The insulin gene is efficiently expressed only in pancreatic beta cells. Using reverse transcriptase-polymerase chain reaction analysis, we show that insulin mRNA levels are at least 10^4-fold higher in beta cells than non-beta cells. To examine the underlying mechanisms, we expressed beta cell transcription factors by transfection of non-beta cells. Separate expression of BETTA2, E2A, or PDX1 led to modest (<10-fold) activation of the insulin promoter, whereas co-expression of the three proteins produced synergistic, high level activation (160-fold). This level of activity is ~25% that observed in transfected beta cell lines. Of the three factors studied, BETTA2 appears to play a dominant role. Efficient transcription required a C-terminal activation domain of BETTA2 and an N-terminal region, which does not function as an independent activation domain. The myogenic basic helix-loop-helix (bHLH) protein MyoD was unable to bind and activate the promoter, even when its DNA binding region was replaced with that of BETTA2. Our results demonstrate the central importance of BETTA2 in insulin gene transcription and the importance of sequences outside the canonical DNA binding domain in permitting efficient DNA binding and cell-specific activity of the insulin gene promoter.

Expression of the insulin gene in adult mammals is restricted with great specificity to the pancreatic beta cells (1). The mechanisms involved are primarily transcriptional and operate through a number of well studied cis elements located in the proximal promoter region of the gene (2, 3). Several transcription factors have been shown to bind to these cis elements and are implicated in regulation of insulin gene transcription. The best characterized of these are the basic helix-loop-helix (bHLH) proteins E2A (4, 5) and BETTA2 (NeuroD1) (6, 7) and the homeodomain protein PDX1 (IPF1/STF1/IDX1) (8–12). The E2A proteins E12/E47 and the products of the (6, 7) and the homeodomain protein PDX1 (IPF1/STF1/IDX1) (8–12). The E2A proteins E12/E47 and the products of the two sites (E1 and E2) on the insulin promoter (6, 15). Likewise PDX1 binds to the A1 and A3/4 regions of the insulin promoter. The phenotypes of mice bearing disrupted alleles of BETTA2, PDX1, and other potential insulin gene transcription factors Pax4, Pax6, Nkx2.2, and Nkx6.1 indicate that these proteins may play an important role in beta cell differentiation also (reviewed in Refs. 16 and 17).

Previous studies have focused on the activity of individual factors or a limited subset of the known factors, primarily using artificial promoter fragments (6, 11, 12, 18, 19). The aim of the present study was to analyze a more physiologically relevant situation, namely the action of several of the known factors (E2A, BETTA2, and PDX1) on the insulin promoter. We show that in transfected non-beta cells, combined expression of these three factors leads to dramatically elevated expression as compared with levels observed in the presence of each factor alone. This level corresponds to ~25% of the promoter activity observed in transfected beta cells. In turn, this represents at least a 100-fold lower specificity as compared with differential steady state insulin mRNA levels as measured by reverse transcriptase-polymerase chain reaction (RT-PCR) reaction. We have further examined the sequence requirements of the BETTA2 protein for this activity and find that efficient activation requires distinct functional domains of the BETTA2 molecule. Substitution with domains derived from the muscle bHLH protein MyoD cannot generate efficient activation of the insulin promoter, in part because binding to the promoter fragment is inefficient.

EXPERIMENTAL PROCEDURES

DNA Constructs—Expression vectors encoding E2A (E47) (20) and PDX1 (8) were generated by insertion of the full-length protein-coding sequences to the vector pCOS3 (Stratagene). A BETTA2 (6) expression vector was generated by insertion of the full-length protein-coding sequences to the vector pCGN-HA (21). In both vectors, expression is under the control of the cytomegalovirus (CMV) promoter. Gal4-BETTA2 hybrid constructs were generated by PCR reaction using Pwo DNA polymerase (Roche Molecular Biochemicals). The resulting BETTA2 fragments were introduced into an expression vector encoding the Gal4 DNA binding domain under the control of the CMV promoter. The constructs were named according to the N- and C-terminal amino acid.

DNA fragments encoding deleted and substituted BETTA2 were generated by PCR and introduced into pCGN-HA. The mouse MyoD cDNA (22) was used for generating hybrid proteins with BETTA2. pOK1 (23) contains 410 base pairs of the rat insulin 1 gene promoter upstream of the chloramphenicol acetyltransferase (CAT) reporter. To generate pRLNT-CAT, the SV40 promoter of the plasmid pGL3 promoter (Promega) was replaced by the 410 base pairs of rat insulin 1 gene promoter. The plasmids 5Gal4.E1b.CAT (24) and pSV-LUC (25) have been described previously. The insulin-M-CAT reporter plasmid was constructed by substituting the insulin E box motifs in pOK1 by the muscle creatine kinase (MCK) E box motifs using PCR with primers containing the MCK E box sequence 5'-AACACCTG-3' (26). The relevant regions of plasmids constructed by PCR were verified by DNA sequencing.

Cell Culture and Transfections—Cells were grown in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal calf serum, penicillin (100 unit/ml), and streptomycin (100 μg/ml). The hybrid cell lines Rin-
m × L were established and cultured as described (27). The cell line HeLa (Tet-off) PDX1 was generated by stable transfection of the line HeLa Tet-off (CLONTECH) with pTet-splice-PDX1, a plasmid containing the PDX1-coding sequence under the control of TRE (tetracycline response element) in the vector pTet-splice (28); HeLa (Tet-off) cells were transfected with the plasmids pTet-splice-PDX1 (30 μg) and pSV2-HYG (1.5 μg). Clones were selected in the presence of 200 μg/ml hygromycin, 100 μg/ml G418, and 2 μg/ml tetracycline (tet). Surviving clones were tested for their ability to express PDX1 in a tet-repressible manner. Cells were grown in the absence or presence (2 μg/ml) of tet for 24–48 h, and PDX1 expression was tested by immunoblotting using a monoclonal antibody directed against PDX1. One of the clones obtained, designated HeLa (Tet-off) PDX1, showed no detectable PDX1 protein in the presence of 2 μg/ml tet but strongly inducible PDX1 in the absence of tet (data not shown); the clone also showed closely similar ability to activate the transfected insulin CAT reporter plasmid pOK1 as compared with that obtained following transient transfection of HeLa cells with a PDX1 expression vector, pCDNA3-PDX1 (data not shown).

HeLa (Tet-off), HIT cells (hamster insulinoma (29)) and 293T cell lines were transfected using the calcium phosphate co-precipitation procedure (30). HeLa (Tet-off) PDX1 cells were plated 16 h before transfection and cultured in the absence of tet to induce PDX1 expression. Cells were cotransfected with 5 μg of CAT reporter plasmid and the indicated amount of expression plasmids and luciferase (pRSV-LUC) internal control plasmid. CAT activity was measured by the phase extraction procedure (31) and normalized according to the luciferase activity of the cotransfected internal control plasmid. Each experiment was repeated at least three times. Results shown are mean ± S.E.

**Western Blot Analysis**—Nuclear extracts were prepared from transfected cells as described previously (4). Fifty μg of protein extract were separated on 10% SDS-polyacrylamide electrophoresis gels and transferred electrophoretically to nitrocellulose sheets. The membrane was probed with the indicated primary antibodies followed by a secondary antibody conjugated to horseradish peroxidase and visualization using the enhanced chemiluminescence reaction.

**Electrophoretic Mobility Shift Assay (EMSA)**—Double strand oligonucleotides (5 pmol) were end-labeled with [γ-32P]dATP using DNA polymerase I, Klenow fragment. The activity obtained was typically 2–3 × 10^6 cpm/ml. The protein extract (4 μg) was incubated for 10 min on ice in binding buffer (25 mM Heps, pH 7.9, 150 mM KCl, 10% glycerol, 5 mM dithiothreitol) containing 600 ng of poly(dI-dC) (Sigma) and 600 ng of poly(dAdT) (Sigma) in a final assay volume of 14 μl. Subsequently, probe was added (1 μl, ~20,000 cpm), and incubation was allowed to continue for an additional 20 min. Samples were subsequently resolved on 6% polyacrylamide gels (30%:0.8 acrylamide:bisacrylamide) at 184 V (constant voltage) for 1 h at 4 °C. Running buffer contained 30 μM Tris, 195 μM glycine (pH 8.5). All binding and electrophoresis steps were performed at 0 °C to 4 °C.

The following sequences were used:

**RT-PCR and Southern Analysis**—Poly(A)-containing RNA isolated from Rin-m cells (rat insulinoma cells), L cells (mouse fibroblasts), and hybrid Rin-m × L cells were used to make cDNA using reverse transcriptase (RT) and oligo(dT). Yeast tRNA was added to RT reactions to activate the transfected insulin CAT reporter plasmid pOK1 as compared with that obtained following transient transfection of HeLa cells with a PDX1 expression vector, pCDNA3-PDX1 (data not shown).

HeLa (Tet-off), HIT cells (hamster insulinoma (29)) and 293T cell lines were transfected using the calcium phosphate co-precipitation procedure (30). HeLa (Tet-off) PDX1 cells were plated 16 h before transfection and cultured in the absence of tet to induce PDX1 expression. Cells were cotransfected with 5 μg of CAT reporter plasmid and the indicated amount of expression plasmids and luciferase (pRSV-LUC) internal control plasmid. CAT activity was measured by the phase extraction procedure (31) and normalized according to the luciferase activity of the cotransfected internal control plasmid. Each experiment was repeated at least three times. Results shown are mean ± S.E.

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The following sequences were used:

**1.** 5'-GATCCGGCATGCGAGAGACG-3'

**2.** 5'-TAAAGAGATGATGATGCAGC-3'

**RT-PCR and Southern Analysis**—Poly(A)-containing RNA isolated from Rin-m cells (rat insulinoma cells), L cells (mouse fibroblasts), and hybrid Rin-m × L cells were used to make cDNA using reverse transcriptase (RT) and oligo(dT). Yeast tRNA was added to RT reactions to ensure that at least 100 ng of RNA was present in each reaction. The PCR reaction was performed with the following oligonucleotide primers:

**1.** 5'-AAGAAGCCATACAGCAAGACG-3'

**2.** 5'-TGGCGGAGCTCGGTGACG-3'

The genome of rat and mouse contains two non-allelic insulin genes, denoted insulin I and insulin II; in both species, the insulin I gene contains a single intron, whereas the insulin II gene contains 2 introns (1). The above primers correspond to sequences found in exons 1 and 2 (insulin I gene) or exons 1 and 3 (insulin II gene), respectively, and are complementary to sequences of rat and mouse insulin genes. The cDNA should give a PCR product of 329 to 334 base pairs depending on the species and gene (insulin gene I or II), whereas genomic DNA should give significantly larger products.

**PCR reaction products were fractionated by electrophoresis on 1.75% agarose gel and blotted to nylon sheets (Nyttran). An oligonucleotide (MW23 5'-TAAAGAGATGATGATGCAGC-3' complementary to a region of the insulin mRNA between the primers was end-labeled with [γ-32P]dATP and T4 kinase (NEN Life Science Products) and used as a hybridization probe for Southern blots containing PCR products.**

**RESULTS**

Northern analysis revealed high levels of insulin mRNA in beta cells but undetectable levels in non-beta cell lines and insulinoma × fibroblast hybrid cell lines (27, 32). We have now used a more sensitive method, RT-PCR, capable of detecting much lower concentrations of insulin mRNA. The analysis was performed with RNA isolated from the beta cell line Rin-m, the fibroblast cell line L, and hybrid cells Rin-m × L (Fig. 1). Under the conditions of the analysis, signal intensity was dependent on the amount of input RNA in the range 50 ng–50 μg of poly(A) RNA (Fig. 1, lanes 1–7). The procedure is sensitive enough to permit detection of insulin mRNA in RT-PCR reactions derived from 0.5 pg of Rin-m poly(A) RNA (band indicated by lower arrow in Fig. 1, lane 6). Yet with 50 ng of poly(A) RNA from Rin-m × L cells or L cells, little or no signal was observed (a signal arising from residual genomic DNA was detected, indicated by the upper arrow in Fig. 1, lanes 8–10). Thus, insulin mRNA levels in hybrid cells and fibroblasts are at least 10^5-fold reduced as compared with Rin-m cells. A similar differential was obtained upon comparison of the hamster beta cell line, HIT with fibroblasts (data not shown).

To evaluate the role of the proteins PDX1, E2A, and BET2 as potential insulin gene transcription factors, we expressed these proteins in a derivative of HeLa cells, HeLa (Tet-off) PDX1, and monitored the activation of a cotransfected reporter plasmid containing the natural rat insulin I gene promoter (−410 to −1). Whereas separate expression of each transcription factor led to fairly modest activity (0.3–4.9%), co-expression of all three led to a striking 160-fold synergistic increase in expression (Fig. 2). Expression of BET2 alone in combination with either E2A or PDX1 led to intermediate expression levels (20–60%) (Fig. 2). The activity observed with BET2 and PDX1 was unexpectedly high, since BET2 is thought to bind DNA inefficiently as a homodimer (6), and endogenous E2A levels are apparently rate-limiting under the conditions of the transfection (since expression of BET2 alone produces substantially lower expression than BET2 + E2A). BET2 seems to play a particularly important role in activation of the insulin gene promoter, since pairwise expression of these factors showed
moter (pOK1) together with expression plasmids (0.1 μg) encoding BETA2 and E2A. Expression of PDX1 was regulated by removal of tetracycline from the culture medium. The CAT activity was normalized to the activity of the internal control luciferase gene. The relative activity (mean ± S.E.) is shown as a percentage of that observed in the presence of E2A + BETA2 + PDX1.

much lower activation when BETA2 was absent as compared with that observed when either E2A or PDX1 were absent (Fig. 2). We obtained essentially identical results using parental HeLa cells as compared with HeLa (tet off) PDX1 (data not shown).

Insulin gene promoter activity in HeLa cells was compared with that observed in transfected beta cells (HIT). For this purpose, to correct for differences in transfection efficiency between cell types, we normalized expression to that observed with the CMV promoter, which functions efficiently in both cell types; similar results were obtained upon normalization with the Rous sarcoma virus promoter (data not shown). The maximal activity of the insulin gene promoter in HeLa cells, observed upon expression of E2A, BETA2, and PDX1 (~160-fold activation) was ~25% that of the insulin gene promoter activity observed in the HIT cell line (Fig. 3).

Because of its central importance in activation of the insulin promoter in this system, we examined the regions of the BETA2 protein required to produce high level expression. We generated plasmids encoding truncated versions of BETA2 (Fig. 4A). Truncations lacking either the C- or N-terminal portions showed significantly reduced ability to activate the insulin gene promoter (Fig. 4B). Like the wild type BETA2 protein, they showed very low activity when expressed alone (data not shown). C-terminal truncations showed more dramatic reduction (5-fold) than N-terminal truncations (2.5-fold) (Fig. 4B). EMSA analysis indicated that wild type and truncated BETA2 proteins were expressed at comparable levels (Fig. 4C). Thus both N- and C-terminal domains of BETA2 are necessary for efficient transcription by the insulin promoter.

To determine whether the observed effects of the truncations were due to the presence of transcription activation domains, expression plasmids were generated encoding portions of the BETA2 protein fused to the DNA binding domain of the yeast transcriptional activator Gal4. Plasmids were cotransfected to 293T human epithelial cells together with a CAT reporter plasmid containing the insulin promoter (prIns-LUC) alone or together with expression plasmids (0.1 μg) encoding BETA2 and E2A in the presence or absence of tet (to induce PDX1). HIT cells were transfected with pOK1. The luciferase activity was normalized to the activity generated by the CMV promoter (using the plasmid pCMV-LUC). The activity (mean ± S.E.) is shown relative to the basal activity of the insulin gene promoter in HeLa cells.

![Fig. 2](image1.jpg)

**Fig. 2.** The cooperative activation of the insulin gene promoter by BETA2, E2A, and PDX1. HeLa (Tet-off) PDX1 cells were cotransfected with a CAT reporter plasmid containing the insulin promoter (pOK1) together with expression plasmids (0.1 μg) encoding BETA2 and E2A. Expression of PDX1 was regulated by removal of tetracycline from the culture medium. The CAT activity was normalized to the activity of the internal control luciferase gene. The relative activity (mean ± S.E.) is shown as a percentage of that observed in the presence of E2A + BETA2 + PDX1.

![Fig. 3](image2.jpg)

**Fig. 3.** Activation of the insulin gene promoter in HeLa and HIT cells. HeLa (Tet-off) PDX1 cells were transfected with a luciferase reporter plasmid containing the insulin promoter (prIns-LUC) alone or together with expression plasmids (0.1 μg) encoding BETA2 and E2A in the presence or absence of tet (to induce PDX1). HIT cells were transfected with pOK1. The luciferase activity was normalized to the activity generated by the CMV promoter (using the plasmid pCMV-LUC). The activity (mean ± S.E.) is shown relative to the basal activity of the insulin gene promoter in HeLa cells.

![Fig. 4](image3.jpg)

**Fig. 4.** Mapping of domains of BETA2 involved in activating the insulin promoter. A, schematic map of truncated BETA2 (numbers refer to the amino acids (aa) of wild type BETA2). B, activation by wild type and truncated BETA2. HeLa (Tet-off) PDX1 cells were cotransfected with a CAT reporter plasmid containing the insulin promoter (pOK1) together with CMV expression plasmids (0.1 μg) encoding E2A and wild type and truncated BETA2 transcription factors in the absence of tet (to express PDX1). The CAT activity was normalized to the activity of the internal control luciferase gene. The relative activity (mean ± S.E.) is shown as a percentage of the activity observed in the presence of E2A + BETA2 + PDX1. C, protein expression levels as indicated by EMSA. Nuclear extracts were analyzed by EMSA using radiolabeled E1 DNA probe.

The cooperative activation of the insulin gene promoter by BETA2, E2A, and PDX1. HeLa (Tet-off) PDX1 cells were cotransfected with a CAT reporter plasmid containing the insulin promoter (pOK1) together with expression plasmids (0.1 μg) encoding BETA2 and E2A. Expression of PDX1 was regulated by removal of tetracycline from the culture medium. The CAT activity was normalized to the activity of the internal control luciferase gene. The relative activity (mean ± S.E.) is shown as a percentage of that observed in the presence of E2A + BETA2 + PDX1.

A) Schematic map of truncated BETA2 (numbers refer to the amino acids (aa) of wild type BETA2). B) Activation by wild type and truncated BETA2. HeLa (Tet-off) PDX1 cells were cotransfected with a CAT reporter plasmid containing the insulin promoter (pOK1) together with CMV expression plasmids (0.1 μg) encoding E2A and wild type and truncated BETA2 transcription factors in the absence of tet (to express PDX1). The CAT activity was normalized to the activity of the internal control luciferase gene. The relative activity (mean ± S.E.) is shown as a percentage of the activity observed in the presence of E2A + BETA2 + PDX1. C) Protein expression levels as indicated by EMSA. Nuclear extracts were analyzed by EMSA using radiolabeled E1 DNA probe.
that efficient transcription requires a trans-activation domain located at the C terminus of the BETA2 protein and a domain at the N terminus, which does not possess an independent activation function.

To further examine the sequence requirements for BETA2 activity, we generated additional expression plasmids based on comparison of the BETA2 protein with corresponding regions of the well studied MyoD protein. The rationale for this is that MyoD, like BETA2, is a bHLH protein that activates transcription as a heterodimeric complex with E2A and related class A bHLH proteins (13, 33) and in this process cooperates with other muscle-specific proteins (34). The intact MyoD protein was unable to efficiently activate the native insulin promoter (Fig. 6, A and B), most likely due to weak binding of MyoD-E2A heterodimers to the E box sequences of the insulin promoter. To test this, we generated a modified insulin promoter (insulinM) in which the two copies of the E box were replaced with sequences from the MCK promoter (Fig. 6A). Indeed, MyoD showed strongly increased ability to activate the modified promoter as compared with the wild type promoter (Fig. 6B).

As a further test of this, we replaced the DNA binding domain of MyoD with that of BETA2 (Fig. 7A). Unexpectedly, this construct showed sharply reduced transcriptional activity as compared with BETA2 (Fig. 7B). The reduction was not attributable to altered expression levels (Fig. 7C) but did correlate with impaired DNA binding activity (Fig. 7D). Previous studies with MyoD have indicated that three key amino acids, Ala-114, Thr-115 (located in the basic region), and Lys-124 (located in the junction between basic domain and the helix 1 of the HLH domain), play an essential role in myogenic activation (35). We tested the significance of the corresponding junction residue Gly in BETA2 by mutating it to Lys (as found in MyoD). No significant effect on the trans-activation activity was observed (Fig. 7). Taken together, the results indicate that the ability of BETA2 to bind DNA efficiently can be significantly affected by sequences outside the canonical DNA binding domain; the sequence of BETA2 is compatible with efficient DNA binding, whereas that of MyoD is not.

To examine the effect of the residues in BETA2 corresponding to Ala-114 and Thr-115 in MyoD, we generated two point-mutated variants of the BETA2 protein, substituting the amino acids Ala-105 and Asn-106 of BETA2 to Asn (as in E2A) and Thr as in MyoD, respectively (Fig. 8, A and B). These two BETA2 proteins bind DNA with similar efficiency as the wild type protein (Fig. 8D). However, in contrast to MyoD, in neither case was a substantial change in promoter activity observed (Fig. 8C). Thus it appears that the residues in BETA2 corresponding to key myogenic amino acids of MyoD are not crucial for activating insulin gene promoter activity in our system.
The aim of this study was to better characterize the cell-specific expression of the insulin gene and begin to evaluate quantitatively the role of known transcription factors in the process. We used RT-PCR analysis to compare steady state mRNA levels in cell lines. The data indicate a differential of at least 10^5-fold. In theory, this could include a contribution of selective mRNA stability. However, since differential insulin mRNA stability in beta versus non-beta cells has not been reported, it has been assumed that transcriptional regulation contributes the majority of the difference.

Most studies performed on the role of specific transcription factors on insulin gene transcription have used nonnatural promoter fragments. We have now established a system based on transfection of the natural insulin promoter and reconstitution of efficient activation in a non-beta cell line. We have been able to demonstrate that simultaneous expression of the three insulin gene transcription factors BETA2, PDX1, and E2A in HeLa cells leads to highly efficient transcription of the insulin promoter. Transcription is activated 160-fold to a level 25% that observed in transfected pancreatic beta cells. The increased activity in beta cells may be due to the presence of additional beta cell transcription factors, e.g., Pax6 and RIPE3b1 (16). By comparison, we have shown that the differential expression of insulin mRNA levels in pancreatic beta cells as compared with non-beta cells is at least 10^5-fold. The significantly higher specificity observed for the endogenous insulin gene as compared with a transfected gene is likely to reflect additional levels of control exerted on the natural endogenous gene, for example repression by chromatin.

To begin to dissect the mechanisms involved in generating this high level of transcription, we have focused on the role of BETA2, which appears to play a dominant role in the system. In addition to the bHLH region of the protein known to be essential for dimerization and DNA binding, we observe that

**DISCUSSION**

FIG. 7. Domains of BETA2 required for insulin gene promoter activation. A, schematic map illustrating proteins tested. B, trans-activation by BETA2 and BETA2/MyoD fusion proteins. Expression plasmids were generated encoding HA epitope-tagged wild type BETA2 hybrid proteins in which portions of BETA2 are substituted with corresponding regions of MyoD. The indicated expression plasmids (0.1 μg) were transfected into HeLa (Tet-off) PDX1 cells in the absence of tet (for expression of PDX1) together with the insulin CAT reporter plasmid (pOK1). Normalized CAT activity (mean ± S.E.) is shown relative to the activity observed in the presence of E2A, PDX1, and wild type BETA2. C, expression of BETA2/MyoD variants. Western blot analysis of 50 μg of nuclear extract protein using monoclonal antibodies directed against the hemagglutinin epitope. D, DNA binding of BETA2/MyoD variants. DNA binding was determined by EMSA using a radiolabeled E1 DNA probe. The data shown is representative of three independent experiments.

**FIG. 8.** Mapping of amino acids in the basic region of BETA2 required for promoter activation. A, schematic map of the basic region of E2A, BETA2, and MyoD illustrating the specific amino acids substituted in BETA2. B, comparison of amino acid sequences of the basic regions of E2A (E12 splice variant (13)), BETA2, and MyoD showing the myogenic amino acids of MyoD and the corresponding mutated amino acids of BETA2. C, expression plasmids were generated encoding wild type and mutated BETA2. The indicated expression plasmids (0.1 μg) were transfected into HeLa (Tet-off) PDX1 cells in the absence of tet (for expression of PDX1) together with the insulin CAT reporter plasmid (pOK1) (5 μg). Normalized CAT activity (mean ± S.E.) is shown relative to the activity observed in the presence of E2A, PDX1, and wild type BETA2. D, DNA binding as determined by EMSA. Nuclear extracts were analyzed by EMSA using radiolabeled E1 DNA probe.
domains located both at the N and C terminus are required for full activation of the insulin promoter. The C-terminal domain can function as an independent activation domain, whereas the N-terminal domain cannot. Possibly the N terminus of BETA2 contains a transcription activation domain that functions only in the context of the native BETA2 molecule or may contribute to recruitment of the heterodimerization partner E2A, which itself possesses two independent activation domains (20). These data confirm and extend the results of Sharma et al. (19) who show activation domain activity in the C terminus of BETA2 using Gal4 fusions. To further explore the functions of BETA2, we compared its properties with those of the related muscle bHLH protein MyoD. The MyoD protein showed inefficient activation of the insulin promoter, at least in part because of its weak ability to bind the insulin promoter E box. Indeed, MyoD efficiently activated a modified insulin promoter bearing preferred MyoD binding sites. On the other hand, a hybrid MyoD-BETA2 bearing the DNA binding domain of BETA2 is unable to efficiently activate the native insulin promoter because of inefficient DNA binding. Thus domains outside the canonical DNA binding domain can have a dominant effect on BETA2 DNA binding. This is reminiscent of observations made previously for the muscle bHLH family and other transcription factors together with analysis of additional, mechanistic analysis of the interactions between these and other transcription factors factors together with analysis of additional, less well studied topics such as chromatin structure.

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REFERENCES

1. Steiner, D. P., Chan, S. J., Welsh, J. M., and Kwok, S. C. M. (1985) Annu. Rev. Genet. 19, 463–484
2. Walker, M. D., Edlund, T., Boulet, A. M., and Rutter, W. J. (1983) Nature 306, 557–561
3. German, M., Ashcroft, S., Docherty, K., Edlund, T., Edlund, H., Goodison, S., Imura, H., Kennedy, G., Madsen, O., Melloul, D., Moss, L., Olson, K., Permutt, A., Philippe, J., Robertson, R. P., Rutter, W. J., Sherer, P., Stein, R., Steiner, D., Tsai, M.-J., and Walker, M. D. (1995) Diabetes 44, 1092–1004
4. Aronheim, A., Ohlson, H., Park, C. W., Edlund, T., and Walker, M. D. (1991) Mol. Cell. Biol. 11, 1734–1738
5. Naya, F. J., Stellrecht, C. M., and Tsai, M. J. (1995) Genes Dev. 9, 1009–1019
6. Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., and Weintraub, H. (1995) Science 268, 836–844
7. Ohlson, H., Karlsson, K., and Edlund, T. (1993) EMBO J. 12, 4251–4259
8. Leonard, J., Peers, B., Johnson, T., Ferreri, K., Lee, S., and Montminy, M. R. (1993) Mol. Endocrinol. 7, 1275–1283
9. Miller, C. P., McGehee, R. E., and Habener, J. F. (1994) EMBO J. 13, 1145–1150
10. Peers, B., Leonard, J., Sharma, S., Teitelman, G., and Montminy, M. R. (1994) Mol. Endocrinol. 8, 1798–1806
11. Serup, P., Jensen, J., Andersen, F. G., Jorgensen, M. C., Blume, N., Holst, J. J., and Madsen, O. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9015–9020
12. Murre, C., Bain, G., Vlandiakj A. M., Engel, L., Furnari, B. A., Massari, M. E., Matthews, J. R., Quong, M. W., Rivera, R. R., and Stuiver, M. H. (1994) Biochem. Biophys. Acta 1218, 129–135
13. Sander, M., and German, M. S. (1997) J. Mol. Med. 75, 327–340
14. Park, C. W., and Walker, M. D. (1992) J. Biol. Chem. 267, 15642–15649
15. Edlund, H. (1998) Diabetes 47, 1817–1823
16. St-Onge, L., Wehr, R., and Gruss, P. (1999) Curr. Opin. Genet. Dev. 9, 295–300
17. Gossen, M. S., Wang, J. H., Chadwick, R. B., and Rutter, W. J. (1992) Genes Dev. 6, 2165–2176
18. Sharma, A., Moore, M., Marcara, E., Lee, J. E., Qiu, Y., Samaras, S., and Stein, R. (1999) Mol. Cell. Biol. 19, 704–713
19. Aronheim, A., Shiran, R., Rosen, A., and Walker, M. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8063–8067
20. Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) Cell 51, 987–1000
21. Tanaka, R., and Herr, W. (1990) Cell 60, 375–386
22. Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) Cell 51, 393–402
23. Karlsson, O., Edlund, T., Moss, J. B., Rutter, W. J., and Walker, M. D. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8819–8823
24. Lifson, J. J., and Green, M. R. (1989) Nature 338, 34–44
25. De Wet, J. R., Wood, K. V., DeLuca, M., Helinoki, D. R., and Subramani, S. (1987) Mol. Cell. Biol. 7, 725–737
26. Davis, R. L., Cheng, P. F., Lassar, A. B., and Weintraub, H. (1990) Cell 60, 733–746
27. Leshkowitz, D., and Walker, M. D. (1991) Mol. Cell. Biol. 11, 1547–1552
28. Yin, D. X., Zhu, L., and Schimke, R. T. (1996) Anal. Biochem. 235, 195–201
29. Santner, R. F., Cook, R. A., Crisel, R. M. D., Sharp, J. D., Schmidt, R. J., Williams, D. C., and Wilson, C. P. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4339–4345
30. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Caslin, L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1373–1376
31. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1988) Current Protocols in Molecular Biology, Greene/ Wiley-Interscience, New York
32. Besnard, C., Monthioux, E., Loras, P., Jami, J., and Daegelen, D. (1991) J. Cell. Physiol. 146, 349–355
33. Weintraub, H. (1993) Cell 75, 1241–1244
34. Black, B. L., Molkentin, J. D., and Olson, E. N. (1998) Mol. Cell. Biol. 18, 69–77
35. Davis, R. L., and Weintraub, H. (1992) Science 256, 1027–1030
36. Chakraborty, T., and Olson, E. N. (1991) Mol. Cell. Biol. 11, 6103–6108
37. Dean, M., and Allikmets, R. (1995) Am. J. Hum. Genet. 57, 1254–1255
38. Huang, J., Weintraub, H., and Kedes, L. (1998) Mol. Cell. Biol. 18, 5478–5484
39. Lefstin, J. A., and Yamamoto, K. R. (1998) Nature 392, 885–888
40. Ptashne, M., and Gann, A. (1998) Curr. Biol. 8, 812–822
41. Mutoh, H., Naya, F. J., Tsai, M. J., and Leiter, A. B. (1998) Genes Dev. 12, 820–830
42. Peers, B., Sharma, S., Johnson, T., Kamps, M., and Montminy, M. (1995) Mol. Cell. Biol. 15, 7091–7097