF and one for ODC-NF-1. Surprisingly, the activity of the promoter construct with this internal deletion was on average 1.46 ± 0.53 fold that of the full -254 construct (three independent transfections done in triplicate; $p = 0.007$) when transfected into 3T3F442A adipocyte cells. However, this internal deletion had no effect on promoter activity in the C2C12F3 cell line. This indicates that the deleted segment may contain sites of interaction with negative regulatory factors, such as ODC-NF-1, which is known to repress Sp1 activation of the ornithine decarboxylase promoter in a tissue-specific manner [44]. These results also indicate that the region between -254 and -215 contains important positive promoter elements since a major loss in activity was observed in deleting the promoter from -254 to -174. Alternatively, the increase in activity associated with the internal deletion may have been the result of relocation of the -254 to -215 region closer to the transcription initiation site.

In conclusion, functional analysis of the human HKII promoter deletions indicates that two sets of positive regulatory sequence elements located between 304 bp and 215 bp from the major transcription start site account for approximately 80 % of the basal HKII promoter activity. One set is located in the 5' half and the other in the 3' half of this approximately 100 bp segment, the sequence of which is highly conserved (88 % identity) between human and rat. A putative CRE is located in this region. In addition, the highly GC-rich region between nucleotides -215 and -184 contains a negative regulatory element, at least in 3T3 cells.

Recently, activities of nested deletions of promoter constructs of the rat HKII were analysed by transfection of rat L6 myotubes [48]. In contrast to the human HKII promoter, maximal basal activity of the proximal rat promoter in L6 myotubes was achieved by constructs containing only 157 bp. A similar length of the human promoter had only 15 % activity in C2C12F3 cells.

Activity of the proximal HKII promoter in response to insulin and glucose. We investigated whether insulin or glucose would induce activity of the HKII promoter by transient transfection of C2C12F3 and 3T3F442A cells with luciferase vectors containing 964, 406, 304, 174 and 97 bp (and one containing 4.0 kb in case of 3T3F442A) of the proximal HKII promoter as described under Materials and methods. Small (less than 1.5-fold) and variable inductive effects of insulin and glucose were observed which could not be used to localize insulin response elements. The steady-state level of HKII mRNA increased by only two-fold upon treatment of 3T3F442A cells with insulin using the same protocol used in transfection experiments (data not shown).

This result was unexpected since insulin was previously shown to increase the steady-state levels of HKII mRNA by six- and three-fold in fully differentiated 3T3F442A and C2C12 cells, respectively [5]. However, it was not determined whether insulin had any effect on the transcription rate of the HKII gene in these cell lines. In vivo, hyperinsulinaemia was observed to be associated with elevated HKII mRNA levels in rat skeletal muscle and adipose tissue [12], and in human skeletal muscle [13]. On the other hand, hyperglycaemia was not found to influence HKII expression in these tissues in the rat [12].

Our results suggest that either the insulin and glucose response elements are located outside of the 4.0 kb region of the proximal promoter, or that the cell lines we transfected were not sufficiently differentiated to give a significant response.

Various *cis*-acting sequences that mediate insulin-dependent regulation of transcription have been reported in promoters of genes encoding phosphoenolpyruvate carboxykinase (PEPCK) [45], *c-fos* [51, 52], amylase [53], glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [54, 55], glucagon [56, 57], malic enzyme [58], delta 1-crystallin [59], prolactin [60], β -actin [61], insulin-like growth factor I-binding protein [62], tyrosine aminotransferase [46], thyrotropin receptor [63] and fatty acid synthase [64]. There seems to be no unique sequence found so far to mediate the effects of insulin. Two putative sequences found in the promoter of HKII gene resemble insulin responsive sequences found in other genes [51–64]. However, neither of these sites is totally conserved between human and rat.

Osawa et al. [65] recently observed that insulin induced by 3.5-fold the activity of a reporter gene driven by 487 bp of the proximal rat HKII promoter transfected into rat L6 myotubes but did not define the insulin response elements within this construct. Interestingly, these authors also showed that the induction of HKII by insulin was blocked by treatment with either wortmannin or rapamycin, suggesting that a phosphatidylinositol 3-kinase (PI 3-kinase)/p70/p85 ribosomal S6 protein kinase (p70^{s6k})-dependent pathway may transduce the action of insulin on HKII gene expression. In addition, both insulin and glucose were shown to activate the rat HKII promoter (4.3 kb) in transient transfection of AS-30D hepatoma cells [23].

More reliable analysis of the level at which the HKII gene is regulated by glucose and insulin in various tissues and of the *cis*-DNA elements that mediate response to these agents in vivo would best be accomplished using transgenic mice that harbour various lengths of the HKII promoter driving expression of a reporter gene.

Acknowledgements. This work was supported by NIH grant HL-30086 to S.D. and by a grant from the Nordisk Insulinfond to M.L. and M.M.

References

1. Wilson JE (1995) Hexokinases. *Rev Physiol Biochem Pharmacol* 126: 95–198
2. Katzen HM, Schimke RT (1965) Multiple forms of hexokinase in the rat: tissue distribution, age dependency and properties. *Proc Natl Acad Sci USA* 54: 1218–1225
3. Katzen HM (1967) Multiple forms of mammalian hexokinase and their significance in the action of insulin. *Adv Enzyme Regul* 5: 335–355
4. Katzen HM, Soderman DD, Cirillo VJ (1968) Tissue distribution and physiological significance of multiple forms of hexokinase. *Ann NY Acad Sci* 151: 351–358
5. Printz RL, Koch S, Potter LR, O'Doherty RM, Tiesinga SM, Granner DK (1993) Hexokinase II mRNA and gene structure, regulation by insulin and evolution. *J Biol Chem* 268: 5209–5219
6. Thelen AP, Wilson JE (1991) Complete amino acid sequence of the type II isoenzyme of rat hexokinase, deduced from the cloned cDNA. *Arch Biochem Biophys* 286: 645–651
7. Deeb SS, Malkki M, Laakso M (1993) Human hexokinase II: sequence and homology to other hexokinases. *Biochem Biophys Res Comm* 197: 68–74
8. Malkki M, Laakso M, Deeb SS (1994) Structure of the human hexokinase II gene. *Biochem Biophys Res Comm* 205: 490–496
9. Printz RL, Ardehali H, Koch S, Granner DK (1995) Human hexokinase II mRNA and gene structure. *Diabetes* 44: 290–294
10. Lehto M, Xiang K, Stoffel M et al. (1993) Human hexokinase II: localization of the polymorphic gene to chromosome 2. *Diabetologia* 36: 1299–1302
11. Osawa H, Printz RL, Whitesell RR, Granner DK (1995) Regulation of hexokinase II gene transcription and glucose

- phosphorylation by catecholamines, cyclic AMP, and insulin. *Diabetes* 44: 1426–1432
12. Postic C, Leturque A, Rencurel F et al. (1993) The effect of hyperinsulinaemia and hyperglycaemia on GLUT 4 and hexokinase II mRNA and protein in rat skeletal muscle and adipose tissue. *Diabetes* 42: 922–929
 13. Mandarino LJ, Printz RL, Cusi KA et al. (1995) Regulation of hexokinase II and glycogen synthase mRNA, protein, and activity in human muscle. *Am J Physiol* 269:E701–E708
 14. Braithwaite SS, Plazuk B, Colca JR, Edwards II CW, Hoffman C (1995) Reduced expression of hexokinase II in insulin-resistant diabetes. *Diabetes* 44: 43–48
 15. Rothman D L, Shulman R G, Shulman GI (1992) ³¹P magnetic resonance measurement of muscle glucose 6-phosphate. Evidence for reduced insulin-dependent muscle glucose transport or phosphorylation activity in non-insulin-dependent diabetes mellitus. *J Clin Invest* 89: 1069–1075
 16. Granner DK, O'Brien R M (1992) Molecular physiology and genetics of NIDDM. Importance of metabolic staging. *Diabetes Care* 15: 369–395
 17. Vestergaard H, Bjorbaek C, Hansen T, Larsen FS, Granner DK, Pedersen O (1995) Impaired activity and gene expression of hexokinase II in muscle from non-insulin-dependent diabetes mellitus patients. *J Clin Invest* 96: 2639–2645
 18. Echwald SM, Bjorbaek C, Hansen T et al. (1995) Identification of four amino acid substitutions in hexokinase II and studies of relationships to NIDDM, glucose effectiveness, and insulin sensitivity. *Diabetes* 44: 347–353
 19. Vidal-Puig A, Printz RL, Stratton IM, Granner DK, Moller DE (1995) Analysis of the hexokinase II gene in subjects with insulin resistance and NIDDM and detection of Gln¹⁴² → His substitution. *Diabetes* 44: 340–346
 20. Laakso M, Malkki M, Deeb SS (1995) Amino acid substitutions in hexokinase II among patients with NIDDM. *Diabetes* 44: 330–334
 21. Lehto M, Huang X, Le Beau MM et al. (1995) Human hexokinase II gene: exon-intron organization, mutation screening in NIDDM, and its relationship to muscle hexokinase activity. *Diabetologia* 38: 1466–1474
 22. Ichiara J, Shinohara Y, Kogure K, Terada H (1995) Nucleotide sequence of the 5'-flanking region of the rat type II hexokinase gene. *Biochim Biophys Acta* 1260: 365–368
 23. Mathupala SP, Rempel A, Pedersen L (1995) Glucose catabolism in cancer cells. Isolation, sequence and activity of the promoter for type II hexokinase. *J Biol Chem* 270: 16918–16925
 24. Nakashima RA, Paggi MG, Scott LJ, Pedersen PL (1988) Purification and characterization of a bindable form of mitochondrial bound hexokinase from the highly glycolytic AS-30D rat hepatoma cell line. *Cancer Res* 48: 913–919
 25. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY pp 7.79–7.83
 26. Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159
 27. Laakso M, Malkki M, Kekäläinen P, Kuusisto J, Deeb SS (1995) Polymorphisms of the human hexokinase II gene: lack of association with NIDDM and insulin resistance. *Diabetologia* 38: 617–622
 28. DeWet JR, Wood KV, De Luca M, Helinski DR, Subramani S (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* 7: 725–737
 29. Nordeen SK (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques* 6: 454–458
 30. Rastinejad F, Blau H (1993) Genetic complementation reveals a novel regulatory role for 3' untranslated regions in growth and differentiation. *Cell* 72: 903–917
 31. Green H, Kehinde O (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* 7: 105–113
 32. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254
 33. Yang W-S, Nevin DN, Peng R, Brunzell JD, Deeb SS (1995) Mutation in the promoter of the lipoprotein lipase (LPL) gene in a patients with familial combined hyperlipidemia and low LPL activity. *Proc Natl Acad Sci USA* 92: 4462–4466
 34. Dynan WS, Tjian R (1983) The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV 40 early promoter. *Cell* 25: 79–87
 35. Jones KA, Kadonaga JT, Rosenfeld PJ, Kelly TJ, Tjian R (1987) A cellular DNA binding protein that activates eukaryotic transcription and DNA replication. *Cell* 48: 79–89
 36. Mitchell P, Wang C, Tjian R (1987) Positive and negative regulation in vitro: enhancer-binding protein AP-2 is inhibited by SV-40 T antigen. *Cell* 50: 847–861
 37. Faist S, Meyer S (1992) Compilation of vertebrate-encoded transcription factors. *Nucl Acids Res* 20: 3–26
 38. Kageyama R, Pastan I (1989) Molecular cloning and characterization of a human DNA binding factor represses transcription. *Cell* 59: 815–825
 39. Dorn A, Bollekens J, Staub A, Benoist C, Mathis D (1987) A multiplicity of CCAAT-box binding proteins. *Cell* 50: 863–872
 40. Szabo SJ, Gold J, Murphy TL, Murphy KM (1993) Identification of cis-acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NF-ATc. *Mol Cell Biol* 13: 4793–4805
 41. Framson P, Bornstein P (1993) A serum response element and a binding site for NF-Y mediate the serum response of the human thrombospondin 1 gene. *J Biol Chem* 268: 4989–4996
 42. Ronchi A, Bellorini M, Mongelli N, Mantovani R (1993) CCAAT box binding protein NF-Y (CBS, CP1) recognizes the minor groove and distorts DNA. *Nucl Acid Res* 23: 4565–4572
 43. Glimcher LH, Kara CJ (1992) Sequences and factors: a guide to MHC class-II transcription. *Annu Rev Immunol* 10: 13–49
 44. Li R-S, Abrahamsen MS, Johnson RR, Morris DR (1994) Complex interactions at a GC-rich domain regulate cell type-dependent activity of the ornithine decarboxylase promoter. *J Biol Chem* 269: 7941–7949
 45. Forest CD, O'Brien RM, Lucas PC, Magnuson MA, Granner DK (1990) Regulation of phosphoenolpyruvate carboxykinase gene expression by insulin. Use of stable transfection approach to locate an insulin responsive sequence. *Mol Endocrin* 4: 1302–1310
 46. Ganss R, Weih F, Schutz G (1994) The cyclic 3' 5' - monophosphate- and the glucocorticoid-dependent enhancers are targets for insulin repression of tyrosine aminotransferase gene transcription. *Mol Endocrin* 8: 895–903
 47. O'Brien RM, Lucas PC, Yamasaki T, Noisin EL, Granner DK (1994) Potential convergence of insulin and cAMP signal transduction systems at the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter through CCAAT/enhancer protein (c/ebp). *J Biol Chem* 269: 30419–31428
 48. Osawa H, Sutherland C, Robey RB, Printz RL, Granner DK (1996) Identification and characterization of basal and

- cyclic AMP response elements in the promoter of the rat hexokinase II gene. *J Biol Chem* 271: 17296–17303
49. Batzer M A, Deininger PL, Hellman-Blumberg U et al. (1996) Standardized nomenclature for Alu repeats. *J Mol Evol* 42: 3–6
 50. Kapitonov V, Jurka JJ (1996) The age of Alu subfamilies. *J Mol Evol* 42: 59–65
 51. Stumpo DJ, Stewart TN, Gilman MZ, Blackshear PJ (1988) Identification of c-fos sequences involved in induction by insulin and phorbol esters. *J Biol Chem* 264: 1611–1614
 52. Messina JL (1990) Insulin's regulation of c-fos gene transcription in hepatoma cells. *J Biol Chem* 265: 11700–11705
 53. Johnson TM, Rosenberg MP, Meisler MH (1993) An insulin-responsive element in the pancreatic enhancer of the amylase gene. *J Biol Chem* 268: 464–468
 54. Nasrin N, Ercolani L, Denaro M, Kong XF, Kang I, Alexander M (1990) An insulin responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein induced by insulin in cultured cells and by nutritional manipulations in vivo. *Proc Natl Acad Sci USA* 87: 5273–5277
 55. Alexander-Bridges M, Dugast I, Ercolani L, Kong XF, Giere L, Nasrin N (1992) Multiple insulin-responsive elements regulate transcription of the GAPDH gene. *Adv Enz Reg* 32: 149–159
 56. Kneipel W, Jepeal L, Habener JF (1990) A pancreatic islet cell-specific enhancer-like element in the glucagon gene contains two domains binding distinct cellular proteins. *J Biol Chem* 265: 8725–8735
 57. Philippe J, Morel C, Cordier-Bussat M (1995) Islet specific proteins interact with the insulin-responsive element of the glucagon gene. *J Biol Chem* 270: 3039–3045
 58. Garcia-Jimenez C, Benito B, Jolin T, Santisteban P (1994) Insulin regulation of malic enzyme gene expression in rat liver: evidence for nuclear proteins that bind to two putative insulin response elements. *Mol Endocrin* 8: 1361–1369
 59. Alemany J, Borrás T, De Pablo F (1990) Transcriptional stimulation of the crystallin gene by insulin-like growth factor I and insulin requires DNA cis elements in chicken. *Proc Natl Acad Sci USA* 87: 3353–3357
 60. Stanley FM (1992) An element in the prolactin promoter mediates the stimulatory effect of insulin on transcription of the prolactin gene. *J Biol Chem* 267: 16719–16726
 61. Onyia JE, Halladay DL, Messina JL (1995) One of the three C/CArGG box/serum response elements of the β -actin gene is an insulin-responsive element. *Endocrinology* 136: 306–315
 62. Suwanickul A, Morris SL, Powell DR (1993) Identification of an insulin-responsive element in the promoter of the human gene for insulin-like growth factor binding protein-1. *J Biol Chem* 268: 17063–17068
 63. Shimura Y, Shimura H, Ohmori M, Ikuyama S, Kohn LD (1994) Identification of a novel insulin-responsive element in the rat thyrotropin receptor promoter. *J Biol Chem* 269: 31908–31914
 64. Moustaid N, Beyer RS, Su HS (1994) Identification of an insulin responsive element in the fatty acid synthase promoter. *J Biol Chem* 269: 5629–5634
 65. Osawa H, Sutherland C, Robey RB, Printz RL, Granner DK (1996) Analysis of signalling pathway involved in the regulation of hexokinase II gene transcription by insulin. *J Biol Chem* 271: 16690–16694