Glucose Metabolism in Cancer

IMPORTANCE OF TRANSCRIPTION FACTOR-DNA INTERACTIONS WITHIN A SHORT SEGMENT OF THE PROXIMAL REGION OF THE TYPE II HEXOKINASE PROMOTER*

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A common signature of many cancers is a high glucose catabolic rate frequently dependent on the overexpression of Type II hexokinase (HKII), a mitochondrial bound enzyme that also suppresses cell death. As the tumor HKII promoter plays a significant role in HKII overexpression, studies reported here were undertaken to identify both the major regions and transcription factors involved under tumor-like conditions. Reporter gene assays following transfection of hepatoma cells with decreasing segments of the HKII promoter traced its known strength to the proximal region (~281 to ~35). Mutational analyses showed that in this short region GC boxes 1, 2, 5, and 6, a CCAAT box, an inverted CCAAT box, and CRE are involved in promoter activation. Other studies demonstrated binding of transcription factors Sp1, Sp2, and Sp3 to GC boxes 1 and 6, Sp1 and Sp2 to GC boxes 2 and 5, NF-Y to CCAAT boxes, and CREB, ATF1, and CREM to CRE. In addition, transfection studies involving Sp1, Sp2, Sp3, CREB, and NFY (dominant negative form) provided evidence that these proteins are promoter activators. Finally, alignment of available HK proximal promoters showed strong conservation only among HKII sequences. These findings implicate signaling pathways directed to a short segment of the proximal region of the HKII promoter as major contributors to HKII overexpression in many cancers.

The most common biochemical phenotype of highly malignant, rapidly growing tumors is their ability to utilize glucose at high rates (1–5). In such tumors hexokinase, the first enzyme of the glycolytic pathway, is markedly elevated (>100-fold) and essential for maintaining the high glycolytic phenotype (6, 7). Among the four mammalian hexokinase types (HK I-IV),1 HKII is frequently the predominant overexpressed form in such tumors (8–11). Significantly, HKII is bound to the outer mitochondrial membrane (6, 12) where it rapidly produces glucose-6-phosphate, a glycolytic substrate and a major precursor for biosynthesis (13). At this location, HKII also prevents bax-mediated cell death (14). Thus, HKII has multifunctional roles in cancer, i.e. to enhance glycolytic ATP production, particularly under hypoxic conditions, to enhance biosynthesis, and to help immortalize the cell population. For these reasons, and the fact that the most common technique (positron emission tomography) used for cancer detection (15–19) is based largely on hexokinase activity, there is a compelling need to better understand those molecular events involved in HKII overexpression.

Recent studies (11, 20–24) have led us to conclude that not one but several events are involved in the marked overexpression of HKII in highly glycolytic hepatomas. Among these events, gene amplification (21) and promoter activation (11, 20, 22, 23) appear to be major contributors. In the model highly glycolytic hepatoma cell line studied, i.e. the AS-30D (6, 25), the HKII gene is amplified at least 5-fold relative to control hepatocytes in which HKII is barely detectable (21). Moreover, transfection of the hepatoma cells with the HKII promoter (4.3 kilobase pairs), isolated from the same cells, shows that various metabolites activate the promoter 1.3–3.6-fold relative to a pyruvate or lactate background in a medium deficient in glucose and lacking serum (11, 20, 22, 23). Activators include glucose, insulin, glucagon, dibutyryl cAMP, p53, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, and hypoxic conditions. Notably, it is glucose rather than a downstream glycolytic intermediate that is the promoter activator (20), and synergy results when either dibutyryl cAMP or hypoxic conditions are used together with glucose (20, 23). In our most recent study, we have found also that in hexokinase-deficient hepatocytes the promoter for HKII is methylated within a Cpg island that includes the transcription start site but is hypomethylated in hexokinase-abundant hepatoma cells (24). As transfected hepatocytes, in contrast to transfected hepatoma cells, fail to result in HKII promoter activation in those cases tested (11), this suggests that demethylation events within the region of the proximal promoter may be a prerequisite for its activation by some transcription factors. Studies reported here were undertaken to gain greater insight into activation of the HKII promoter under more tumor-like conditions (i.e. availability of serum and glucose) than those used in previous studies (11, 20–23). Specifically, we wanted to identify under such conditions which region of the promoter that makes the major contribution to its activation and identify also the major transcription factors involved. This contrasts with our previous work on the promoter described above that used media deficient in glucose and lacking serum to assess the contributions of individual metabolic agents to promoter activation (11, 20–23). In the study reported here, the post-transfection incubation medium contained both a serum supplement and glucose to provide an environment that more closely resembles that of a hepatoma cell located in a well vascuilarized tumor or within the peritoneal cavity. Signifi-
cantly, serum is rich in hormones and other growth factors that initiate signal transduction pathways, the end products of which may modulate HKII promoter activity. Results of studies presented below show that under these tumor-like conditions the remarkable strength of the Type II hexokinase promoter resides within a short segment (~281 to ~35) of the proximal region where a number of transcription factors known to be linked to signal transduction pathways are involved.

MATERIALS AND METHODS

Animals, Cell Lines, and Cell Media—Female Sprague-Dawley rats (100–150 g) were purchased from Charles River Breeding Laboratories. Their care and use were approved by the Johns Hopkins University Animal Care and Use Committee. AS-30D hepatoma cells (25) exhibiting a high glycolytic rate (6) were propagated in the peritoneal cavity of these animals as previously described (11, 26). The cells in ascites form were harvested 6–8 days post-transplantation. For reporter gene assays, cells were first purified and transfected in RPMI 1640 medium (Invitrogen). Female Sprague-Dawley rats (150 g) were purchased from Charles River Breeding Laboratories. Additional 5’/3’ restriction sites, PCR products were inserted into the pGL2-Basic vector (Promega) that contains the gene for luciferase. The expression

plasmids pPacO and pPacSp1 were provided by Dr. R. Tjian, University of California, Berkeley, and pPac-HA-Sp1 (1–293), pPac-HA-Sp2, and pPac-HA- Sp3 were from Dr. H. Rotheneder, University of Vienna, Austria. Those for NF-йA and DN-NF-йA were from Dr. R. Mantovani, University of Milano, Italy, and those for CREB and A-CREB were from Dr. D. Ginty, Johns Hopkins University, School of Medicine, Baltimore, MD. 

Antibodies—Anti-Sp1 (SC-420x and SC-59), anti-Sp2 (SC-643x), anti-Sp3 (SC-644), anti-Sp4 (SC-645x), anti-CREB* (SC-186x), anti-ATF1 (SC-270), and anti-CREM (SC-440x) were from Santa Cruz Biotechnology Inc. The anti-CREB antibody (C-8977) was from Sigma, and the polyclonal antibody against NF-йB was from Ny-A-Y from Dr. R. Mantovani. 

Site-directed Mutagenesis of the HKII Promoter—To generate the site-specific mutants mE2F, mGC1, mGC2, mGC3, mGC4, mGC5, mCRE, mGC6, and mGC7 that contain mutations in potential regulatory elements within the proximal sequence of the HKII promoter, the ~29 deletion construct was utilized as a template. Site-directed mutagenesis was performed using the Clontech Transformer™ site-directed mutagenesis kit. A sequence (CCCCGGAGGTACCCTAGCTCTACGGC) containing a mutated SacI site (in bold) was used as a primer to select mutant plasmids. Mutagenic primers for these site-specific mutants are listed in Table I. Plasmid plasmids were denatured and annealed with primers, followed by synthesis, ligation, and transformation into the Escherichia coli BHM71-18 mutS strain. Plasmids were isolated, digested with SacI (New England Biolabs), and then transformed and isolated for sequencing.

To generate other site-specific mutants (mCCAAT, mGC5, mCRE, and mGC6), PCR was performed using forward primers (Table I) and the same reverse primer (TGGCCGGAGGTACCTAGCTCTACGGC) containing a mutated SacI site (in bold) was used as a primer to select mutant plasmids. Mutagenic primers for these site-specific mutants are listed in Table I. Plasmid plasmids were denatured and annealed with primers, followed by synthesis, ligation, and transformation into the Escherichia coli BHM71-18 mutS strain. Plasmids were isolated, digested with SacI (New England Biolabs), and then transformed and isolated for sequencing.

Transient Transfections and Reporter Gene Assays—Transient transfections of AS-30D hepatoma cells with either the deletion or site-specific mutants, and the pSV-й-Gal plasmid, a й-galactosidase vector (Promega), were carried out by electroporation using a modified version of an earlier procedure (11). Plasmid DNA for transfection was purified using the Qiagen plasmid maxi kit (Qiagen, Inc). Molar amounts equal to 10 μg of the full-length promoter-luciferase reporter gene construct (~4369 construct) were used for all deletion constructs including the site-specific mutants, pGL2-basic vector (no promoter), and pSV-Luc vector containing the luciferase gene under control of the SV40 promoter/enhancer (pGL2 control from Promega). The й-galactosidase vector
were end-labeled in a final volume of 20 μl of pre-warmed Dulbecco’s modified Eagle’s medium (37°C, 5% CO₂) with 10% FBS, 15% heat-inactivated anticytokine mixture. Cells were incubated for 20–24 h, harvested, washed with phosphate-buffered saline, and extracted with 100 μl of 1× Reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were assayed as described below, and the luciferase activity of each sample normalized with respect to β-galactosidase activity. For transient co-expression in AS-30D hepatoma cells of the plasmids pPacSp1, pPac-HA-Sp1-(1×), pPac-Sp1-(1×), A, lysed, and centrifuged at 1,000 g for 10 min. The packed cells were then suspended in buffer containing 6.7 mM Tris-HCl, 3.3 mM sodium acetate, and 1 mM EDTA, pH 7.9. After drying, gels were subjected to autoradiography at −70°C with intensifying screens on Hyperfilm™ ECL™ film (Amerham Biosciences). RESULTS Orientation—The 4.3-kilobase pair tumor HKII promoter isolated previously in this laboratory from the highly glycolytic model AS-30D hepatoma cell line (11) has a well defined transcription start site with a flanking region predicted by the search program MOTIF (29) to contain a number of putative response elements (Fig. 1A). It is a strong promoter nearly equivalent to that of the SV-40 tumor promoter (11), and is activated by a number of metabolically related agents (11, 20). The 4.3-kilobase pair of the HKII promoter has not been found. In studies reported here we addressed two important questions. What region of the promoter contributes most to its activation under conditions in which hepatoma cells are exposed to a tumor-like medium, and what response elements and transcription factors are involved?

In a Medium Supplemented with FBS and Glucose the Strength of the HKII Promoter in the Highly Glycolytic Model AS-30D Hepatoma Cell Line Resides within a Short Segment (−281 to −35) of the Proximal Region—To determine what region of the HKII promoter contributes most to its activation under tumor-like conditions, studies were conducted using both a luciferase reporter gene construct containing the original 4,369-bp HKII promoter isolated earlier (11) and a series of deletion mutants derived there from. Some of these mutants were constructed previously (23), whereas others were newly constructed. Following transfection, hepatoma cells were incubated for 20–24 h in Dulbecco’s modified Eagle’s medium supplemented with FBS and glucose and then assayed for luciferase activity. Results presented in Fig. 1B (upper panel) and expressed relative to that observed for the −4,369 HKII “control” construct show that deletion of >90% of the promoter, i.e., from −4369 to −281, has little or no effect on its activity. Some loss of activity is observed in the region between −2072 and −1072, perhaps because of a silencer, but is fully recovered by further deletions. Moving into the heart of the proximal region of the HKII promoter, its activity is almost completely lost by deleting the region from −281 to −35. Herein resides the response element cluster flanking the transcription start site as depicted in simplified form in Fig. 1A, and in more detail in Fig. 1C. Additional controls for this experiment include the pGL2 basic vector containing the luciferase gene but no promoter that, as expected, shows insignificant activity in the reporter gene assay, and the pSV-Luci construct (luciferase gene under control of the SV-40 promoter) that as previously

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FIG. 1. Response element features of the rat hepatoma HKII promoter and deletion analyses to localize those regions that contribute significantly to its strength. A, schematic representation of the hepatoma HKII promoter showing a cluster of response elements near the transcription start site. The GC boxes, CCAAT box, inverted CCAAT box, and cyclic AMP response element are indicated as GC, CCAAT, Inv CCAAT, and CRE, respectively. B, effect of deletion mutations on HKII promoter activity. AS-30D hepatoma cells were transiently transfected
noted (11) exhibits an activity nearly equivalent to the HKII promoter-luciferase construct.

A second set of experiments presented in Fig. 1B (lower panel) focused on shorter deletions in the “active center” of the HKII promoter, i.e., within the proximal region from −281 to −35. Here, 6 GC boxes (GC1, GC2, GC3, GC4, GC5, and GC6) reside as well as response elements for E2F, CCAAT, inverted CCAAT, and CRE. Deletions from −281 to −179, −157 to −89, and −89 to −35 all result in sharp reductions in promoter activity. Only the deletion from −179 to −157 that includes the GC3 response element fails to affect promoter activity. Taken together, this initial set of experiments revealed that under tumor-like conditions, the HKII promoter participates in the transcriptional activities of several transcription factors and is active in both liver and non-liver cells.

**Mutational Analysis of the Region (−281 to −35) Flanking the Transcription Start Site of the HKII Promoter Shows That Four GC Boxes, a CCAAT Box, an Inverted CCAAT Box, and One CRE Contribute to Its Activation**—To determine which of the potential responsive elements within the −281 to −35 region of the HKII promoter (Fig. 1C) contribute significantly to its high transcriptional activity in hepatoma cells, site-specific mutations were generated in the six GC boxes, the CCAAT box, the inverted CCAAT box, CRE, and E2F. The deletion construct −329 of the HKII promoter served as template for introducing site-specific mutations into E2F, 4 of the 6 GC boxes, and the inverted CCAAT box (Fig. 2A), whereas the deletion construct −89 served as template for introducing site-specific mutations into the other 2 GC boxes, the CCAAT box, and in CRE (Fig. 2B). The reporter gene activity of each site-specific mutant was compared with that of the template construct from which it was derived and reported as relative luciferase activity. The results obtained show that mutations in each of the 4 GC boxes (GC1, GC2, GC5, and GC6), the inverted CCAAT box, the CCAAT box, and CRE reduce significantly HKII promoter activity. In contrast, mutations in either E2F, or the other GC boxes (GC3 and GC4) are without significant effect. Of those mutations that did result in reductions in the total promoter activity, the GC2 mutant was the most effective, whereas other mutants resulted in moderate decreases. These results indicate that within the transcriptionally active segment of the HKII promoter (i.e. −281 to −35) several different transcription factors binding to the indicated elements above (GC1, GC2, GC5, GC6, CRE, inverted CCAAT, and CCAAT) contribute to promoter activity.

**The Four GC Boxes Contributing to the Optimal Activity of the HKII Promoter Bind Transcription Factors in the Sp Family That Act as Promoter Activators**—On the basis of the above experiments, by electroporation with 10 µg of the HKII construct −4369 (“full-length” promoter) or equimolar amounts of truncated HKII promoter constructs and 15 µg of a pSV-β-galactosidase plasmid. Equimolar amounts of pGL2-basic (no promoter) vector and pSV-Luc vector containing a luciferase gene under control of the SV40 promoter/enhancer were used for transfection as negative and positive controls, respectively. Cells were cultured for 20–24 h and harvested. Luciferase (LUC) and β-galactosidase activities were assayed, and the luciferase activity of each sample was normalized with β-galactosidase activity to provide a normalized luciferase activity. The normalized activity of the template HKII construct −329 (or −89) was represented as 100%. The activities of the mutant constructs were compared with this and presented as relative luciferase activities. A, relative promoter activities of the −329 HKII construct and its site-specific mutants. Experiments were carried out as indicated above using the −329 HKII construct and mutant constructs mE2F, mGC1, mGC2, mGC3, mGC5, mNSS CCAAT, and mGC6. Data are presented as the mean ± S.D. *, p < 0.01; **, p < 0.001. B, relative promoter activities of the −89 HKII construct and its site-specific mutants. Experiments were carried out as indicated above using the −89 HKII construct and mutant constructs mCCAAT, mGC5, mCRE, and mGC6. Data are presented as the mean ± S.D. **, p < 0.001.

**Fig. 2. Effect of site-specific mutations on HKII promoter activity.** Site-specific mutations were made in either the −329 or −89 HKII promoter construct within each of the response elements flanking the transcription start site. AS-30D hepatoma cells were transiently transfected by electroporation with equimolar amounts of the site-specific mutants and 15 µg of the pSV-β-galactosidase construct. Molar amounts equal to 10 µg of full-length HKII promoter-luciferase reporter gene construct were used for transfection. Cells were cultured for 20–24 h and harvested. Luciferase (LUC) and β-galactosidase activities were assayed, and the luciferase activity of each sample was normalized by β-galactosidase activity to provide a normalized luciferase activity. The normalized activity of the template HKII construct −329 (or −89) was represented as 100%. The activities of the mutant constructs were compared with this and presented as relative luciferase activities. A, relative promoter activities of the −329 HKII construct and its site-specific mutants. Experiments were carried out as indicated above using the −329 HKII construct and mutant constructs mE2F, mGC1, mGC2, mGC3, mGC5, mNSS CCAAT, and mGC6. Data are presented as the mean ± S.D. *, p < 0.01; **, p < 0.001. B, relative promoter activities of the −89 HKII construct and its site-specific mutants. Experiments were carried out as indicated above using the −89 HKII construct and mutant constructs mCCAAT, mGC5, mCRE, and mGC6. Data are presented as the mean ± S.D. **, p < 0.001.
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Fig. 3. EMSAs implicating binding of hepatoma transcription factors Sp1, Sp2, and Sp3 to both the GC1 and GC6 boxes and Sp1 and Sp2 to the GC2 and GC5 boxes. A, EMSA studies to identify Sp family members that bind to the GC1 box. A 32P-labeled GC1 probe (Table II) was incubated without (lane 1) or with hepatoma nuclear extracts (NE) (lanes 2–11). Competition (comp) analysis was performed in the presence of a 40- and 200-fold molar excess of unlabeled wild type (WT) GC1 oligonucleotides (lanes 3 and 4), or unlabeled mutant (MT) GC1 oligonucleotides (lanes 5 and 6). The effect of specific antibodies (Ab) was determined in the presence of labeled oligonucleotide, nuclear extract, and one of the following: anti-Sp1 (lane 7), anti-Sp2 (lane 8), anti-Sp3 (lane 9), or anti-Sp4 (lane 10). B, EMSA studies conducted to identify factors that bind to the GC6 box. 32P-Labeled GC6 probe (Table II) was incubated with nuclear extracts (lanes 1–8). Competition analysis was performed in the presence of a 200-fold molar excess of unlabeled wild type GC6 oligonucleotides (lane 2), or unlabeled mutant GC6 oligonucleotides (lane 3). The effect of specific antibodies was determined in the presence of labeled oligonucleotide, nuclear extract, and one of the following: anti-Sp1 (lane 5), anti-Sp2 (lane 6), anti-Sp3 (lane 7), or anti-Sp4 (lane 8). C, EMSA studies to identify Sp family members that bind to the GC2 box. 32P-Labeled GC2 probe (Table II) was incubated with nuclear extracts (lanes 1–7). Competition (comp) analysis was performed in the presence of a 200-fold molar excess of unlabeled wild type GC2 oligonucleotides (lane 2), or unlabeled mutant (MT) GC2 oligonucleotides (lane 3). The effect of specific antibodies was determined in the presence of labeled oligonucleotide, nuclear extract, and one of the following: anti-Sp1 (lane 4), anti-Sp2 (lane 5), anti-Sp3 (lane 6), or anti-Sp4 (lane 7). D, EMSA studies to identify Sp family members that bind to the GC5 box. 32P-Labeled GC5 probe (Table II) was incubated with nuclear extracts (lanes 1–5). The effect of specific antibodies was determined in the presence of labeled oligonucleotide, nuclear extract, and one of the following: anti-Sp1 (lane 2), anti-Sp2 (lane 3), anti-Sp3 (lane 4), or anti-Sp4 (lane 5). Specific bands that represent DNA-protein complexes are indicated by arrows I, II, and III. Bands supershifted in whole or in part by anti-Sp1 or anti-Sp2 are indicated by SSII and SSIII, respectively.

mutational analysis identifying active response elements within the -281 to -35 region of the HKII promoter, it became of interest to identify potential transcription factors that bind to these elements. Experiments were designed first to determine to what extent the 4 GC boxes interact with members of the Sp family of transcription factors (Sp1, Sp2, Sp3, and Sp4), as these homologous proteins are known to recognize some GC or GT boxes located within gene promoters (30, 31). The method used to detect possible GC-Sp interactions entailed the use of an EMSA in the absence and presence of antibodies specific for Sp family members. Results presented in Fig. 3A were obtained in an experiment to determine whether one or more of the Sp family members interact with the GC1 box. Here, a double stranded 32P-labeled oligonucleotide that included the sequence of the GC1 box (Table II) was mixed with a nuclear extract from AS-30D hepatoma cells and subjected to electrophoresis. Lane 1 shows the control without labeled oligonucleotide that as expected contains no labeled bands. In contrast, lane 2 shows several 32P-labeled oligonucleotide-protein complexes, the 32P-labeled oligonucleotide of which can be competed off with unlabeled “wild type” oligonucleotide (lanes 3 and 4) but not with the oligonucleotide bearing a mutation in the sequence of the GC1 box (lanes 5 and 6). When an antibody to Sp1 is present (lane 7) it causes part of the top band (I) to undergo an upward shift (SS or “supershift”) resulting in band SSII. An antibody to Sp2 (lane 8) causes a larger upper shift resulting in band SSIII, whereas an antibody to Sp3 (lane 9) causes the lighter bands (II and III) below the upper band (I) to disappear, perhaps shifting it upward to overlap with band I. In contrast to the obvious band shifts observed with antibodies to Sp1, Sp2, and Sp3, an antibody to Sp4 (Lane 10) causes no band shift relative to the control without antibody (lane 11).

A similar set of experiments was carried out to determine to what extent the other 3 GC boxes required for optimal HKII promoter activity interact with the Sp family of transcription factors. These experiments show that similar to GC1, GC6 also interacts with Sp1, Sp2, and Sp3 but not Sp4 (Fig. 3B), whereas GC2 (Fig. 3C) and GC5 (Fig. 3D) both interact with Sp1 and Sp2 but not Sp3 and Sp4. (It should be noted that in some cases antibody binding causes band disappearance rather than a band shift, e.g. Fig. 3B (lane 5), Fig. 3C (lanes 4 and 5), and Fig.
sequences are indicated by underlined lowercase letters. Where indicated, $m$ = mutant, $F$ = forward, $R$ = reverse.

3D (lanes 2 and 3). In such cases, the antibody may bind to a site on the protein factor that is essential for DNA binding, thus totally blocking the ability of the factor to bind to DNA and resulting in the complete absence of a DNA–protein complex from the gel.)

On the basis of the above experiments, it became important to determine to what extent the Sp family members can function as activators of the HKII promoter. Therefore, Drosophila SL2 cells lacking endogenous Sp factors (32) were transiently co-transfected with both the −281 construct of the HKII promoter and variable amounts of the expression plasmids encoding Sp1, Sp2, or Sp3. As shown in Fig. 4, A and C, Sp1 and Sp3 strongly activate the HKII promoter in a dose-dependent manner, whereas Sp2 gives a significant but weaker response (Fig. 4B). A control experiment (Fig. 4A, far right) shows as expected that the HKII promoter is not activated by a truncated form of Sp1 (pPacSp1−10) lacking the DNA binding domain (33), confirming that activation by the Sp factors is specific.

Finally, it can be concluded from the above studies that the 4 GC boxes (GC1, GC2, GC5, and GC6) residing near the transcription start site of the HKII promoter interact with Sp family members, and that this interaction activates the promoter.

The Single CRE Contributing to the Optimal Activity of the HKII Promoter Binds Transcription Factors in the CREB Family That Act as Promoter Activators—Having established that 4 of the 6 GC boxes found in the narrow proximal segment (−281 to −35) of the HKII promoter bind transcription factors in the Sp family that can activate the HKII promoter, our attention turned to the single CRE also located in this region and responsible in part for the maximal promoter activity. As CRE has been reported to bind CREB and family members ATF1 and CREM (reviewed in Ref. 34), we first carried out EMSA experiments to determine whether a nuclear extract from AS-30D hepatoma cells contains one or more proteins that bind a double stranded 32P-CRE oligonucleotide (Table II). As shown in Fig. 5A, 3 bands are observed (lane 1), all of which disappear upon addition of excess unlabeled oligonucleotide (lane 2), and only one of which remains when unlabeled oligonucleotide mutated in the CRE sequence is added (lane 3). This finding indicates that 2 of the 3 bands (lane 1) contain proteins that bind nonspecifically to the CRE oligonucleotide, and that only the central band (I) contains a protein specific for the CRE sequence.

To determine whether band I consisted of a transcription factor complex containing members of the CREB family, the assay was repeated in the presence of an antibody (CREB$^*$) reactive with the three family members CREB, ATF1, and CREM, and then with antibodies specific for each member. Results presented in Fig. 5B shows that the CREB$^*$ antibody disrupts band I (lane 2), observed as a very dense band in the control without antibody (lane 1). Moreover, the disruption by the CREB$^*$ antibody is not observed for the control antibody IgG (lane 3). This experiment suggested that one or more members of the CREB family of transcription factors is present in band I. Experiments presented in Fig. 5C show that a specific antibody to CREB, ATF1, or CREM causes an upward shift.

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**Oligonucleotides used in electrophoretic mobility shift assays**

Response elements are indicated with bold letters. The mutated sequences are indicated by underlined lowercase letters.

**Table II**

| Oligonucleotide | Sequence |
|-----------------|----------|
| GC1-F | 5’ GCCGCCGCTCGCGCGCTAGGCT 3’ |
| GC1-R | 3’ GCCGCCGAGGCGGGATCGGA 5’ |
| mGC1-F | 5’ CGCCCGGAGTCAAGCCCCCTAGGCT 3’ |
| mGC1-R | 3’ GCCGCCGAGGCGGGATCGGA 5’ |
| GC2-F | 5’ GCCTCTTCCCCGGCCGCTTCT 3’ |
| GC2-R | 3’ GCCAAGAGGCGGGATCGGA 5’ |
| mGC2-F | 5’ GCCTCTTCCCCGGCCGCTTCT 3’ |
| mGC2-R | 3’ GCCAAGAGGCGGGATCGGA 5’ |
| GC5-F | 5’ AATGAGCCGCACCAGTCAC 3’ |
| GC5-R | 3’ TACTGCGCGGTCGAGT 5’ |
| mGC5-F | 5’ AATGAGCCGCACCAGTCAC 3’ |
| mGC5-R | 3’ TACTGCGCGGTCGAGT 5’ |
| GC6-F | 5’ CGCTCTGGGCGGCGCGAAGAG 3’ |
| GC6-R | 3’ GACAGAACCCCGCGGGTTTCTC 5’ |
| mGC6-F | 5’ CGCTCTGGGCGGCGCGAAGAG 3’ |
| mGC6-R | 3’ GACAGAACCCCGCGGGTTTCTC 5’ |
| CCAAT-F | 5’ TCCGCGACCAATAGGCGGC 3’ |
| CCAAT-R | 3’ AGGGCGTGGTACGCGGGG 5’ |
| mCCAAT-F | 5’ TCCGCGACCAATAGGCGGC 3’ |
| mCCAAT-R | 3’ AGGGCGTGGTACGCGGGG 5’ |
| InvCCAAT-F | 5’ CTGGGGCGTGATGCTGGGTA 3’ |
| InvCCAAT-R | 3’ GACGCCGCACTAAAGCCATC 5’ |
| mInvCCAAT-F | 5’ CTGGGGCGTGATGCTGGGTA 3’ |
| mInvCCAAT-R | 3’ GACGCCGCACTAAAGCCATC 5’ |
| CRE-F | 5’ GACGCCGCACTAAAGCCATCCTCTG 3’ |
| CRE-R | 3’ CTCGGCGGGCTGGAGTACAGGAAAC 5’ |
| mCRE-F | 5’ GACGCCGCACTAAAGCCATCCTCTG 3’ |
| mCRE-R | 3’ CTCGGCGGGCTGGAGTACAGGAAAC 5’ |
Fig. 5. EMSAs to assess binding of nuclear factors to the CRE site located in the proximal region of the HKII promoter and detection of CREB family members in hepatoma cells. A, EMSA studies to detect proteins in the nuclear extract that bind CRE. These were performed after incubation of a 32P-labeled CRE probe (Table II) with nuclear extracts from the AS-30D hepatoma cells without competitor (lane 1) and with competitor (comp), a 200-fold molar excess of unlabeled wild type (WT) (lane 2), or mutant (lane 3) CRE oligonucleotide. Proteins within band I are considered to have specificity for the CRE site, whereas proteins within bands marked NS are considered to be nonspecific. B, EMSA studies to determine whether the specific band I contains CREB family members. Experiments were performed as in A without antibody (lane 1), with an antibody to CREB* (lane 2), or with a nonspecific antibody (lane 3). (*The CREB* antibody used here is known to exhibit cross-reactivity with CREB, and family members ATF1 and CREM.) C, EMSA studies to determine whether the specific band I contains the transcription factors CREB, ATF1, or CREM. Experiments were performed as in A in the presence of labeled (WT) and unlabeled mutant (MT) CRE oligonucleotides without antibody (lane 1), or with a specific antibody against CREB (lane 2), a specific antibody against ATF1 (lane 3), and finally a specific antibody against CREM (lane 4). Band I, reactive with the 3 different antibodies, is indicated an arrow. SS refers to band I after it has been supershifted by antibody. D, detection of CREB and family members in AS-30D hepatoma cells. Total cell extracts of hepatoma AS-30D cells (50 and 100 µg) were subjected to electrophoresis and then to Western blot analysis with an antibody specific for CREB (left panel), an antibody specific for ATF1 (center panel), and an antibody specific for CREM (right panel). Enhanced chemiluminescence was used to detect the signal.

(SS or supershift) of band I (lanes 2–4) indicating that this band contains all 3 transcription factors. This experiment was carried out against a background of the oligonucleotide (MT) mutated in the CRE sequence to prevent interference by the nonspecific bands. Finally, results presented in Fig. 5D verify the presence in AS-30D hepatoma cells of proteins that react with a CREB specific antibody (left gel), an antibody specific for ATF1 (center gel), and an antibody specific for CREM (right gel).

To confirm that CREB can function as an activator of the HKII promoter in AS-30D hepatoma cells, these cells were transiently co-transfected with the −329 deletion construct of the promoter containing the luciferase reporter gene and variable amounts of an expression plasmid encoding CREB. Fig. 6A shows that the newly expressed CREB protein causes a significant activation of the HK II promoter (center and right bars) relative to that observed when the expression plasmid containing CREB is omitted (left bar). To determine whether the HKII promoter activity observed when the expression plasmid containing CREB is omitted is due in part to the presence of endogenous CREB family members in the hepatoma cells, these cells were transfected with variable amounts of an expression plasmid containing CREB referred to as “A-CREB.” This form interacts with CREB and family members ATF1 and CREM and prevents their binding to DNA (35). Results presented in Fig. 6B show that co-transfecting the −329 deletion construct of the HKII promoter and variable amounts of an expression plasmid containing A-CREB in AS-30D hepatoma cells causes promoter activity (far left bar) to be inhibited by about 70% (far right bar). These findings provide evidence that the transcription factor CREB and the related proteins ATF1 and CREM are present in the AS-30D model hepatoma cell line, that they do interact with the single CRE that contributes in part to HK II promoter activity, and that endogenous levels of these proteins contribute to HKII promoter activation.

The CCAAT and Inverted CCAAT Boxes Contributing to the Optimal Activity of the HKII Promoter Bind the Transcription Factor NF-Y That Acts as a Promoter Activator——In the final set of experiments, we focused on determining whether the transcription factor NF-Y is present in nuclear extracts from the AS-30D hepatoma cells, and, if so, whether it is an activator of the HKII promoter in these cells. Both the CCAAT box and inverted CCAAT box are known to bind NF-Y, a heterotrimeric complex consisting of NF-YA, NF-YB, and NF-YC (reviewed in Ref. 36). Fig. 7A shows that when a double stranded 32P-labeled oligonucleotide containing the CCAAT box is mixed
with hepatoma nuclear extracts and subjected to EMSA analysis, 4 bands are resolved (lane 1). All 4 bands (I, II, III, and IV) disappear on addition of unlabeled CCAAT oligonucleotide (lane 2) but not on addition of oligonucleotide mutated in the CCAAT box (lane 3). The same 4 bands disappear also on addition of an unlabeled oligonucleotide containing the inverted CCAAT box (lane 4), whereas an oligonucleotide mutated in the inverted CCAAT box causes significant disappearance of band II, but not bands I, III, and IV (lane 5). This suggested that common binding proteins for the CCAAT box and inverted CCAAT box are present in bands I, III, and IV, and, therefore, that one or more of these bands contains the transcription factor NF-Y. Consistent with this conclusion, the results of EMSA analysis (Fig. 7B) conducted with a 32P-labeled oligonucleotide containing the CCAAT box, and an antibody available to NF-YB, show disappearance of most of band I (lane 8) with a small remaining amount shifting upward (SS or supershift). Disappearance of band I is not observed using a control antibody, IgG (lane 7), indicating that the interaction of band I with the NF-YB antibody is specific.

To determine whether NF-Y is involved in HKII promoter activation in AS-30D hepatoma cells, these cells were co-transfected with the −329 deletion construct of the HKII promoter and variable amounts of expression plasmids encoding dominant negative NF-YA (DN-NF-YA) or wild type NF-YA. DN-NF-YA, differing in only 3 amino acids from NF-YA, interferes with the formation of a functional complex between DNA and NF-Y (37). Fig. 8 shows that expression of DN-NF-YA reduces the HKII promoter activity in hepatoma cells (central bar) about 40% (far right bar) indicating that part of the HKII promoter activity is because of activation by endogenous NF-Y. In contrast, expression of wild type NF-YA (control) fails to show any effect on HKII promoter activity (two left bars) as it cannot function in the absence of NF-YB and NF-YC. These findings provide evidence that the transcription factor NF-Y is present in nuclear extracts of AS-30D hepatoma cells, and that it activates the HKII promoter by binding to both the CCAAT box and the inverted CCAAT box.

The DNA Sequence of That Segment of the HKII Promoter Required for Its Activity in Hepatoma Cells Is Highly Conserved Among HKII Promoters but Not Among Promoters of Other Hexokinases—As the results obtained from the previously described experiments showed that under tumor-like conditions a short segment (−281 to −35) of the proximal region of the HKII promoter accounts for its full activity, we inquired whether this property is unique to HKII promoters. For this reason, we aligned the sequences of the proximal regions of HKII promoters from 3 different sources, i.e. the rat (11), mouse (38), and human (39), with sequences reported previously for HKI (40), HKII (41), and HKIV (42, 43), all from the rat. The results based on the program CLUSTAL W (44) and presented in Fig. 9 show clearly that the HKII proximal promoter sequences, including those comprising the relevant response elements discussed above, are highly conserved. Specifically, the rat sequence exhibits 95.8 and 75.2% identity, respectively, with those reported for the mouse and human. However, this high degree of sequence identity does not extend to the other hexokinase promoters of the rat where the HKI, HKII, and HKIV proximal promoter regions exhibit only 36.5–44.7% identity with that of HKII. Moreover, with the exception of the HKI
promoter that contains two GC boxes that bind members of the Sp family (45), other response elements characteristic of the proximal region of the HKII promoters are either absent or poorly conserved in HK promoters I, III, and IV. These findings suggest that the selective overexpression of HKII in highly malignant hepatomas is related directly to its unique response element organization within the proximal region of its gene promoter.
DISCUSSION

Results reported here provide valuable new insights into how gene regulation of HKII at the promoter level may contribute to its overexpression in many cancers and therefore to the high glycolytic phenotype. Specifically, we have examined the activity of the HKII promoter within freshly isolated hepatoma cells under conditions, i.e., availability of serum and glucose, that closely reflect those that many different types of tumors are likely to experience. The results obtained can be divided into four sets. In the first, deletion analysis revealed that a very narrow region (−281 to −35) flanking the transcription start site is responsible for the full activity of the HKII promoter. This region is rich in known or putative response elements including E2F, 6 GC boxes, CRE, a CCAAT box, and an inverted CCAAT box. A second set of results based on site-specific mutational analysis showed that 4 of the GC boxes (GC1, GC2, GC5, and GC6), CRE, the CCAAT box, and the inverted CCAAT box contribute in part to HKII promoter activity. In a third set of studies, evidence was obtained that each of the 3 transcription factors in the Sp family (Sp1, Sp2, and Sp3) bind to at least 2 of the 4 GC boxes; that transcription factors in the CREB family (CREB, ATF1, and CREM) all bind to CRE; and that the transcription factor NF-Y binds to the CCAAT box and the inverted CCAAT box. In a final set of experiments, evidence was obtained either directly or indirectly (e.g. using dominant negative forms) that each of the above transcription factors can contribute to HKII promoter activation.

The above results suggest that two major signal transduction pathways, one involving CREB family members and NF-Y, that were demonstrated earlier to be activators of the muscle HKII promoter (46–48), and another involving Sp family members, may play prominent roles in promoting HKII overexpression in tumors. However, as HKII is not overexpressed in muscle (8, 11) where signal transduction pathways involving CREB and NF-Y are operative (47), this would suggest that in tumors the Sp family members may further enhance the rate of transcription. Although we have shown here that Sp1, Sp2, and Sp3 can activate the HKII promoter in Drosophila cells deficient in Sp family members, future experiments will be necessary to assess the roles of these transcription factors in HKII overexpression in tumor cells.

Additional support for signal transduction pathways altering gene transcription via Sp family members is exemplified by a number of studies demonstrating the capacity of Sp1 to undergo phosphorylation and dephosphorylation (49–52). Among these reports, most relevant to the work described here is a study with two other glycolytic enzymes, pyruvate kinase and aldolase, both of which show high activity levels in the rat hepatoma cell line FTO2B that also expresses the high glycolytic phenotype (52). Here, evidence is provided that in the presence of glucose, dephosphorylation of Sp1 is facilitated resulting in the transcriptional activation of pyruvate kinase and aldolase. Although the transcriptional activity of HKII was not examined in the latter study, it would be predicted to be activated in the glucose-supplemented medium used in experiments described here as Sp1 would be dephosphorylated and bind to GC boxes 1, 2, 5, and 6.

Results of experiments described here may be related to the epigenetic regulation of the HKII gene described in an earlier study from this laboratory (24). In this study, we showed that the HKII gene contains a CpG island that encompasses the transcription start site, a region that we now know includes the HKII promoter segment (−281 to −35) shown in this study to be required for the activity of the promoter in a serum/glucose-based medium. Moreover, this island was shown to be methylated at only 6 sites in the −281 to −35 region in hepatocytes where HKII is nearly silent, and to be unmethylated at all of these sites in the AS-30D model hepatoma where HKII is markedly activated (24). Significantly, one of these methylated sites (−55) resides in GC box 6, a second (−70) resides in the CRE site, and a third (−202) lies near GC box 2. Therefore, it seems likely that in the hepatocyte → hepatoma transition process demethylation of the methylated cytosines within the −281 to −35 region of the HKII promoter is important for activating gene transcription.

Finally, these studies support the view that among the hexokinase genes, HKII may be the most highly regulated, and that the heart of this regulation resides in the narrow segment (−281 to −35 bp) within the proximal region of its promoter. These studies also raise an intriguing question about the role of the remaining 90% of the HKII promoter. One possibility is that it participates in response to stress, e.g. when oxygen is limiting. In this regard, we demonstrated in a previous study (23) that the HKII promoter is activated by hypoxic conditions, and that part of the hypoxic response involves its distal region and the remainder involves its proximal region.

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