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Functional analysis of a newly identified TAAT-box of the rat insulin-II gene promoter

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Abstract Transcriptional regulation of insulin gene expression is achieved by an interplay of tissue-specific and ubiquitous *cis*- and *trans*-acting elements. E-box like motifs and TAAT-motifs were shown to play a crucial role in initiating insulin gene transcription. Studying the AT-rich region of the rat insulin-II promoter between nucleotides −212 and −196, we observed a base difference at −211, an adenosine instead of a cytidine, compared to the previously reported sequence (EMBL Accession No. J00748). Sequence analysis of promoter fragments from different rat strains showed that adenosine at position −211 represents the wild type (EMBL Accession No. X82162). This base exchange leads to the formation of an additional TAAT-motif, i.e. TAAT3, at the complementary DNA strand directly upstream of the previously studied TAAT2 motif, formerly named CT-2. Here we show that the newly identified motif TAAT3 is involved in (i) transcriptional control in vivo, (ii) in vitro DNA/protein interactions, and that (iii) TAAT1, TAAT2 and TAAT3 are binding sites for the homeodomain-containing factor IPF-1.

Key words: Rat insulin II gene; Gene expression; Transcriptional control; Insulinoma; DNA–protein interaction; Promoter

1. Introduction

The expression of the insulin gene in adult organisms is restricted to pancreatic β-cells. Multiple elements exhibiting both positive and negative effects contribute to its transcriptional control (for review see [1–3]). At least two known *cis*-elements seem to play a crucial role in both the tissue-specific and the glucose-responsive regulation of insulin gene transcription: E-box like sequences with a consensus sequence GCCANNTG, and TAAT-boxes. The E-box like motifs such as IEB2/Far- and IEB1/Nir-boxes [4,5] in the rat insulin-I promoter, ICE [6] and part of RIPE3 [7] in the rat insulin-II promoter, and the GC-I motif in the human insulin promoter [8] are binding sites for factors of the basic helix-loop-helix family. Whereas this class of transcriptional control elements is relatively well studied in insulin gene regulation, the investigation of the TAAT-motifs, representing binding sites for homeodomain-containing factors, was so far mainly restricted to the rat insulin-I gene [9,10] and the human insulin gene [8,11–15].

In a previous report we showed the impact of two TAAT-motifs between nucleotides −82/−76 and −202/−196 (CT-1 and CT-2, respectively) of the rat insulin II promoter in transcription initiation in insulin producing cells [16]. Both elements were mutationally sensitive in transient expression studies and showed a similar retardation pattern in electrophoretic mobility shift assays. In a very recent study Peshavaria et al. [17] confirmed the data for the distal region between −201 and −196 bp and showed that it represents a binding site for the homeodomain containing factor STF-1/IPF-1/IDX-1. Furthermore, studying the region containing the CT-2 element we observed a difference in the nucleotide sequence at promoter position −211: an adenosine instead of the so far described cytidine. This base exchange would lead to the formation of an additional TAAT-motif at the lower DNA-strand directly upstream of the upper-strand distal TAAT-motif (CT-2). In this study we demonstrate that the newly identified TAAT-motif, called here TAAT3, represents the 'wild type' situation of this promoter region and, further, that TAAT3 is involved in (i) transcriptional control in vivo, (ii) in vitro DNA–protein interactions, as demonstrated by DNase I footprinting analysis and methylation interference assays, and (iii) represents a binding site for the homeodomain-containing factor IPF-1.

2. Material and methods

2.1. DNA-preparation and sequencing

Genomic DNA from Wistar and Sprague–Dawley rats was prepared as described in [18]. DNA from BB- and DA-rats were gifts from Dr. I. Klöting (Institut für Diabetes, EMAU Greifswald, Karlsburg). To amplify DNA-fragments of the rat insulin-II promoter using PCR we used as the upstream primer 5′-CTCTCTGAGACAATGTC-3′ and as the downstream primer 5′-GTTACTGAACCCCACT-3′, and *Ultma*-polymerase (Perkin Elmer) or Taq-Polymerase (Amersham) as thermostable DNA-polymerases. Generated PCR products were subcloned directly into the T-tailed vector pT7Blue (Novagen) and were verified by either cycle sequencing using the *Dye Termination Technique* and the 373A-sequencer (Applied Biosystems) or the T7-DNA-polymerase sequencing kit (Pharmacia). The nucleotide sequence data reported in this paper was deposited under accession number X82162 in EMBL/GenBank/ DDJB.

2.2. Plasmid construction

Both plasmids p211A and p211C contain the rat insulin-II gene promoter (−218bp/+129bp) fused to the reporter gene CAT, but differ in the promoter sequence at position −211 in containing an adenosine (p211A) or a cytidine (p211C). p211A is identical with pBRINSII-218CAT [16]. p211C was generated on the basis of pBRINSII-218CAT by site-directed mutagenesis using the MutaGene In Vitro Mutagenesis Kit (Bio-Rad) and with the mutagenic primer 5′-CTTAGGGTAAT-TAGAGTCTTAAGAGGGGCT-3′. After performing mutagenesis the promoter fragment was subcloned into the promoterless vector pBSV0CAT [19] and the sequence was verified.

2.3. Cell culture and transfection

Insulin-producing cells, HIT M2.2.2, were grown in RPMI 1640 supplemented with 40 units/ml penicillin, 40 μg/ml streptomycin, 2 mM glutamine, and 10% fetal calf serum. Cells were transfected using the calcium phosphate coprecipitation technique as described in [20] using 20 μg CsCl-purified CAT expression vector and 5 μg pRSVβgal [21] per 100 mm dish. Cells were harvested 48 h after transfection, and cell

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lysates analyzed for CAT- and β-gal activities [22,23]. The β-galactosidase values were used to normalize the individual test CAT levels.

2.4. In vitro DNA–protein interaction

Crude nuclear extracts were prepared from HIT M2.2.2 and βTC-3 as described [24] except that the nuclear proteins were finally dialyzed against two changes of 500 volumes of buffer (20 mM HEPES-KOH pH 7.9, 25% (v/v) glycerol, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.5 mM ε-aminocapronic acid, and 2.5 μg/ml trasylol) at 4°C. In vitro DNase I footprinting assays as well as methylation interference assays were performed according to [8]. Electrophoretic mobility shift assays (EMSAs) were carried out as described in [16,25]. Single-stranded deoxyoligonucleotides were provided by Dr. P. Oppermann, IBI EMAU Greifswald, Germany. Anti-IPF-1-antibodies (a generous gift from H. Edlund, Umeå, Sweden) were used in EMSAs as a 1:10 diluted solution of the original antiserum [26].

3. Results

3.1. The AT-rich region (−212/−196) of the rat insulin-II promoter contains an additional TAAT-motif

Studying the role of the AT-rich region between nucleotides −212 and −196 of the rat insulin-II promoter, we observed a sequence difference at position −211 compared to the previously reported sequence [27], i.e. an adenosine instead of a cytidine (compare Accession No. X82162 with J00748). Due to the formation of a putative additional TAAT-motif at the lower DNA strand adjacent to the previously studied CT-2-box [16] here named TAAT2, we performed a more detailed sequence analysis. To verify that this base exchange represents the 'wild type' sequence, we analyzed genomic DNA from several different rat strains: Wistar, Sprague–Dawley, BB-rats, and from DA-rats (both males and females in each case). The DNA region of interest was amplified by the PCR technique using separate reactions with either Taq polymerase from *Th. aquaticus* (Amersham) or Ultma-polymerase from *Th. maritima* (Perkin Elmer). To verify the DNA sequences we analyzed both DNA strands by using two different sequencing techniques: Sanger's technique [28] using the T7-DNA-polymerase sequencing kit (Pharmacia) and cycle sequencing using the 'Dye Termination Technique' (Applied Biosystems). The resulting sequence is illustrated in Fig. 1B as p211A and in Fig. 2. The findings obtained demonstrate that the sequence presented here

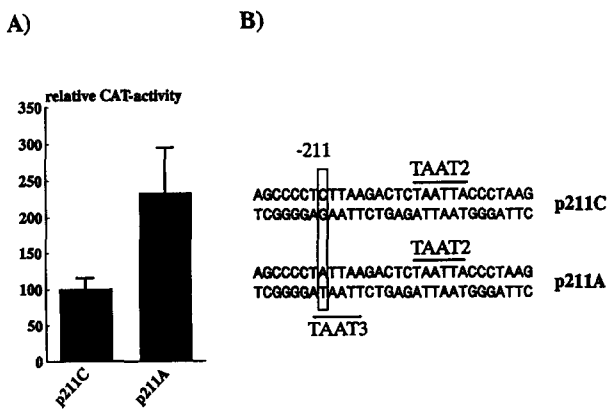

Fig. 1. (A) Transient expression of p211C and p211A in the insulin-producing cell line HIT M2.2.2. CAT values represent averages of at least three individual experiments and are expressed as percentages of p211C (= 100%). (B) Sequences represent the region between nucleotides −218 and −189 of the promoter fragments used in p211C and p211A. The base difference at position −211 is shown and the TAAT-motifs are delineated.

Fig. 2. Results summarized from in vitro footprinting assays performed between nucleotides −278 and −163. The regions protected from DNase I digestion is bracketed, purines involved in methylation interference between nucleotides −218 and −186 are shown as ● when fully involved, or as ○ when partially involved. Position and sequences of the E-box-like motif and the TAAT-motifs within this region are shown.

does represent the wild type situation for the rat insulin-II gene. One consequence of this observation is the formation of an additional TAAT-motif, i.e. TAAT3 (p211A, Fig. 1B) at the lower strand directly upstream of the previously described TAAT-motif TAAT2. To prove the impact of TAAT3 on transcriptional control we performed both transient expression analysis as well as in vitro analysis of DNA–protein interactions.

3.2. The newly identified TAAT-motif is involved in transcriptional control in vivo

To study the functional relevance of the newly identified TAAT-motif TAAT3 on the regulation of gene expression, we performed transient expression studies in the insulin-producing cell line HIT M2.2.2. In these experiments we used expression constructs based on pBRINSII-218CAT [16] containing (i) the sequence containing a cytidine at −211 within its promoter fragment (Fig. 1B, p211C) and (ii) the 'wild-type' sequence containing an adenosine at this position (Fig. 1B, p211A). The results presented in Fig. 1A demonstrate that the single-base difference caused a 2.3-fold increase in CAT expression in HIT M2.2.2 cells and, therefore, show that the newly described TAAT-motif is involved in transcriptional control in vivo.

3.3. The newly identified TAAT-motif is involved in DNA–protein interactions in vitro

Performing in vitro footprinting experiments by methylation interference and DNase I treatment showed that both TAAT-motifs between −212/−207 bp and −201/−196 bp were protected by DNA/protein interaction (Fig. 2). To further study the involvement of the identified TAAT-motifs of the rat insulin-II promoter in in vitro DNA–protein interactions, we used the following double-stranded oligonucleotides in electrophoretic mobility shift assays: R2AT211A and R2AT211C (representing the DNA sequences between nucleotides −218 and −189 according to GenBank Acc. no. X82162 and J00748, respectively), R2TAAT2 (i.e. R2AT211A but with mutated TAAT3-motif), R2TAAT3 (i.e. R2AT211A but with mutated TAAT2-motif), and oligonucleotides containing the TAAT1-motifs of the rat insulin-I and -II promoters (R2TAAT1 and R1TAAT1, respectively), and the entire FLAT-element of the rat insulin-I promoter (R1FLAT). The sequences of these oligonucleotides are shown in Fig. 3.

Using nuclear extracts prepared from the insulin-producing cell line HIT M2.2.2 in electrophoretic mobility shift assays, the oligonucleotide containing the AT-rich region of the rat

R2AT211A	AGCCCCTATTAAGACTCTAATTACCCTAAG TCGGGGATAATTCTGAGATTAATGGGATTC	-218/-189
R2AT211C	AGCCCCTcTTAAGACTCTAATTACCCTAAG TCGGGGAgAATTCTGAGATTAATGGGATTC	-218/-189
R2TAAT3	AGCCCCTATTAAGACgacgAcTACCCTAAG TCGGGGATAATTCTGctgcTgATGGGATTC	-218/-189
R2TAAT2	AGCCCCTgcTgAGACTCTAATTACCCTAAG TCGGGGAcgAcTCTGAGATTAATGGGATTC	-218/-189
R2TAAT1	TGCTGATCCACCCTTAATGGGACAAACAGC ACGACTAGGTGGGAATTACCCTGTTTGTCG	-94/-65
R1FLAT	CCTTGTTAATAATCTAATTACCCTAGGTCT GGAACAATTATTAGATTAATGGGATCCAGA	-227/-198
R1TAAT1	CCTCCTAGAGCCCTTAATGGGCCAAACGGC GGAGGATCTCGGGAATTACCCGGTTTGCCG	-94/-65

Fig. 3. Position and sequence of oligonucleotide probes used for the analysis of protein binding in EMSAs and competition studies. Oligonucleotides contain TAAT-motifs of the rat insulin-I promoter (R1TAAT1, R1FLAT) and normal (R2TAAT1, R2AT211A) and mutated (R2AT211C, R2TAAT2, R2TAAT3) regions of the rat insulin-II promoter. Positions of mutations within mutant oligonucleotides are shown as lower-case letters.

insulin-II promoter between nucleotides −216/−187, i.e. R2AT211A, led to the formation of three retarded complexes A, B, and C (Fig. 4A, lane 1). Surprisingly, the oligonucleotide containing the originally described sequence composition, i.e. R2AT211C, generated the same retardation pattern as seen from R2AT211A (Fig. 4A, lane 6). However, while changing the incubation conditions, especially the concentration of KCl, we observed a marked difference in the retardation patterns of both probes: whereas increasing amounts of KCl from 16 mM to 200 mM led to a stronger formation of complex B for the wild-type variant R2AT211A (Fig. 4A, lanes 1–5) the same conditions caused at best a weak increase in the formation of complex B for R2AT211C (Fig. 4A, lanes 6–10). To study the protein-binding properties of the newly identified TAAT-motif on its own, we synthesized an oligonucleotide where the adjacent upper-strand TAAT-motif was mutated, i.e. R2TAAT3 (Fig. 3). In EMSAs R2TAAT3 formed only a single retarded complex (Fig. 4B, lane 4), very similar to complex B of R2AT211A (Fig. 4B, lane 1). The formation of this complex was abolished when using oligonucleotides containing the TAAT2-motif (R2TAAT2) or the TAAT1-motif (R2TAAT1) as competitors (Fig. 4B, lanes 6 and 7, respectively). Furthermore, R2TAAT3 as a competitor was able to reduce the formation of all complexes A, B, and C (Fig. 4B, lane 3).

3.4. *The AT-rich region between −212 and −196 bp of the rat insulin-II promoter contains two binding sites for IPF-1*

IPF-1 was shown to be the favored *trans*-acting factor binding to the proximal TAAT-motif P1 of the rat insulin-I promoter but not to the distal E2 element (i.e. the FLAT-element [10]), where IEF2 binds when using nuclear extracts of the insulin-producing cell line βTC-1 [9].

To study whether the AT-rich region at −212/−196 bp of the

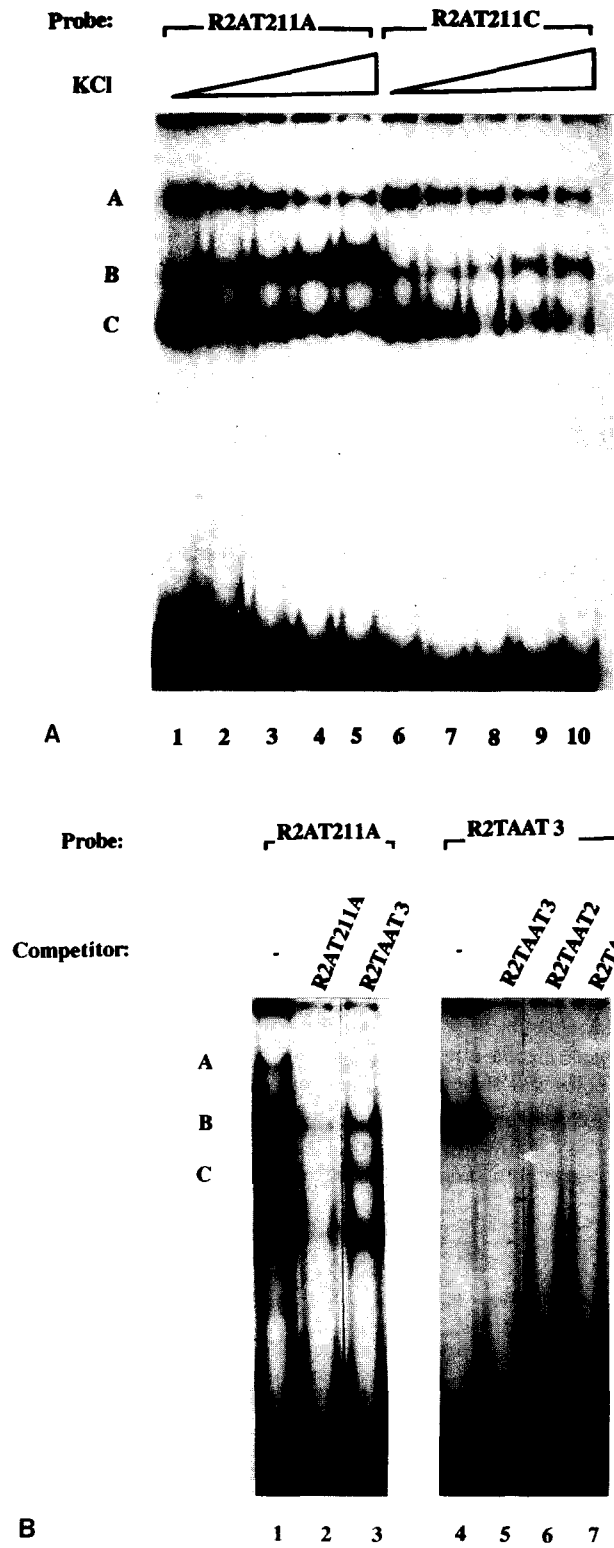

Fig. 4. Binding of HIT M 2.2.2 nuclear proteins to oligonucleotides R2AT211A, R2AT211C, and R2TAAT3. (A) Labeled oligonucleotides R2AT211A (lanes 1–5) and R2AT211C (lanes 6–10) were incubated with nuclear proteins in the presence of increasing amounts of KCl: 16 mM (lanes 1 and 6), 50 mM (lanes 2 and 7), 100 mM (lanes 3 and 8), 150 mM (lanes 4 and 9), and 200 mM (lanes 5 and 10), respectively. (B) Labeled oligonucleotides R2AT211A (lanes 1–3) and R2TAAT3 (lanes 4–7) were incubated in the presence of 150 mM KCl. The indicated unlabeled competitor oligonucleotides were used in approximately 100- fold molar excess.

rat insulin-II promoter is a target of IPF-1, we performed electrophoretic mobility shift assays using nuclear extracts prepared from βTC-3, a cell line very similar to βTC-1 [29]. As a positive control we used an oligonucleotide containing the proximal TAAT-motif (−80/−74 bp) of the rat insulin-I promoter (R1TAAT1), and as a negative control the FLAT-element (R1FLAT), i.e. the rat insulin-I promoter AT-rich region between −222/−208 bp. The EMSAs revealed similar retardation pattern for oligonucleotides containing the rat insulin-I IPF-1 binding site (R1TAAT1), the proximal TAAT-motif (R2TAAT1) and the AT-rich region between −212 and −196 bp (R2AT211A) of the rat insulin-II promoter (Fig. 5A, lanes 1, 5, 7, respectively). Addition of antibodies raised against the DNA-recognition part of IPF-1 lead to a loss in DNA-binding to both rat insulin-II AT-rich regions (Fig. 5A, lanes 6, 8). On the contrary, in the rat insulin-I promoter only the proximal TAAT-motif showed a loss in binding of IPF-1 when using the antibody (Fig. 5A, lane 2). When the IPF-1 binding properties of the two TAAT-motifs of the rat insulin-II AT-rich region were studied separately, both TAAT-motifs were able to bind protein complexes, which disappeared upon inclusion of antibodies raised against IPF-1 (Fig. 5B, lanes 4, 5). As expected, the FLAT-element of the rat insulin-I promoter formed retarded complexes different from those seen with the equivalent AT-rich region of the rat insulin-II promoter (Fig. 5A, lane 3), and these complexes were insensitive to treatment with anti-IPF-1 antibodies (Fig. 5A, lane 4).

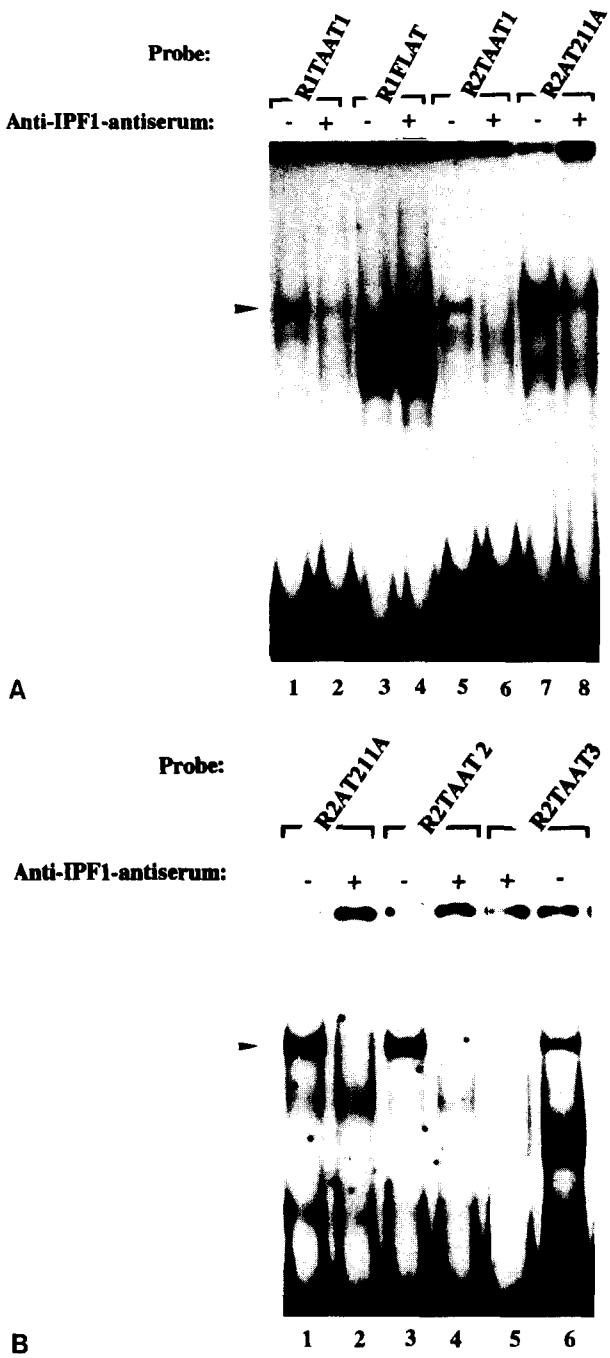

Fig. 5. Binding of βTC-3 nuclear proteins to oligonucleotide probes corresponding to TAAT-motifs of rat insulin-I and -II promoters (see Fig. 3). Protein binding to the indicated labeled probes was performed with (+) or without (−) a pre-incubation of the nuclear extracts with anti-IPF-1 antiserum for 10 min at room temperature. The arrowheads indicate the position of IPF-1-containing complexes. (A) Binding of treated and untreated nuclear proteins to oligonucleotides containing the proximal or distal TAAT-motifs of rat insulin-I promoter (R1TAAT1 and R1FLAT, respectively), and rat insulin-II promoter (R2TAAT1 and R2AT211A, respectively). (B) Binding of treated and untreated nuclear proteins to oligos containing the intact distal AT-rich region with the combination of both TAAT2 and TAAT3 motifs (R2AT211A), or to mutant oligos containing either the TAAT2-motif (R2TAAT2) or TAAT3-motif (R2TAAT3) alone.

4. Discussion

Previous studies on insulin gene regulation by different groups have confirmed the involvement of TAAT-motifs in transcriptional control (8–17,30–32). TAAT-motifs were shown to be the binding core for homeodomain-containing transcription factors [33]. Several *trans*-acting factors were reported to bind to insulin TAAT-motifs: IUF-1 [11,12,14], *isl-1* [31], IPF-1 [26] and IEF-2 [9], *lmx-1* and *cdx-3* [32], STF-1/IPF-1 [15], STF-1/IPF-1/IDX-1 [17]. In a previous report we demonstrated that TAAT-motifs of the rat insulin-II promoter between nucleotides −83/−76 and −204/−197 are mutationally sensitive and contribute to transcriptional control [16]. Furthermore, we found a difference in the nucleotide composition within the AT-rich region at −212/−196 at position −211 compared to the originally described sequence [27], i.e. an adenosine instead of a cytidine. One consequence of that sequence difference is the formation of an additional TAAT-motif on the complementary strand directly upstream of the well studied TAAT2-motif [16]. Here we present evidence that this sequence represents the 'wild type' situation for the rat insulin-II promoter for at least four different rat strains: Wistar, Sprague–Dawley, DA, and BB-rats. As demonstrated by in vitro DNA–protein interaction analysis this region is protected from DNase I digestion and is also involved in methylation interference (Fig. 2). Although the observed sequence difference at position −211 did not lead to a changed retardation pattern in EMSA at 16 mM KCl, the formation of these complexes is clearly different under increasing concentrations of KCl (Fig. 4A). This might be the reason for the more than 2.3-fold difference in CAT expression using constructs with both sequence variants (Fig. 1A). The differences in retardation patterns and binding affinities of the same oligonucleotide probes in figures 4 and 5 is caused by the use of nuclear extracts of different species, hamster insulinoma (Fig. 4) and mouse insulinoma (Fig. 5). Con-

cerning the involvement of the TAAT2- motif in transcriptional control, our previous observation [16] was confirmed by Peshavaria et al. [17] showing furthermore that this motif is a binding site for a homeodomain-containing protein very similar to STF-1/IPF-1/IDX-1 and related to the endoderm-specific *Xenopus laevis* homeodomain protein XlHbox8. Since the insulin promoter factor 1, i.e. IPF-1, was demonstrated to be identical to a part of XlHbox8 [26] and to be the favored binding factor for the proximal rat insulin-I TAAT-motif, i.e. to the P1-region [9], we investigated the binding of IPF-1 to the AT-rich region of the rat insulin-II promoter located between nucleotides −212 and −196. Using nuclear extracts prepared from βTC-3 as well as antibodies raised against IPF-1 in electrophoretic mobility shift assays we demonstrate that not only the previously reported TAAT-motifs TAAT1 and TAAT2, but also the newly identified motif TAAT3, is a binding target of IPF-1. Additionally our studies confirm the findings by Ohlsson et al. [9] showing that the two TAAT-containing regions within the rat insulin-I promoter, i.e. P1 and E2 (FLAT), represent binding sites for different *trans*-acting factors. Our observations are also in agreement with those of German et al. [10] that different *trans*-acting factors are involved in complex-formation of the AT-rich regions of the rat insulin-I and -II promoters. The fact, that TAAT2/TAAT3 is a binding site for IPF-1, and the absence of a correct Far-element (GCCATCTG) in the correct juxtaposition to the AT-rich region of the rat insulin-II promoter, may contribute to the marked difference observed in the transcriptional control of both rat insulin genes, i.e. the promoter region of the rat insulin-II gene similar to the rat insulin-I FF-minienhancer does not exhibit enhancer properties ([10], own observations, data not shown). On the other hand we found that IPF-1 binding is not restricted to the promoters of different insulin genes. IPF-1 is also a *trans*-acting factor binding to TAAT-motifs of further β-cell-active promoters, such as the rat glucokinase upstream promoter, the rat amylin gene promoter, and the human glucose transporter-2 gene promoter (I.B. Leibiger, unpublished data). Theses findings support our previous assumption, based on EMSA studies, that TAAT-motifs of the genes mentioned above may use similar, if not identical, *trans*-acting factors [25]. In this way, IPF-1, or related factors such as IUF-1 [11], STF-1 [34], or IDX-1 [35], may represent one of the central factors responsible for β-cell-active and islet-specific transcriptional control, and, furthermore, may be involved in the coordinated regulation of pancreatic β-cell expressed genes.

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