Hexokinases catalyze the phosphorylation of glucose and initiate cellular glucose metabolism. Hexokinase II (HKII) is the principal hexokinase isoform in skeletal muscle, heart, and adipose tissue. Isoproterenol and exogenous cyclic AMP (cAMP) increase HKII gene transcription in L6 myotubes. Various segments of the HKII promoter that direct the expression of the chloramphenicol acetyltransferase reporter gene were transfected into L6 myotubes to identify basal and cAMP response elements. The 5′-flanking region that extends 90 base pairs upstream of the transcription start site includes a CCAAT box and a CAMP response element (CRE); both contribute to basal promoter activity and each provides an independent, maximal response to cAMP. An inverted CCAAT motif, or Y box, located just upstream of the CCAAT box, contributes to basal promoter activity but is not involved in the CAMP response. Homo- and heterodimers composed of the CRE-binding protein and activating transcription factor-1 bind specifically to the CRE. The Y box and the CCAAT box specifically bind the factor NF-Y (also known as CBF).

The four mammalian hexokinases (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1; designated HKI to HKIV) convert glucose to glucose 6-phosphate (for review see Ref. 1). The hexokinases thus initiate glucose metabolism, and this maintains the downhill glucose concentration gradient that results in glucose entry into cells through facilitative glucose transporters (2, 3). HKII is the principal hexokinase isoform in insulin-responsive tissues such as skeletal muscle, heart, and adipose tissue (4, 5). The cloning of HKII cDNA permitted deduction of the primary amino acid sequence of the protein (6, 7), provided insight into the complete structure of the HKII mRNA and gene (6), and led to studies of the regulation of the gene (6, 8). The rate of HKII gene transcription is increased by insulin, catecholamines, and cyclic AMP (cAMP) in L6 myotubes (6, 8), and this induction results in increased HKII mRNA, protein synthesis, and glucose phosphorylation. This is interesting in view of observations regarding the regulation of hexokinases in various physiologic circumstances. For example, skeletal muscle glucose utilization is increased by exercise (9), an effect that can be explained in part by the observation that a single bout of acute exercise increases HKII gene transcription, mRNA, and protein in rat skeletal muscle (10, 71). Other physiologic stimuli, such as cold stress, swimming, or the administration of β-adrenergic agonists, increase total hexokinase activity in rat and mouse brown adipose tissue and muscle (11–14). The mechanism of these effects is unknown but could involve cAMP.

The regulation of gene transcription by cAMP has been analyzed quite extensively (15–18). Hormones such as β-adrenergic agonists that couple cell surface receptors to adenylylate cyclase result in increased intracellular levels of cAMP. Cyclic AMP, in turn, activates protein kinase A, which enters the nucleus and phosphorylates cyclic AMP response element-binding protein (CREB) on Ser133 (19, 20). Phosphorylated CREB, bound to the cyclic AMP response element (CRE), enhances transcription through linked promoters (21, 22). Activating transcription factor-1 (ATF-1), a protein closely related to CREB, also binds CREs and mediates cAMP responsiveness in certain genes (23, 24). A CCAAT box and an inverted CCAAT box mediate cAMP responsiveness in several promoters that lack typical CREs, but the factors that bind to these elements were not identified (25–27).

We initiated an analysis of the HKII promoter in L6 cells, a cell line representative of rat skeletal muscle, to further understand how hormones influence HKII gene expression. The basal promoter consists of about 160 bp of 5′-flanking sequence that includes an inverted CCAAT box (henceforth referred to as the Y box), a CCAAT box, and the CRE. The CCAAT box and the CRE are both involved in cAMP responsiveness and are functionally redundant in this regard. The Y box does not play a role in cAMP responsiveness but does contribute to basal promoter activity. Several known transcription factors bind to these sequences, notably CREB and ATF-1 to the CRE and NF-Y to both the Y and the CCAAT boxes.

**MATERIALS AND METHODS**

The Rat HKII Promoter Sequence—The EcoRI–EcoRI fragment from pGK51.1 was inserted into pBluescript II SK+ and named pgHK51.1 (6); this contains ~5.5 kb of 5′-flanking sequence of the rat HKII gene. The pgHK51.1 plasmid was used as the DNA template for deoxy sequencing reactions performed using Sequenase 2.0™ (U.S. Biochemical Corp.) and synthetic oligonucleotide primers.

Plasmid Constructs—The BamHI fragment of pgHK51.1, which contains the rat HKII promoter between ~5.5 kb and ~147 bp (numbers relative to the transcription start site), was inserted into the BamHI
wild type (wt) and mutated (mt) oligonucleotide sequences containing an HKII element or a consensus sequence element (uppercase letters), along with flanking sequences (lowercase letters), are shown in the 5′ to 3′ orientation. Underlines indicate the mutated regions.

### Table I

| Oligonucleotide          | Sequence                            |
|--------------------------|-------------------------------------|
| Rat HKII CRE 28wt        | atgccgcgcgCCACGCTGACTgtctggg        |
| Rat HKII CRE 28mt-2       | ctttccggcgtCAATTCGACTgtctggg        |
| Rat HKII CCAAT 28wt       | ctttccggcgtCAATTTTGCTGACTgtctggg    |
| Rat HKII CCAAT 28mt-1     | ctttccggcgtCAATTTTGCTGAGAACTggcagaag|
| Rat HKII Ybox 28wt        | cttggGGGCTGTGACTGGTCGGGctctggagcgg  |
| Rat HKII Ybox 28mt-1      | acatctttcCTGATGTTGaaattgctgctggagcgg|
| Murine MHC class II E     | acatctttcCTGATGTTGaaattgctgctggagcgg|
| Ybox 28wt                | gccttccggcgtGAATTCGACTgtctggg       |
| Rat somatostatin CRE 30wt | agctttccggcgtGAATTCGACTgagagagagag|
| C/EBP 25wt               | atgccgcgcgCCACGCTGACTgtctggg        |

*TABLE I*—Oligonucleotides used for electrophoretic mobility shift assays. Wild type (wt) and mutated (mt) oligonucleotide sequences containing an HKII element or a consensus sequence element (uppercase letters), along with flanking sequences (lowercase letters), are shown in the 5′ to 3′ orientation. Underlines indicate the mutated regions.

Effect of 5′ Deletion Mutations on the Basal Promoter Activity and PKA Inducibility—We cloned about 5.5 kb of 5′-flanking sequence of the rat HKII gene, sequenced the proximal 1176 bp of sequence, and constructed a series of 5′ deletion mutants that consist of the HKII promoter fused to the CAT reporter gene. These constructs were transiently transfected into differentiated L6 cells to localize functional cis-acting DNA elements responsible for mediating basal promoter activity and cAMP responsiveness. The PKA expression vector was co-transfected with the plasmid pRSV-CAT(An) as a reporter and either 5 μg of pRSV-CAT(An) as the effector or pRSVNeo (36) as a control for DNA amount when no effector was used. The plasmid pRSV-luciferase (2.5 μg) was also transfected as an internal control to ensure that changes in promoter activity were not due to differences in transfection efficiency (data not shown). Luciferase activity was assayed as cell extracts as described (37).

Electrophoretic mobility shift assays (EMSA) were performed using cationic poly-L-ornithine coupled with dimethyl sulfoxide shock of L6 cell nuclear extract at room temperature for 15 min except for the CRE probes, which were incubated for 30 min at 4°C. Poly(dI-dC):poly(dI-dC) (50 ng/μl), Nonidet P-40 (0.5%), and a 100-fold molar excess of the single-stranded sense oligonucleotide of each probe were added to non-specific binding. Competition experiments were performed in the presence of a 100-fold molar excess of unlabeled oligonucleotide pairs and titration experiments were performed to determine the affinity of each DNA element for its binding protein(s). Polyclonal anti-CREB antibody (α-244) was the generous gift of Dr. M. Montminy (Salk Institute; La Jolla, CA) (44); the monoclonal anti-NF-YA (monoclonal antibody YA7) and polyclonal anti-NF-YB antibodies were the generous gifts of Dr. R. Mantovani (University of Milan) (45), and the monoclonal anti-ATF-1 antibody (sc-243 X) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies (anti-CREB, 1:400; anti-NF-Y, 1:100; or anti-ATF-1, 1:20 dilution) when used were added to each EMSA as indicated, and incubation conditions were as described for the binding assays. Reaction samples were electrophoretically separated on 6% nondenaturing polyacrylamide gels at room temperature or at 4°C when the CRE probes were employed. The relevant bands were visualized by autoradiography and analyzed by scanning densitometry.

Statistical Analysis—All data are presented as the mean ± S.E. Electrophoretic mobility shift assay (EMSA) results were analyzed by single-factor factorial analysis of variance. The following oligonucleotides were used as probes or competitors for EMSAs listed in Table I. Oligonucleotides that contain the rat somatostatin CRE (40), a high affinity CCAAT/enhancer-binding protein (C/EBP) binding site (41), and the murine Eα Y box (42) have been employed previously.
HKII promoter constructs when cAMP responsiveness was evaluated. The basal promoter activities of all constructs that contained at least 90 bp of 5′-flanking sequence were significantly greater than those of their shorter counterparts (Fig. 1). Construct 9A, with 90 bp of flanking sequence, was thus taken as the standard and assigned a relative basal activity of 100%. Construct 6A, which contains the additional sequence between −157 and −90, showed enhanced basal activity compared with 9A (190 ± 10%; p < 0.05). The largest drop-off of basal activity occurred when the region between −90 and −78 was deleted (100% for 9A versus 27 ± 1% for 7A, p < 0.05), and another drop-off of basal activity occurred when the region between −78 and −60 was deleted (27 ± 1% for 7A versus 14 ± 4% for 10A; p < 0.05). The low promoter activity observed for 10A (∼60 bp) was also found for the shorter construct 5A (∼41 bp).

Regions involved in cAMP (PKA) responsiveness were sought using the same 5′ deletion constructs (Fig. 1). A 2–4-fold increase of CAT expression in response to PKA (PKA inducibility is described as the ratio of PKA-treated to basal value) was noted in constructs that contained at least 78 bp of 5′-flanking sequence; the lower total PKA response of 7A as compared with 9A is due to a reduction of the basal promoter strength in the former construct. Complete loss of PKA inducibility occurred when the sequence upstream from −60 was removed (0.8 ± 0.1-fold induction). We conclude from these findings that the sequence within −157 is important for basal promoter activity and that the sequence within −78 is required for cAMP (PKA) responsiveness.

Analysis of the Rat HKII Promoter Sequence—Examination of the sequence between nucleotides −157 and −60 relative to the transcription initiation site, a region important for basal promoter activity and cAMP (PKA) responsiveness, allowed us to identify several putative eukaryotic transcription factor binding motifs including a Y box, a CCAAT box, and a CRE (Fig. 2A) (16, 46). A 14-bp Y box containing sequence −148 to −135 is identical at 13 positions with the reverse complement of the region identified as a CCAAT box, which is located between nucleotides −89 and −76 (see Fig. 2A, A and B). Interestingly, this CCAAT box is closer to the Y box consensus sequence than it is to a C/EBP consensus sequence. In this sense, the HKII CCAAT box may be more properly termed a "proximal inverted Y box," but to avoid confusion with nomenclature, we use the term "CCAAT box" for this element. The putative CRE, located between nucleotides −73 and −66, shares a 6 of 8 bp identity with a consensus CRE core sequence (Fig. 2, A and B). These three elements are all located upstream of the putative TATA box, which is located at nucleotides −30 and −26 (Fig. 2A).

Effect of Mutations of the Y Box, CCAAT Box, and CRE on Basal Promoter Activity and PKA Inducibility—Site-directed mutagenesis was employed to determine if the putative Y box, CCAAT box, and CRE are involved in basal and cAMP-induced expression of the rat HKII gene (Fig. 3). The 6A construct showed enhanced basal promoter activity when compared with...
Effect of mutations of the Y box, CCAAT box, and CRE on basal activity and PKA inducibility of the rat HKII gene promoter. Each HKII CAT plasmid construct (10 μg) was transiently transfected into L6 myotubes with 5 μg of pRSVCATα (PKA-treated) or pRSVNeo (basal), and the CAT activity of each plate of cells was measured as described under "Materials and Methods." The data are expressed as described in the legend to Fig. 1. The basal and PKA-treated CAT activity of each construct is shown as mean ± S.E. (%), determined in at least three independent experiments using three replicate plates for each construct. The PKA inducibility (PKA-treated/basal) of each construct is shown as the -fold induction ± S.E. The relative positions of the Y box, CCAAT box, CRE, and TATA box in the HKII promoter are illustrated. Arrows indicate the CCAAT motifs. The bases mutated are underlined.

9A (190 ± 10%, p < 0.05, as described above and illustrated in Fig. 3), and the ratio of PKA-treated to basal was lower for 6A than 9A (1.7 ± 0.2 versus 3.5 ± 0.4-fold induction; p < 0.05; Fig. 3) because of this. The Y box, located between the 5' end points of the 6A and 9A constructs, was mutated to generate the 6A[Y boxmt-1] construct. The basal activity of this construct, like that of 9A, is lower than that of 6A. Accordingly, since the total PKA responses are similar, the PKA-treated/basal ratio of both is higher than that of 6A. The effect of this mutation was identical to that seen when the region between −157 and −90 was deleted, and it effectively converted the promoter activity of 6A to that observed for 9A.

The putative CCAAT box was mutated within 9A to generate 9A[CCAATmt-1] (Fig. 3). This mutation resulted in a reduction of basal activity identical to that seen when the region between −90 and −78 was deleted (compare with construct 7A, Fig. 1). No change in PKA inducibility (3.2 ± 0.7-fold) was detected because PKA-treated CAT activity was proportionally decreased along with the reduction of the basal activity (Fig. 3). Mutation of the putative CRE within 9A to generate 9A[CREmt-2] resulted in a reduction of basal and PKA-treated CAT activity but no loss of PKA inducibility (3.0 ± 0.5-fold). The promoter activity of 9A[CREmt-2], which contains an intact CCAAT box, was markedly different from that of the 10A (−60 bp) deletion construct. The 10A construct lacks both the CRE and CCAAT box, has little basal promoter activity with respect to 9A, and also shows no response to PKA. A double mutation of both the CCAAT box and the CRE within 9A (9A[CCAATmt-1/CREmt-2], see Fig. 3) resulted in a reduction of both basal and PKA-treated activity equivalent to that measured in 10A (−60 bp) and caused complete loss of PKA inducibility (1.0 ± 0.1-fold). Thus, the CCAAT box and CRE both play a role in cAMP (PKA) responsiveness, and they are functionally redundant in this regard. In contrast, the Y box, CCAAT box and CRE contribute independently to basal promoter activity.

Effect of Orientation or Spacing of the CCAAT Box with Respect to the CRE on Basal Promoter Activity and PKA Inducibility—The CCAAT box region (−90 through −80) was inverted to determine whether the orientation of this element contributed to the basal or PKA response. The inversion of the CCAAT box enhanced basal activity compared with 9A (190 ± 10%, p < 0.05) but did not affect PKA inducibility (Fig. 4). The spacing between the CCAAT box and CRE is important for the PKA response. Insertion of 5 bp between the CCAAT box and the CRE resulted in a significant reduction in both basal activity (50 ± 10%, p < 0.05) and PKA inducibility (1.9 ± 0.3-fold, p < 0.05) with respect to 9A (Fig. 4). A 10-bp insertion had no effect on the PKA inducibility, but this did enhance the basal activity of the promoter (170 ± 10%, p < 0.05). These observations suggest that the orientation or spacing of the CCAAT box with respect to the CRE affects basal activity, whereas spacing alone is critical for PKA induction.

Protein Binding to the HKII CRE—Using a probe that contains the rat HKII CRE (see Table I) and L6 cell nuclear extracts, three specific DNA-protein complexes (1, 2, and 3) were identified by EMSA (see Fig. 5A, lane a). All three
complexes were nearly eliminated when the HKII CRE (100-fold molar excess) was used to compete against itself for protein binding (see Fig. 5A, lane b) or when the somatostatin CRE was employed (Fig. 5A, lane h). These complexes were not observed when an L6 cell cytoplasmic extract was used (data not shown). Other weaker complexes were occasionally observed, but these probably do not reflect the binding of specific complexes to the rat HKII CRE based on the following criteria: 1) the complexes were observed with both nuclear and cytoplasmic extracts; 2) the formation of these complexes was not specifically competed for by rat HKII CRE; and 3) formation of the complexes was reduced by the inclusion of single-stranded DNA in the incubation. The three CRE-specific complexes were not observed when a probe containing the same mutation (9A CRE mt-2, Table I) was employed in EMSA (data not shown); nor did this mutant CRE compete with the wild type CRE probe for protein binding (Fig. 5A, lane c). Thus, a CRE mutation that results in the loss of promoter activity also results in a loss of specific protein binding to this DNA element. The HKII CCAAT and Y boxes, the MHC class II E box, and the CREB-ATF-1 sequence did not compete for the formation of these three complexes (Fig. 5A, lanes d–g, i, and j).

Anti-CREB antibody disrupted formation of complexes 1 and 2 (Fig. 5B, lane αCREB), and anti-ATF-1 antibody disrupted formation of complexes 2 and 3 (Fig. 5B, lane αATF-1). Nonimmune serum had no effect on DNA-protein complex formation (Fig. 5B, control). These results suggest that the HKII CRE binds CREB and ATF-1 and that these two factors account for the three specific complexes observed. We provisionally identify complex 1 as a CREB homodimer, complex 2 as a CREB-ATF-1 heterodimer, and complex 3 as an ATF-1 homodimer.

Protein Binding to the HKII CCAAT and Y Boxes—The HKII CCAAT box, when inverted, is nearly identical to the HKII Y box (see Fig. 2B), which suggests that a common factor may bind to both elements. HKII CCAAT and Y box probes were used with L6 cell nuclear extracts to identify specific DNA-protein complexes by EMSA. A single specific complex was observed with both probes (Fig. 6, compare panels A and B, lane a). This complex was not observed when cytoplasmic extracts prepared from L6 cells were used in the EMSA (data not shown). Other weaker complexes were occasionally observed, but these complexes do not appear to be specific for the HKII CCAAT and Y box elements (see above for criteria used to determine nonspecific binding). Protein binding was nearly eliminated when a 100-molar excess of the HKII Y box was used to compete with itself or with the HKII CCAAT box probe (Fig. 6, A and B, lane f). Similarly, the HKII CCAAT box effectively competed with itself for protein binding but was a less effective competitor with the HKII Y box probe (Fig. 6, A and B, lane d). CCAAT or Y box probes that contain mutations shown to reduce HKII promoter activity (see above and Table I), formed no specific DNA-protein complexes (data not shown). The mutant HKII CCAAT and Y boxes were also unable to compete for DNA binding with either of the wild type probes (Fig. 6, A and B, lanes e and g); thus, the loss of promoter activity observed with these mutations correlates with loss of specific protein binding. Also, the ability of the Y box to compete for protein binding to the CCAAT box suggests that both elements bind the same protein.

The promoter for the murine MHC class II Eα gene contains a well characterized Y box that binds the transcription factor NF-Y (47, 48). However, a Y box is also an inverted CCAAT box; thus, one of the CCAAT box binding factors, such as C/EBP, C/EBF, γC/EBF, or YB-1, may bind to the HKII Y box and/or CCAAT box. To determine which factor(s) binds to the rat HKII elements, we first showed that an Eα Y box probe binds a factor from an L6 cell nuclear extract and forms a complex similar to that observed for the HKII elements (Fig. 6C, lane a). A 100-fold molar excess of the Eα Y box competed for factor binding to the HKII CCAAT and Y box probes to itself (Fig. 6, A–C, lane j). A 100-fold molar excess of the HKII CCAAT or Y box also competes for factor binding to the Eα Y box probe (see Fig. 6C, lanes d and f, respectively), and the mutant HKII CCAAT and Y boxes failed to compete (Fig. 6C, lanes e and g). As shown in Fig. 6D, the HKII elements competed with the Eα box for protein binding in a concentration-dependent fashion. The HKII and Eα Y box probes compete about equally well, but the HKII CCAAT box is about 50% less effective. The single nucleotide difference between the Y box consensus sequence and an inverted HKII CCAAT box (Fig. 2B), may explain why the HKII CCAAT box is a weaker competitor for this common factor (49, 50). A 100-fold excess of the CREB element, the HKII CRE, or the somatostatin CRE did not compete for specific protein binding to the HKII CCAAT box, HKII Y box, or the Eα Y box probes (Fig. 6, A–C, lanes b, h, and i).

The Eα Y box binds NF-Y, a multisubunit DNA-binding protein (51). The similar DNA-protein complex formation observed between the Eα Y box and the HKII Y and CCAAT boxes suggested that the HKII elements may also bind NF-Y. A monoclonal antibody raised against the A subunit and a polyclonal antibody raised against the B subunit were used to determine whether NF-Y forms the complex observed with the
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FIG. 6. Protein binding to the rat HKII gene CCAAT and Y boxes. Panels A, B, and C represent typical results obtained from at least three independent experiments using L6 cell nuclear extracts and rat HKII CCAAT 28wt, rat HKII Y box 28wt, and murine MHC class II Eα Y box 28wt (murine Eα Y box 28wt) probes, respectively. Each probe was incubated with L6 cell nuclear extracts in the absence or presence of a 100-fold molar excess of each competitor, as described under “Materials and Methods.” Each lane contains double-stranded oligonucleotide competitors in the same order: no competitor (lane a); rat HKII CRE 28wt (lane b); rat HKII CRE 28mt-2 (lane c); rat HKII CCAAT 28wt (lane d); rat HKII CCAAT 28mt-1 (lane e); rat HKII Y box 28wt (lane f); rat HKII Y box 28mt-1 (lane g); rat somatostatin CRE 30wt (lane h); C/EBP 25wt (lane i); murine MHC class II Eα Y box 28wt (lane j). All the oligonucleotides used for probes or competitors are described in Table I. The specific complex representing protein binding to each probe is indicated by an arrow. D. Comparison of the affinity of the HKII CCAAT and Y boxes to the Eα Y box for protein complex formation. The murine MHC class II Eα Y box 28wt (Eα Y) probe was incubated with L6 cell nuclear extract and the rat HKII CCAAT 28wt (HKII CCAAT), rat HKII Y box 28wt (HKII Y), or Eα Y competitors as described under “Materials and Methods.” The amount of each competitor was titrated from a 3–100-fold molar excess. The intensity of the bands was measured by densitometry. Protein binding, expressed as the percentage bound with competitors relative to that with no competitors, is shown as mean ± S.E. as determined in three independent experiments.

FIG. 7. Effect of anti-NF-Y antibodies on protein binding to the HKII gene CCAAT and Y boxes. Either the rat HKII CCAAT 28wt (HKII CCAAT), Y box 28wt (HKII Y), or murine MHC class II Eα Y box 28wt (Eα Y) probe was incubated with L6 cell nuclear extract or without anti-NF-YA monoclonal antibody (YAT) or anti-NF-YB polyclonal antibody. Specific bands representative of the proteins binding to the probes are indicated by an arrow. The figure represents typical results obtained from three independent experiments. The HKII CCAAT and Y boxes and L6 nuclear extracts. Anti-NF-YA antibody resulted in similar disruptions of complex formation with the HKII CCAAT or Y box probes and the Eα Y box probe (Fig. 7, aNF-YA7 lanes). Anti-NF-YB antibody completely disrupted complex formation by the HKII CCAAT or Y box probes and the Eα Y box probe (Fig. 7, aNF-YB lanes). Nonimmune serum had no effect on the complex formation by any of the probes (Fig. 7, control lanes). These results suggest that the HKII CCAAT box and Y box bind the transcription factor NF-Y and that this factor may be important for both the basal and cAMP-inducible expression of the HKII gene.

Discussion

The promoter for the rat HKII gene was isolated and used to construct HKII-CAT fusion genes as a means of identifying cis-acting DNA elements important for HKII gene expression. The HKII-CAT fusion gene constructs were transiently expressed following transfection into differentiated L6 cells. Ichihara et al. (52) used a reporter gene similar to the 4A construct employed in this paper to direct the expression of a CAT fusion gene in the SR-3Y1–2 transformed fibroblast cell line. Mathupala et al. (53) recently reported that approximately 4.3 kb of the rat HKII promoter fused to a luciferase reporter gene directed basal expression in AS-30D hepatoma cells and in cultured hepatocytes. The basal promoter activity directed by this construct was comparable in magnitude in both cell types; however, glucose, insulin, or glucagon increased fusion gene expression only in the hepatoma cells (53).

We chose to study HKII gene expression in L6 cells, a cell line representative of skeletal muscle, which is a primary site of expression of the gene (4, 5). We have previously used these cells to study the regulation of HKII gene transcription by insulin and cAMP (6, 8) and now extend these earlier studies by identifying three cis-acting DNA elements in the HKII promoter, a Y box, a CCAAT box and a CRE, that are required for basal gene expression. Two of these elements, the CCAAT box and CRE, are required for the induction of the gene by cAMP and have redundant, nonadditive roles in mediating this response.

The Y box element, originally defined as an inverted CCAAT motif (CTGATTGGYY) in the major histocompatibility complex (MHC) class II genes, plays an important role in tissue-specific gene expression (for review, see Refs. 47 and 48). The binding protein NF-Y, also called CBF or CP-1 (42, 54), stimulates gene transcription when bound to the Y box (55–57). NF-Y is highly conserved among species and is composed of several subunits, referred to as NF-YA (CBF-B), NF-YB (CBF-A), and CBF-C; all these subunits bind to DNA as a complex (51, 58–60). Although the MHC class II genes are expressed in a cell-specific pattern, NF-Y is ubiquitously expressed (51, 61, 62), and it also binds to Y box motifs in non-class II genes including those that encode thymidine kinase (63), albumin (50, 64), globin (65, 66), β-actin...
The Y box, CCAAT box, and CRE contribute to the basal activity of the HKII gene promoter, and the CCAAT box and CRE provide a redundant response to cAMP. Homo- and heterodimers of CREB/ATF-1 bind to the CRE. NF-Y binds to the Y and CCAAT boxes, probably as a trimeric complex of A, B, and C subunits (58–60).

The HKII CRE binds both CREB and ATF-1, factors known to affect basal and cAMP responses in a variety of genes (23, 24). Mutations of the HKII gene CRE that result in reduced binding of CREB and ATF-1 also result in decreased basal activity of the promoter. These mutations, however, had no effect on the cAMP response. Cyclic AMP induction is only lost when both the HKII CCAAT box and the CRE are mutated. Since mutations that affect only the CCAAT box have no effect on the cAMP response, the HKII CCAAT box-NF-Y complex and the CRE-CREB/ATF-1 complex have a redundant role in the cAMP response. To our knowledge, NF-Y has not been associated with CAP responsiveness; nor has it been directly implicated as a regulator of genes involved in glucose metabolism. In this regard, it should be noted that an inverted CCAAT box mediates CAP responsiveness of the human tryptophan hydroxylase gene and fatty acid synthase gene through an interaction with an unknown nuclear factor (25, 27). In addition, a CCAAT box mediates the CAP response of the G-protein α2 subunit gene, which lacks a typical CRE (26). It will be interesting to see if NF-Y is involved in these effects.

The apparent role of NF-Y in the CAP response of the HKII gene was not expected. CREB and ATF-1 bound constitutively to CREs and support basal transcription. The phosphorylation of these proteins by PKA results in an enhanced rate of gene transcription (15, 16, 24, 69). PKA-mediated phosphorylation of NF-Y has not been reported, but the protein does contain several consensus phosphorylation sites (data not shown). Phosphorylation could increase the affinity of binding of NF-Y to the HKII CCAAT box, which has a lower binding affinity for the protein than does the consensus Y box. Alternatively, phosphorylation could enhance the transcriptional potential of constitutively bound NF-Y, as happens with CREB.

The orientation of the CCAAT box relative to the CRE did not alter the PKA response, but the introduction of a half turn in the DNA helix between the CCAAT box and the CRE reduces the PKA induction, and insertion of a full turn maintains the response. This suggests that a specific helical orientation of the binding factors NF-Y and CREB/ATF-1 is critical for the PKA response in the rat HKII gene. A similar stereospecific alignment of the Y box and X box is required for MHC class II A promoter function (70).

As summarized in Fig. 8, NF-Y bound to the CCAAT box and CREB/ATF-1 bound to the CRE play important roles in both basal and cAMP-induced expression of the rat HKII gene. A third element important for basal HKII gene transcription, the Y box, also binds NF-Y. Finally, a typical TATA box is located near the transcription initiation site. It is not clear which of the combinations of CREB and ATF-1 are involved in the basal and cAMP-mediated responses through the CRE. It is also not clear how NF-Y is involved in these responses. Further experiments should help clarify these points.
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