Gene-specific Transcriptional Activity of the Insulin cAMP-responsive Element Is Conferred by NF-Y in Combination with cAMP Response Element-binding Protein*

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Cyclic AMP stimulates insulin gene transcription through a cAMP response element (CRE). In the present study the insulin CRE-binding proteins and their functions were investigated. A mutational analysis of nuclear protein binding in electrophoretic mobility shift assays in combination with specific antisera showed that in the CRE of the rat insulin I gene the imperfect CRE octamer-like sequence TGACGTCC interacts weakly with CREB and overlaps with two sequence motifs (TTGTTGAC and CCAAT) that bind winged helix-like proteins and the transcription factor NF-Y, respectively. Transient transfection of wild-type and mutant insulin CRE-reporter fusion genes and the inactivation of cellular CREB or NF-Y by overexpression of the dominant negative mutants KCRCB or NF-YA29, respectively, indicate that cAMP inducibility of the insulin CRE is mediated by CREB or closely related proteins; however, NF-Y binding to the insulin CRE confers constitutive, basal activity and decreases the ability of CREB to mediate cAMP-stimulated transcription and calcium responsiveness. Results from these studies demonstrate that NF-Y binds to the insulin CRE and modulates the function of CREB. Together with the nonpalindromic sequence of the CRE octamer motif, the interaction of NF-Y with CREB may be responsible for the gene-specific transcriptional activity of the insulin CRE and explain why it has considerable basal activity but is less responsive to cAMP stimulation than others.

The second messenger cAMP regulates the transcription of many genes by a mechanism involving the activation of protein kinase A and subsequent phosphorylation of the nuclear transcription factor CREB1 (1–3). In addition to protein kinase A, CREB is phosphorylated on serine 119 (in CREB-327) by other kinases including calcium/calmodulin-dependent protein kinases, Ras-dependent RSK2, and a p38/HOG-1-dependent protein kinase (1–7). CREB can thus confer cAMP, calcium, and growth factor responsiveness to genes carrying CRE-binding sites called cAMP response elements (CRE) and typified by the consensus palindromic octamer sequence 5’-TGACGTCA-3’. CREB is expressed ubiquitously and many genes whose transcription is regulated by cAMP contain a CRE, or variants of it, in their 5’-flanking regions. CREB/CRE-directed transcription is thus of general importance in virtually all cells (1–7). Cyclic AMP responsiveness has been shown in individual cases to be conferred by transcription factors other than CREB such as AP-1 (8), AP-2 (9), Pit-1 (10), and SF-1 (11).

Although CREB is a ubiquitous protein, gene-specific differences exist in the responsiveness of different CREs to transcriptional activation (1–3, 12–15). In addition to CREB and the closely related proteins ATF-1 and CREM, the CRE motif is recognized by a number of proteins of the CREB/ATF family of transcription factors that are not activated by cAMP but may compete with CREB for binding to the CRE (1–3). CREs in which the DNA binding or function of CREB is modulated by the presence of another protein at adjacent or overlapping sites have been described (1–3, 13) and in some cases the accessory proteins have been identified including the glucocorticoid receptor (14, 16), YY1 (17), and C/EBP proteins (15, 18–21). Therefore, a given CRE may have unique functional properties depending on its specific DNA sequence both within the core octamer and nucleotides flanking this motif.

Gene-specific transcriptional activities have been reported also for the CRE of the rat insulin I gene (22), although the molecular basis for its unique functional properties has remained unclear. The peptide hormone insulin is synthesized in β cells of the islets of Langerhans. It is a key regulator of blood glucose concentration, and inappropriate regulation of insulin production and secretion causes diabetes mellitus. Cyclic AMP stimulates insulin gene transcription (22, 23). In the absence of other signals, cAMP inducibility of the insulin gene is modest (22–24). However, cAMP stimulates insulin gene transcription synergistically with glucose (25–28). Potent stimulators of β cell cAMP levels are hormones including glucagon-like peptide-1 (29), which appears to be a physiologically important hormonal mediator of the “incretin effect” on insulin secretion and has been proposed as a new therapeutic agent for the treatment of non-insulin-dependent diabetes mellitus (29). Therefore, similar to the glucose competence concept of insulin secretion (29), a synergistic interaction between the hormonally regulated cAMP-dependent signaling system and the glucose-regulated signaling system may give β cells the ability to match the ambient concentration of glucose to an appropriate transcriptional response of the insulin gene (24). Despite its suggested physiological significance, the molecular mechanism of cAMP-induced insulin gene transcription is poorly understood.

Studies of the transcriptional activity of reporter fusion genes in insulinoma cells demonstrated that a CRE in the 5’-flanking region of the rat insulin I gene is required and sufficient for CAMP induction (22, 23). However, the insulin CRE shows unique functional properties; it (i) has considerable basal transcriptional activity, (ii) gives a weak cAMP response, and (iii) is not responsive to membrane depolarization and

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The abbreviations used are: CRE, cAMP response element; CREB, CRE-binding protein; HNF, hepatocyte nuclear factor.

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EXPERIMENTAL PROCEDURES

Plasmid Construction—The plasmid −85InsLuc was prepared by cloning a fragment of the rat insulin I gene promoter (from −85 to +49) with 5′-XhoI and 3′-BglII ends into the XhoI-BglII sites of pXP2 (37). For 4xInsCRE−85InsLuc, 4x1-InsCRE−85InsLuc, 4x2-InsCRE−85InsLuc, 4x3-InsCRE−85InsLuc, 4x4-InsCRE−85InsLuc, 4xExo-CATAT−85InsLuc, and 4xG3B−85InsLuc, four copies of the synthetic oligonucleotides with 5′-GATC overhangs (for sequences see Fig. 1 or further below) were cloned in the forward orientation into the BamHI site of −85InsLuc. The plasmid −410InsLuc was prepared by cloning a fragment of the rat insulin I gene promoter (from −410 to +49) with 5′-XhoI and 3′-BglII ends into the XhoI-BglII sites of pXP2 (37). Four bases in the CRE octamer-like sequence (from −185 to −180) were selectively deleted inside the insulin promoter with the restriction enzyme AvaII and T4 DNA polymerase, yielding the construct −410A−183−180InsLuc. Subcloning and plasmid isolation were performed by standard procedures. All constructs were sequenced by the enzymatic method to confirm the identity and the orientation of the inserts.

Cell Culture and Transfection of DNA—The pancreatic islet cell line HIT-T15 (38) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were trypsinized and transfected in suspension by the DEAE-dextran method as described (30) with 2 μg of indicator plasmid per 6-cm dish. Rous sarcoma virus-chloramphenicol acetyltransferase expression plasmid (0.4 μg/6-cm dish) was added as an internal reporter to check for transfection efficiency. When indicated, 2 μg of RSV-KCRE (39) or, unless indicated otherwise, 3 μg of an expression vector encoding the dominant negative mutant of NF-Y, NF-YA29 (40), were co-transfected per 6-cm dish. These co-transfections were done with a constant DNA concentration, which was maintained by adding Bluescript (Stratagene, La Jolla, CA). Cells were stimulated with forskolin (10 μM) or high KCl (45 mM final concentration) for 6 h before harvest. Cell extracts (30) were prepared 48 h after transfection. A chromatographic chloramphenicol acetyltransferase assay (41) and the luciferase assay (30) were performed as described previously. Thin layer chromatography plates were analyzed with a Fuji PhosphoImager.

Nuclear Extracts—Nuclear extracts were prepared from HIT cells by the method of Dignam et al. (42) with the modification described (22). Electrophoretic Mobility Shift Assay—Using 15 μg of protein from nuclear extracts, the electrophoretic mobility shift assay was performed as described (41). In some binding reactions 2.5 μl of an antiserum directed against the B subunit of NF-Y, α-NF-YB (43), 2 μl of a specific anti-CREB antiserum (R1090) (44), or an equal volume of preimmune serum was added to the binding reaction, and the assay was then performed as described (22).

Oligonucleotides—The sequences of the CRE oligonucleotides of the rat insulin I gene (wild type and mutants 1 to 4) and the rat somatostatin gene are shown in Fig. 1. The sequences of other oligonucleotides were as described previously or read as follows (only one strand with a dash): TTR-HNF-3, containing a fragment of the transthyretin promoter from −111 to −85 that includes an HNF-3-binding site (45); TTR-HNF-4, containing a fragment of the transthyretin promoter that includes an HNF-4-binding site (46); 5′-GATCCG-CCAAAGGTTCATA-3′; G3B, containing domain B of the rat glucagon G3 enhancer-like element from −247 to −234 (41, 45); Glu-C/EBP, containing the C/EBP binding site of the rat glucagon gene from −241 to −212 (47); CGa-CAT, containing a CACAT sequence motif from the proximal promoter of the human gene encoding the α subunit of glycoprotein hormones (45); CTF/NF-I consensus, consensuses sites for CTF/NF-I (48, 49), 5′-GATCCCTTTTGTGTTGAACGCGGATGGA-3′; MSV-CAT, containing the NF-Y-binding CAAT box from the long terminal repeat of the Moloney murine sarcoma virus (50, 51), 5′-GATCCAGCGAACTGGTTAGTTCA-3′; G3B, containing the NF-Y-binding CAAT box from the murine class II genes of the major histocompatibility complex (51); 5′-GATCCATTTCGTTGATGTTG-3AAAATGA-3′.

Materials—A stock solution of forskolin (100 mM) was prepared in dimethyl sulfoxide and further diluted in cell culture medium. Controls received the solvent only.

RESULTS

Mutational Analysis of Nuclear Protein Binding to the Insulin CRE—Using an electrophoretic mobility shift assay, it has been shown previously that three unidentified nuclear proteins bind to the rat insulin I gene CRE (complexes 1, 2, and 3) (Ref. 22; see also Fig. 2A, lane 3). As a first approach to characterize these insulin CRE-binding proteins a mutational analysis was performed. The sequences of the oligonucleotides used are shown in Fig. 1. Oligonucleotides containing the insulin CRE wild-type sequence or mutants 1 to 4 were labeled and incubated with nuclear extracts from the insulin-producing β-cell line HIT. The labeled CRE of the rat somatostatin gene was used for comparison. As shown in Fig. 2A, complex 1 binding to the insulin CRE was also detected using mutant 2 as probe, whereas complexes 2 and 3 were no longer formed. In contrast, when mutants 3 or 4 were used as probes, complex 1 was not detectable, whereas complexes 2 and 3 persisted (Fig. 2A). It has been shown previously (22) and was confirmed in the present study (not shown) that complexes 1, 2, and 3 are not formed with labeled mutant 1. Note that a new protein complex was detected with labeled mutant 3 that comigrated with complex A on the labeled somatostatin CRE (Fig. 2A, compare lanes 2 and 1). A band of similar mobility but lower intensity was detected with labeled mutant 4 (Fig. 2A, lane 5). Labeled mutant 4 showed also an additional band migrating more slowly than complex 1 (Fig. 2A, lane 5); this band was not further investigated. The results of this mutational analysis are summarized in Fig. 2B, which indicates the mutations that abolish the binding of complex 1 or complexes 2 and 3, respectively. The bases that are required for the binding of bands 2 and 3 fall within a sequence that shows high similarity with a consensus motif for binding sites of HNF-3 proteins and related members.
of the winged helix family of transcription factors (match of 10 bases out of 12) (52) (Fig. 2B). The bases that are critical for the binding of complex 1 are very similar to a consensus NF-Y-binding site (match of 8 bases out of 10) (53) (Fig. 2B).

**Evidence That Complexes 2 and 3 Represent Winged Helix-like Proteins**—The significance of these sequence similarities was further investigated. As shown in Fig. 3A, nuclear protein binding to labeled insulin CRE forming complexes 2 and 3 was selectively competed away by an oligonucleotide (TTR-HNF-3) that contained a well characterized binding site for HNF-3 proteins in the transthyretin promoter (46, 52). It was, however, not competed away by an oligonucleotide (TTR-HNF-4) that contained another fragment from the transthyretin promoter (TTR-HNF-4) that contained another fragment from the transthyretin promoter that lacked complex 2 and 3 binding but did selectively compete for protein binding to labeled m3-InsCRE which comigrated with complex 3 of labeled InsCRE (Fig. 3A); at the same time the intensity of the band comigrating with complex 2 was only somewhat reduced by TTR-HNF-3 (Fig. 3A), revealing that the mutation in mutant 3 allowed the binding of two new proteins, one comigrating with complex A of labeled SomCRE as mentioned earlier (Fig. 2A) and one that, although it does not comigrate with, is distinct from complex 2; noteworthy, this complex does comigrate with complex B of labeled SomCRE (see Fig. 2A). The sequence similarity of their binding sites within the insulin CRE to an HNF-3 consensus site and the competition by TTR-HNF-3 suggest that the binding specificity of the proteins forming complex 2 and 3 is related to that of winged helix proteins. Winged helix proteins share a conserved DNA-binding domain and thus exhibit related but distinct DNA-binding specificities (52, 54, 55). The winged helix protein HNF-3β is expressed in islets and also in HIT cells (56–58). However, the addition of antisera directed against HNF-3β, -3α, or -3γ to the binding reaction did not affect nuclear protein binding to the insulin CRE (data not shown). Evidence has been presented recently that another, not yet identified member of the winged helix family of transcription factors binds to the glucagon and somatostatin genes (45); it interacts with domain B of the glucagon G3 element and the...
somatostatin upstream element (45). When an oligonucleotide containing domain B of the glucagon G3 element (G3B) was used as probe, protein complexes were formed that comigrated with complexes 2 and 3 of the insulin CRE (Fig. 3B). Cross-competition of the insulin CRE and G3B for binding to these complexes was observed using a similar molar excess of the competitors (Fig. 3B). Thus, based on their binding specificity, the proteins forming complexes 2 and 3 on the insulin CRE could be members of the winged helix family of transcription factors distinct from HNF-3b but the same as those binding to the glucagon G3 element.

Complex 1 Represents Binding of NF-Y—The binding site of complex 1 within the insulin CRE contains a CAAT motif with strong similarity to an NF-Y consensus site (Fig. 2B). Sequences related to “CAAT boxes” can, however, be recognized by diverse transcription factors with distinct DNA-binding domains including C/EBP (59) and CTF/NF-I (48). As shown in Fig. 4A, complex 1 with the insulin CRE is not competed away by oligonucleotides containing a C/EBP-binding site (Glu-C/EBP) (47) or an CTF/NF-I consensus site (49). Complex 1 binding is, however, competed away by CAAT boxes from the α subunit of the choriongonadotropin gene (CGα-CAAT) (60), the long terminal repeat of the Moloney murine sarcoma virus (MSV-CAAT) (50, 51), and the Ea gene from the murine class II genes of the major histocompatibility complex (Ea-CAAT) (51) (Fig. 4A), all of which are recognized by NF-Y (50, 51, 60). CGα-CAAT competed also for complex 2 and 3 binding (Fig. 4A), consistent with the fact that CGα-CAAT is recognized by NF-Y and the cAMP Response of the Insulin Gene

**Fig. 4. Complex 1 represents binding of NF-Y.** Nuclear extracts were incubated with the labeled oligonucleotides indicated. Specific protein complexes formed with InsCRE are indicated as complexes 1–3. F, free probe. A, competition experiments with unlabeled oligonucleotides containing various CAAT box motives. Competitors were added at a 50-fold (lanes 5, 7, 10, and 12), 150-fold (lanes 4, 6, 8, 11, and 13), 500-fold (lane 2), or 1,500-fold (lane 3) molar excess. B and C, cross-competition between InsCRE and Ea-CAAT, a well characterized NF-Y-binding site. Competitors were added at a 5-, 50-, and 500-fold molar excess (from left to right). D, effect of a specific antiserum directed against the B subunit of NF-Y (α-NF-YB). Preimmune serum (−) or α-NF-YB (+) as indicated on top of the lanes were added to the binding reaction. The asterisk indicates a band which appeared in the presence of α-NF-YB when added to the binding reaction with labeled InsCRE (“super-shifted band”).
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FIG. 5. Mutational analysis of sequences within the insulin CRE required for basal and cAMP-induced transcription. Four copies of the insulin CRE oligonucleotide with wild-type sequence (Ins-CRE) or the insulin CRE mutants 1 to 4 (m1-InsCRE, m2-InsCRE, m3-InsCRE, and m4-InsCRE) were placed in front of the minimal rat insulin I promoter (from −85 to +49) fused to the luciferase reporter gene. −, promoter alone. The plasmids were transfected into HIT cells. The figures on top of the bars indicate the fold stimulation by forskolin (10 μM). Luciferase activity is expressed relative to the mean value, in each experiment, of the activity measured in the control (promoter alone, no treatment). Values are mean ± S.E. of four independent experiments, each done in duplicate.

The winged helix-like proteins binding to G3B (45). Cross-competition was observed between InsCRE and Eo-CAT as increasing amounts of InsCRE and Eo-CAT competed for complex 1 with labeled InsCRE (Fig. 4B) and protein binding to labeled Eo-CAT (Fig. 4C). Irrespective of whether InsCRE or Eo-CAT were used as probe, Eo-CAT was a stronger competitor than InsCRE (Fig. 4, B and C), suggesting that Eo-CAT binds with somewhat higher affinity. Competition for protein binding to Eo-CAT was also seen with m2-InsCRE, whereas m4-InsCRE (NF-Y site mutated) did not compete (Fig. 4C), indicating that the competition by InsCRE was specific. The addition of an antiserum directed against NF-Y to the binding reaction abolished complex 1 of labeled InsCRE, whereas complexes 2 and 3 remained unaffected (Fig. 4D). Protein binding to labeled Eo-CAT comigrated with complex 1 binding to the insulin CRE and was markedly reduced by the anti-NF-Y antiserum (Fig. 4D). These results, when taken together, strongly suggest that complex 1 represents the binding of NF-Y or a closely related protein to the insulin CRE.

Mutational Analysis of Sequences within the Insulin CRE That Are Required for Function—As a first approach to study the functional significance of the proteins binding to the insulin CRE, the transcriptional activity of the insulin CRE and the mutants 1 to 4 was investigated in transient transfection experiments. Four copies of the insulin CRE wild-type sequence or mutants 1 to 4 were cloned in front of the truncated insulin promoter (from −85 to +49) fused to the luciferase reporter gene. These fusion genes were transiently transfected into HIT cells. As has been shown before (22), the insulin CRE conferred basal activity (12.6-fold increase) as well as modest cAMP responsiveness through m4-InsCRE 12-fold and through m3-InsCRE 148-fold (Fig. 5).

Transcriptional Activity of Binding Sites for NF-Y and Winged Helix-like Proteins in Islet Cells—The above results obtained with mutant 2, which lacks complex 2 and 3 binding, and with mutants 3 and 4, which lack NF-Y binding, suggest that basal activity may be conferred to the insulin CRE by NF-Y; NF-Y may not, however, mediate cAMP responsiveness. Consistent with this assumption the NF-Y-binding site Eo-CAT (binding complex 1) conferred basal activity to the minimal promoter but not cAMP responsiveness (Fig. 6). Basal activity of Eo-CAT was less than that of InsCRE (Fig. 6), which may be explained by a different spacing of the NF-Y-binding sites in the oligomerized Eo-CAT construct which allows functional synergism in a somewhat less efficient way. G3B, which binds the winged helix-like proteins (complexes 2 and 3), did not show any transcriptional activity (Fig. 6). These data suggest that proteins others than NF-Y and the winged helix-like proteins of complexes 2 and 3 confer cAMP responsiveness to the insulin CRE.

The Mutations in Mutants 1 to 4 Alter CREB Binding—It has been shown before that the transcription factor CREB is not detected among the nuclear proteins that bind to the labeled insulin CRE in the electrophoretic mobility shift assay (22). This is confirmed in Fig. 7A showing that the addition of an antiserum directed against CREB to the binding reaction had no effect on nuclear protein binding to the insulin CRE (compare lanes 5 and 6). The anti-CREB antiserum did, however, abolish the binding of complexes A and B to the labeled somatostatin CRE used as a positive control (Fig. 7A, compare lanes 1 and 2). The somatostatin CRE is a well characterized high affinity CREB-binding site (1–3). The base substitution in mutant 3 converts the CRE octamer-like sequence of the insulin CRE (TGACGTCCA) into a perfect CRE octamer consensus sequence (TGACGTCAT) (Fig. 1). It was noted above that two new
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Fig. 7. Effect of the mutations in mutants 1 to 4 on CREB binding to the insulin CRE as revealed by the electrophoretic mobility shift assay. Nuclear extracts were incubated with the labeled oligonucleotides indicated. Specific protein complexes formed with labeled InsCRE are indicated as complexes 1–3. Specific protein complexes formed with labeled SomCRE and containing proteins with CREB-like immunoreactivity are indicated as complexes A and B, F, free probe. A, proteins with CREB-like immunoreactivity are part of the protein complexes binding to labeled m3-InsCRE but not of the protein complexes binding to labeled InsCRE. Effect of a specific anti-CREB antiserum. Preimmune serum (Pre) or anti-CREB antiserum (Anti) as indicated on top of the lanes were added to the binding reaction. The asterisk indicates bands which appeared in the presence of anti-CREB antiserum when added to the binding reaction with labeled SomCRE or m3-InsCRE (super-shifted band). B, effect of the mutations in mutants 1, 2, and 4 on CREB binding to the insulin CRE as detected by the competition of InsCRE for the binding of proteins with CREB-like immunoreactivity to labeled SomCRE. Competitors were added at a 50- and 500-fold molar excess from left to right.

Weak binding of cellular CREB or closely related proteins to the insulin CRE can be detected by the electrophoretic mobility shift assay in competition experiments (22). This is shown in Fig. 7B. The binding of nuclear proteins with CREB-like immunoreactivity to the labeled somatostatin CRE (complexes A and B) is competed away by unlabeled somatostatin CRE and at a higher molar excess also by the insulin CRE (Fig. 7B). Mutant 1 had lost CREB binding (Fig. 7B, compare lanes 6 and 4), and mutant 2 competed somewhat less efficiently for complexes A and B than InsCRE wild-type sequence (Fig. 7B, compare lanes 9 and 4). Mutant 4 was a stronger competitor for CREB binding than InsCRE wild-type sequence (Fig. 7B, compare lanes 10 and 3). These results show that the mutations in mutants 1 to 4 of the insulin CRE change the affinity of the binding to CREB or closely related proteins; mutant 2 and, more so, mutant 1 show a decrease in binding whereas mutants 4 and 3 show an increase in binding.

Functional Significance of CREB and NF-Y Binding to the Insulin CRE as Assessed by Using Dominant Negative Mutants—The binding of CREB or closely related proteins to the insulin CRE, although weak, could be functionally significant. To further explore this possibility co-transfection experiments were performed using an expression vector encoding a dominant negative mutant of CREB, KCREB (39). Whereas forskolin stimulated insulin CRE-mediated transcription in controls, forskolin had no stimulatory effect when in addition KCREB was overexpressed (Fig. 8A), indicating that CREB or closely related proteins are required for cAMP responsiveness of the insulin CRE. Forskolin slightly decreased activity in the presence of KCREB (Fig. 8A), which is similar to what was found using m1- and m2-InsCRE (Fig. 5) and remains unexplained. When taken together with the results from the mutational analysis of the insulin CRE showing that mutations to the insulin CRE that increase CREB binding enhance cAMP responsiveness, whereas mutations to the insulin CRE that decrease CREB binding prevent cAMP responsiveness (see above), the present data strongly suggest that cAMP responsiveness is conferred to the insulin CRE by CREB or closely related proteins.

To further explore the role of NF-Y, a dominant negative mutant of NF-Y, NF-YA29 (40), was used. When increasing amounts of an expression vector encoding NF-YA29 were co-transfected with the insulin CRE reporter fusion gene, basal activity conferred by the insulin CRE (11.7 ± 1.2 relative to the minimal promoter) was decreased to 8.9 ± 0.6 (co-transfection of 0.025 μg/dish of the NF-YA29 plasmid), 8.0 ± 0.8 (0.150 μg), 7.0 ± 1.0 (0.5 μg), and 3.9 ± 0.0 (3 μg) (n = 6 each). These results independently confirm the conclusion drawn from the results of the mutational analysis of the insulin CRE and from the transcriptional activity of Eo-CAAT (see above) that NF-Y binding confers basal activity to the insulin CRE. Co-transfection of increasing amounts of the NF-YA29 expression vector enhanced cAMP responsiveness of the insulin CRE (Fig. 8B). While forskolin stimulated insulin CRE-mediated transcription 3.5-fold in controls, forskolin stimulated transcription up to 11-fold in the presence of the dominant negative NF-Y mutant (Fig. 8B). This effect was specific, because co-transfection of increasing amounts of the NF-YA29 expression vector had no effect on the stimulation by forskolin of transcription mediated by m4-InsCRE which lacks NF-Y binding (Fig. 8B). These data suggest that NF-Y binding to the insulin CRE not only confers basal activity but also decreases cAMP responsiveness.

It has been shown before that the insulin CRE does not
In the present study the insulin CRE-binding proteins and their function were characterized. It is shown that in addition to CREB, which interacts with low affinity with the CRE octamer-like sequence, the transcription factor NF-Y and winged helix-like proteins bind to overlapping sites within the insulin CRE. This combination of CRE-binding proteins forms a composite CRE with gene-specific properties. Cyclic AMP responsiveness is mediated by CREB; however, NF-Y binding to the insulin CRE confers basal activity and modulates the function of CREB. The data offer an explanation why the insulin CRE has considerable basal activity but is less responsive to cAMP stimulation than others.

The winged helix protein HNF-3β is expressed in insulin-(56) and glucagon-producing islet cell lines (57, 58, 61) and regulates glucagon gene transcription through a binding site within the G2 element of the glucagon gene (57, 61). The winged helix-like proteins binding to the insulin CRE are distinct from HNF-3β. Although it is not excluded that they enhance responsiveness of the insulin CRE, the present study does not establish a role for these proteins and, thus, their identity and functional significance remains to be shown. Islet
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Fig. 9. Role of CREB and NF-Y in the regulation of the insulin promoter. A, effect of an internal 4-base deletion inside the CRE octamer-like sequence on insulin promoter activity. HIT cells were transfected with the plasmids −410InsLuc or −410(Δ−183/−180)InsLuc. Forskolin, 10 μM. Luciferase activity is expressed relative to the mean value, in each experiment, of the activity measured in the −410InsLuc control. Values are mean ± S.E. of three independent experiments, each done in triplicate. B, effect of overexpression of the dominant negative mutants KCREB and NF-YA29 on cAMP-induced insulin promoter activity. HIT cells were co-transfected with the reporter plasmid −410InsLuc and expression vectors encoding KCREB or NF-YA29, respectively. Values are mean ± S.E. of the forskolin-induced increase in reporter enzyme activity of a representative experiment with four dishes per group.

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Several lines of evidence strongly suggest that NF-Y binds to the insulin CRE. First, the insulin CRE contains a CCAAT box motif with high similarity to an NF-Y consensus site. Second, this CCAAT box motif is recognized by a nuclear protein (complex 1). Third, complex 1 binding is not competed for by binding sites of the CAAT box-binding proteins CTF/NF-I or C/EBP, but is competed for by several NF-Y binding sites. Fourth, there is cross-competition for comigrating complexes between the insulin CRE and a well characterized NF-Y-binding site, Eo–CAAT. Finally, complex 1 is recognized by an antisense directed against NF-YB. NF-Y (also called CBF and CPI) is a ubiquitous CCAAT box-binding protein and extremely conserved in evolution (40, 53, 65, 66). Like the TATA box, CCAAT boxes are widespread promoter elements typically located between −60 and −100 base pairs 5′ to the start of transcription, suggesting a role in basal transcription (67). Most CCAAT boxes located at this position are NF-Y-binding sites (67). NF-Y has been shown to interact with the TATA box-binding protein (68), to be important for transcription re-initiation (43), and to facilitate in vivo recruitment of upstream DNA binding transcription factors (69). Thus, NF-Y may have a general role in the assembly of proximal promoter complexes. Although, in general, NF-Y is constitutively active, it has been implicated directly or indirectly in the regulation of some promoters by, for example, heme (70), sterol (71, 72), calcium depletion (73), and the viral Tax protein (74). In the insulin promoter the NF-Y-binding CCAAT box is located further upstream at −179 and falls within an element that confers CAMP responsiveness. The CCAAT box motif has been implicated in the cAMP inducibility of several genes (33–36). Some of these CCAAT boxes have been shown to bind NF-Y. Boularand et al. (36) reported that an inverted CCAAT box motif at −67 of the human tryptophan hydroxylase gene promoter was required and sufficient for CAMP inducibility in pinealocytes. This inverted CCAAT box in the mouse gene has been shown to bind NF-Y (75). In addition, it has been demonstrated that a CCAAT box that binds NF-Y can mediate induction by CAMP of the rat hexokinase II gene in L6 myotubes (35). However, the present study clearly demonstrates that NF-Y does not confer CAMP responsiveness to the insulin CRE in islet cells.

Instead, CAMP responsiveness seems to be conferred by CREB. In the absence of other proteins, CREB binds with low affinity to the insulin CRE (Ref. 22 and this study). This may largely be due to the deviation in position 8 of the core octamer-like motif of the insulin CRE (TGACGTCA) from the consensus sequence (TGACGTCc) rather than to the bases flanking the octamer in the insulin CRE. This is suggested by mutant 3 which bound CREB much more strongly than the wild-type or mutant 4. The x-ray crystal structure of the AP-1 DNA complex indicates that the corresponding position in the AP-1 site is close to the start of transcription, whereas CREB binds to a site that is further upstream. The results of the present study, when taken together, strongly suggest that CREB does not confer CAMP responsiveness to the insulin CRE in islet cells.

Whereas NF-Y does not confer CAMP responsiveness, the
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present results define a role for NF-Y in insulin CRE-mediated transcription. Insulin CRE mutants that lack NF-Y binding still responded to cAMP but had lost basal activity. An NF-Y-binding site alone was not cAMP responsive but did confer basal activity in islet cells. NF-Y and NF-YC associate with each other to form a binary complex which then interacts with NF-YA. Formation of this ternary complex is required for binding to DNA (40, 53, 65, 66, 77). Mutants of NF-Y subunits, such as NF-YA29 (40), that still interact with the other subunits but do not bind DNA act as dominant negative mutants (40, 66). Using this technique, the present study shows that inactivation of NF-Y in islet cells decreases basal activity of the insulin CRE while it enhances cAMP responsiveness. Thus, these data indicate that NF-Y modulates the function of CREB. NF-Y decreases the ability of CREB to mediate CAMP-induced gene transcription. It also inhibits depolarization responsiveness, and through NF-Y the insulin CRE gains constitutive basal activity that is conferred to the insulin promoter (22, 23, 26). The interaction of CREB with NF-Y at the insulin CRE could include direct protein-protein interaction. The transcription factor YY1, that binds immediately downstream of the CRE of the mouse c-fos promoter, interacts directly with CREB, and this interaction has been suggested to lead to repression of CRE-dependent transcription (17). A direct interaction between CREB and NF-Y remains to be shown. However, as the basis of functional synergism, evidence has already been presented for complex formation between NF-Y and other transcription factors such as C/EBP proteins on the serum albumin promoter (78) and ATF-2 on the fibroenectin promoter (79). Alternatively, CREB and NF-Y could compete for binding to the insulin CRE as their binding sites overlap. This is reminiscent of CREB-binding sites which overlap with binding sites for the glucocorticoid receptor in the human glycoprotein hormone α-subunit gene (14) or for CREBβ and related proteins in the phosphoenolpyruvate carboxykinase gene (15, 18–21). In these cases a specific negative regulation by glucocorticoids (14) and a tissue-dependent activation by cAMP (15, 18–21), respectively, is assumed to be achieved through these overlapping sites.

To maintain glucose homeostasis, insulin gene transcription appears to be regulated synergistically by glucose metabolism and the hormonally regulated CAMP pathway (24, 25). Studies on many genes have taught that promoter activity depends on a synergistic interaction between multiple promoter-binding proteins. Transcriptional activation in response to extracellular signals thus involves the assembly of multicomponent complexes on enhancers and promoters induced by regulated transcription factors through interaction with other proteins. Consistent with this view, several specific cooperative effects of CREB have been shown including synergistic interactions with HNF-4 in the tyrosine aminotransferase gene (80), liver-enriched transcription factors in the phosphoenolpyruvate carboxykinase gene (15), and steroidogenic factor-1 in the aromatase CYP19 gene (81). Some of the insulin CRE-binding proteins identified in the present study are therefore likely to interact with constitutive or glucose-regulated transcription factors of the insulin promoter. In the absence of glucose, stimulation of insulin gene transcription by cAMP is modest. Together with the nonpalindromic sequence of the CRE octamer motif, the binding of NF-Y to the insulin CRE that inhibits the ability of CREB to mediate cAMP-induced transcription reported in our studies may be wholly or partially responsible for this modest response.

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