Glucose Metabolism in Cancer

EVIDENCE THAT DEMETHYLATION EVENTS PLAY A ROLE IN ACTIVATING TYPE II HEXOKINASE GENE EXPRESSION*

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One of the “signature” phenotypes of highly malignant, poorly differentiated tumors, including hepatomas, is their remarkable propensity to utilize glucose at a much higher rate than normal cells, a property frequently dependent on the marked overexpression of type II hexokinase (HKII). As the expression of the gene for one of the initial events in activating the HKII gene resulted in activating the basal expression levels of HKII mRNA and protein. Finally, stably transfecting the hepatocyte cell line with DNA demethylase also activated basal expression levels of HKII mRNA and protein. These novel observations indicate that one of the initial events in activating the HKII gene during either transformation or tumor progression may reside at the epigenetic level.

One of the most common biochemical phenotypes of highly malignant, poorly differentiated cancer cells is their capacity to metabolize glucose at elevated rates (1–3). This aberrant metabolism serves well the goal of the cancer cell to proliferate both by maintaining a constant supply of energy even when oxygen levels decrease and by providing enhanced levels of biosynthetic precursors. Thus, the transformation/progression process that ultimately leads to the high glycolytic tumor phenotype provides the tumor with a metabolic advantage over its normal tissue of origin.

Significantly, we have demonstrated in earlier studies the essential role that hexokinase plays in sustaining the high glycolytic tumor phenotype (4, 5), particularly the type II isoform that becomes markedly elevated in rapidly growing, highly malignant hepatomas (6, 7). These experimental observations are dramatic considering that liver normally expresses glucokinase (type IV “high K<sub>m</sub>” hexokinase), whereas the type II (“HKII,” gene is located on a single rat chromosome, within a poorly differentiated hepatoma, the expression of HKII may be elevated more than 100-fold (6), whereas the type IV enzyme is undetectable (6, 8). Thus, in the transformation/progression process the genetic machinery has been directed to completely down-regulate the expression of type IV hexokinase and markedly up-regulate that of HKII. The major advantages of doing this are 2-fold (9), one of which is to enhance the glycolytic rate. This role is served optimally by HKII as it has a high affinity for ATP and binds to outer mitochondrial membrane porin (10) where it has more ready access to ATP for phosphorylating glucose (11) and is less sensitive to both product inhibition (4) and proteolytic degradation (12). The second advantage is that, by binding to the mitochondria, HKII acts as an antiapoptotic factor (13), thus protecting the cancer cells against death signals and promoting their immortality.

In a program designed to elucidate the molecular basis for the marked activation of HKII in rapidly growing hepatomas, we have employed the AS-30D cell line growing in ascites form in the peritoneal cavity of rats. This is a hepatocellular carcinoma line derived originally from a solid liver tumor induced by feeding rats the carcinogen dimethylaminoazobenzene (14, 15). This cell line exhibits the high glycolytic phenotype characteristic of aggressive tumors (4) and contains markedly elevated levels of both HKII mRNA (7) and the expressed enzyme bound to the outer mitochondrial membrane (4, 11). From this cell line we have isolated the HKII promoter (4.3 kb) and shown that it is quite promiscuous in its activation response to a number of physiological agents or conditions (7, 16–18). These include hypoxia, glucose, dibutyryl cAMP, a phosphor ester, mutated p53, and the opposing hormones insulin and glucagon. Furthermore, fluorescence in situ hybridization analysis showed that the normal rat liver contains a single rat HKII gene (5) that is ampliﬁed at least 5-fold without noticeable chromosomal aberrations or rearrangements (19). Finally, we have sequenced the normal rat liver promoter and found that it is about 99% identical to the AS-30D hepatoma promoter (GenBank™ accession number AY082375), rendering it unlikely that liver versus hepatoma differences in HKII expression are related to differences in the nucleotide sequence of the two promoters.

Although the above studies demonstrated that a combination
of gene amplification and transcriptional events contribute significantly to the marked expression of HKII in highly glycolytic hepatoma cells, they fail to explain why the expression of the enzyme is nearly silent in normal liver (7). These findings, and the recent progress in the study of the role of epigenetic factors in the silencing and reactivation of gene expression (20–22), led us to generate a working hypothesis. Stated simply, our hypothesis envisions that methylation/demethylation events may be involved in regulating HKII gene expression in hepatocytes and highly malignant hepatomas. The results of experiments described below provide substantial support for this working hypothesis.

EXPERIMENTAL PROCEDURES

Animals and Cells—Rats (Sprague-Dawley, female) were obtained from Charles River Breeding Laboratories. Their care and experimental use was approved by and conducted in accordance with the guidelines of The Johns Hopkins University Animal Care and Use Committee. Rat hepatocytes, freshly prepared by the collagenase perfusion method (25), were kindly provided by Dr. Anna Mae Diehl, Department of Medicine, The Johns Hopkins University School of Medicine. The normal rat liver (clone 9) cells (American Type Culture Collection) were grown in 90% DMEM/Ham’s F-12 (1:1), and 15% HEPES, pH 7.5, L-glutamine, and 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO2. The clone 9 cells were maintained in the exponential growth phase at all times with subculture every 48 h at 1:5 dilution. AS-30D hepatoma cells were grown in the peritoneal cavity of female Sprague-Dawley rats (100–150 g) and were harvested from the ascites fluid 6–7 days post-transplantation as described earlier (6). These cells were harvested by collagenase digestion and purified using a Wizard DNA Clean-Up System (Promega). The bisulfite reaction was completed by 50 μl of TE (10 mM Tris, 0.1 mM EDTA, pH 7.5) and stored at −20 °C for up to 1 month.

Sodium Bisulfite Conversion—Sodium bisulfite deaminates unmethylated cytosine to uracil in single-stranded DNA under conditions where the 5-methylcytosine remains nonreactive. Thus, all cytosine residues remaining after PCR amplification and sequencing represent cytosines that were methylated in the original DNA sequence.

The genomic DNA was isolated from freshly isolated rat hepatocyte and AS-30D hepatoma cells using a genomic DNA isolation kit (Qiagen) according to the manufacturer’s protocol. DNA (10 μg) was digested to completion by BgII (New England Biolabs) at 37 °C and purified using a Wizard DNA Clean-Up System (Promega). The bisulfite reaction was carried out for 16–18 h at 50 °C, pH 5.0, on 1 μg of BgII-digested genomic DNA from either rat hepatocytes or AS-30D cells using CpGenome™ DNA modification kit (Intergen) according to the manufacturer’s instructions. The modified DNA was finally eluted in 50 μl of TE (10 mM Tris, 0.1 mM EDTA, pH 7.5) and stored at −20 °C for up to 1 month.

PCR Amplification of Sodium Bisulfite-modified DNA and Primers—PCR amplifications were performed using the HotStarTaq™ PCR kit (Qiagen). Sodium bisulfite-treated DNA (100 ng) was amplified in a 50-μl reaction mix containing 200 μM each of the four dNTPs, 30 pmol of each primer, 1.5 mM MgCl2, 1× PCR buffer, 1× Q solution, and 2.5 units of HotStarTaq DNA polymerase (Qiagen). All reagents used were those supplied with the kit. The sequences of strand-specific primers containing the modified cytosine bases together with the annealing temperature used for the amplification of sodium bisulfite-treated DNA are summarized in Table I. The general hotstart thermal cycler program used for all the reactions was as follows: 95 °C for 15 min × 1 cycle; 94 °C for 1 min, 48 or 50 °C for 1 min, 72 °C for 1 min × 40 cycles; 72 °C for 10 min × 1 cycle.

Sequence Analysis—The PCR fragments amplified from rat hepatocyte and AS-30D hepatoma modified DNA were cloned using pCR®2.1 TA Cloning™ kit (Invitrogen) according to the manufacturer’s instructions. The positive clones were sequenced in the Biosynthesis and Sequencing Facility, Department of Biological Chemistry, The Johns Hopkins University School of Medicine.

Analysis of Sodium Bisulfate Modification Efficiency—To test the efficiency of bisulfite conversion, the modified DNA was PCR-amplified using modified primers specific for HKII and digested with the restriction enzymes ApoI (R | AATT † Y) or Tsp509I (AATT † Y) (New England Biolabs) that cut only modified DNA. These restriction enzyme sites are only generated when cytosine residues are modified to thymine residues. Subsequently, the efficiency of bisulfite conversion is assessed by complete digestion of a PCR fragment by ApoI or Tsp509I.

DNA Methylation by DNMT Inhibitors, 5′-Aza-cytidine and 5′-Az2′-deoxycytidine—Clone 9 hepatocyte cells that predominantly express high Km glucokinase were used in this study. These cells were seeded at a density of 5 × 105 cells/100-mm dish and maintained in DMEM/Ham’s F-12 (1:1) (Invitrogen) and 10% fetal bovine serum as detailed before. The test populations of cells were treated with either 2.5 and 5 μM 5′azaC (Sigma) or 2.5 and 5 μM 5′azadC (Sigma). The cells were harvested after 96 and 120 h of drug treatment, and total RNA was isolated using RNeasy® kit (Qiagen) according to the manufacturer’s instructions.

RT-PCR Analysis—RT-PCR was performed using TITANIUM™ One-Step RT-PCR kit (Clontech) according to the manufacturer’s protocol. Total RNA (1 μg) from each sample was used for multiplex RT-PCR in a 25-μl reaction mix containing 40 μM each of the following primers: 3 mM MgCl2, 0.2 μM dNTPs, 20 units of recombinant RNase inhibitor (Promega), and 20 pmol each of the HKI-specific primers: HKRTP (5′-GGTTGCGTCCAGGATGAGGC-3′, sense, position 469–490 of HKII cDNA) and HKRTR (5′-CGTTCGATGGTCAATTGAGG-3′, antisense, position 1570–1590 of HKII cDNA), and HKRMB (5′-CAATAGCCCTCCGGACCAAA-3′, sense, position 2243–2270 of HKII cDNA) and HKRMBR (5′-TAAAGTGAAGACCGGAGGT-3′, antisense, position 2713–2740 of HKII cDNA).

Table I

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a Boldface letters indicate primer bases corresponding to cytosine/uracil conversions.

b GenBank™ accession number U198605.
with ethidium bromide, and photographed. PCR products were electrophoresed on 2% agarose gels, stained for 30 s, 65°C for 1 min × 1 cycle; 94°C for 5 min × 1 cycle; 94°C for 30 s, 65°C for 30 s, 68°C for 1 min × 30 cycles; 68°C for 2 min × 1 cycle. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed.

Western Blot Analysis—Clone 9 cells were seeded at a density of 2 × 10⁵ cells/150-mm dish and treated with DNMT inhibitors: 5 azacytidine (2.5 and 5 μM) and 5 azadC (2.5 and 5 μM) for 120 h as described earlier. Total cell lysate (100 μg) from each test sample was separated by 10% SDS-PAGE. Subsequently, the proteins on the gel were transferred in the cold onto a polyvinylidene difluoride membrane (Bio-Rad) in CAPS buffer (10 mM CAPS, 10% v/v methanol, pH 11) at 100 V/2 h. The membranes were then blocked for overnight at 4°C with 5% nonfat dry milk in TBST (20 mM Tris, 136 mM NaCl, 0.1% Tween 20, pH 7.6), incubated with rabbit anti-HKII polyclonal antibody (Santa Cruz Biotechnology) at 22°C for 1 h, followed by 1 h of incubation with a secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences). Finally, HKII protein was detected by an ECL system (Amersham Biosciences) according to the manufacturer’s protocol.

Establishment of a Stable CRLdM Cell Line—The DNA demethylase (dMTase) cDNA, a kind gift from Dr. M. Szyf (McGill University, Montreal, Canada), had been cloned previously in the mammalian expression vector pcDNA 3.1/His (Invitrogen) containing neomycin for selection of stable transfectants (24). Clone 9 cells were maintained in DMEM/Ham’s F-12 (1:1) containing 10% fetal bovine serum, as described earlier. The cells were seeded in 6-well plates at a density of 2 × 10⁶ cells/well. The dMTase expression construct (2 μg) was transfected per well using LipofectAMINE™ 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After transfection for 48 h, the cells were split 1:10 in DMEM/Ham’s F-12 (1:1) containing 10% fetal bovine serum, and cells were maintained in G418 (Geneticin®)-selective antibiotic (Invitrogen). The cells (CRLdM) were selected on G418 for 14 days, and viable colonies were expanded for further experiments.

Nucleotide Sequence Accession Numbers—The GenBank™ accession numbers for the rat HKII promoter sequence from normal liver and hepatoma cells (AS-30D) are AY082375 and U19605, respectively.

RESULTS

A CpG Island Is Located within a Region That Includes the Transcription Start Site of the HKII Promoter, the First Exon, and Part of the First Intron—As an initial test of our hypothesis that methylation/demethylation events may be involved in regulating HKII gene expression in normal liver and hepatoma cells, we carried out a search for CpG dinucleotide rich “CpG islands” using computer algorithm “CpG Island Finder” (25). Such islands frequently contain methylated cytosines in repressed genes (20–22). Significantly, a high density of CpG dinucleotides was found in a response element-rich region straddling the transcription start site (Fig. 1A). This region (−350 to +781 bp) shown in Fig. 1B contains 58.5% GC content with CG(dA)/(CG)gmp ratio > 0.8 and fits the criteria attributed to a classical CpG island (26). This finding applies to both normal liver and the AS-30D model hepatoma as they show >99% sequence identity (GenBank™ accession numbers AY082375 and U19605).

MSRE Analysis Indicates That the Methylation Pattern of the Hepatocyte HKII Promoter and Associated First Exon and Intron Is Different from That of the Hepatoma Model—The above analysis identifying a CpG island (−350 to +781 bp) in the HKII promoter raised the question as to whether this segment might be involved in regulation of stable transfectants (24). Clone 9 cells were maintained in DMEM/Ham’s F-12 (1:1) containing 10% fetal bovine serum, and cells were maintained in G418 (Geneticin®)-selective antibiotic (Invitrogen). The cells (CRLdM) were selected on G418 for 14 days, and viable colonies were expanded for further experiments.

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MSRE Analysis Indicates That the Methylation Pattern of the Hepatocyte HKII Promoter and Associated First Exon and Intron Is Different from That of the Hepatoma Model—The above analysis identifying a CpG island (−350 to +781 bp) in the HKII promoter raised the question as to whether this segment and perhaps other regions of the promoter are differentially methylated in hepatocytes and hepatoma cells. For this reason, we subjected genomic DNA obtained from freshly isolated hepatocytes and AS-30D cells to digestion with several methylation-sensitive restriction enzymes (BstUI, HhaI, HpaII, EagI, and CiaI). The fully digested genomic DNA was subjected to Southern blot hybridization using a probe containing the 4.3-kb HKII promoter with first exon and intron (7). With only one exception (EagI), the results obtained (Fig. 2) clearly showed more bands in the lanes containing restriction enzyme-digested hepatoma DNA than hepatocyte DNA (compare lanes 1 and 2; 3 and 4; 5 and 6; and 9 and 10). Moreover, the bands were in different positions in all cases. Thus, these findings strongly implicate hypermethylation of the HKII promoter in hepatocyte genomic DNA as compared with hepatoma DNA. Also, lanes containing digested hepatoma DNA showed much more intense bands than lanes containing the same amount of hepatocyte DNA consistent with our earlier work showing HKII gene amplification in the AS-30D hepatoma cell line (19).

Bisulfite Modification/Sequence Analyses of the HKII CpG Island Reveals Significant Methylation in Hepatocytes While Detecting None in the Hepatoma Model—The observations noted above provided the impetus for subjecting the HKII CpG island (−350 to +781 bp) to sodium bisulfite modification/sequence analyses. Sodium bisulfite converts cytosine to uracil in single-stranded DNA under conditions whereby 5-methylcytosine remains non-reactive. After PCR amplification and sequencing, all cytosines that remain are the ones that were originally methylated.

Results presented in Fig. 3, A and B, provide examples of how these analyses were conducted for the HKII CpG island in

![FIG. 1. Analysis of the rat HKII promoter and associated first exon and intron for CpG dinucleotide frequency. A, schematic representation of 4.3-kb HKII promoter and associated first exon and intron showing the frequency of CpG dinucleotides. The putative binding sites for some of the common transcription factors have also been marked. The arrow denotes the transcription start site. Each filled circle above the line represents one CpG dinucleotide. The CpG island has been marked by a black line below the schematic representation of the HKII promoter. B, the sequence of the CpG island of the rat HKII gene (GenBank™ accession number AY082375). The putative motifs for binding of different transcription factors as well as all the CpG dinucleotides (filled circles) have been marked in this 1131-bp region (−350 to +781 bp) straddling the transcription initiation site (denoted by +1).](http://www.jbc.org/content/1535/6/15335.full.html)
The genomic DNA was isolated from rat hepatocyte (lanes 1, 3, 5, 7, and 9) and hepatoma (AS-30D) cells (lanes 2, 4, 6, 8, and 10) was subjected to complete digestion using various methylation-sensitive restriction endonucleases including BamHI (lanes 1 and 2), HpaII (lanes 5 and 6), EcoRl (lanes 7 and 8), and ClaI (lanes 9 and 10). The digested DNA was run on a 1% agarose gel, transferred onto a nylon membrane, and probed with the 4.3-kb rat HKII promoter and associated first exon and first intron, as detailed under “Experimental Procedures.” The arrows mark the bands showing different patterns of digestion of the promoter in rat hepatocytes and hepatoma cells.

Fig. 2. MSRE analysis of the HKII promoter and associated first exon and intron. The genomic DNA isolated from rat hepatocyte (lanes 1, 3, 5, 7, and 9) and hepatoma (AS-30D) cells (lanes 2, 4, 6, 8, and 10) was subjected to complete digestion using various methylation-sensitive restriction endonucleases including BamHI (lanes 1 and 2), HpaII (lanes 5 and 6), EcoRl (lanes 7 and 8), and ClaI (lanes 9 and 10). The digested DNA was run on a 1% agarose gel, transferred onto a nylon membrane, and probed with the 4.3-kb rat HKII promoter and associated first exon and first intron, as detailed under “Experimental Procedures.” The arrows mark the bands showing different patterns of digestion of the promoter in rat hepatocytes and hepatoma cells.
Hexokinase Gene Activation by DNA Demethylation in Cancer

350 to methylated CpG dinucleotide mapping of the rat HKII CpG island – 51 of different transcription factors in this CpG island are also shown. The putative motifs for binding of hypermethylated CpG dinucleotides in the rat HKII CpG island. The primary sequence of the HKII CpG island is shown with all CpG dinucleotides marked as vertical lines. The putative motifs for binding of different transcription factors in this CpG island are also shown. Information about the primers used for the amplification of bisulfite-treated DNA and the region amplified are given in Table 1. The black circles represent the CpG dinucleotides that show different methylation patterns in the hepatocyte and hepatoma HKII CpG island. The position of CpG dinucleotide with respect to the transcription initiation site has been marked below each black circle. The bent arrow denotes the transcription initiation site. Middle and lower panels, the methylation profile of the HKII gene within the CpG island found in rat hepatocytes and hepatoma cells. The methylation profile of 15 individual, bisulfite-treated clones from hepatocytes (middle panel) and hepatomas (lower panel) is shown. Only the CpG dinucleotides that show differences in methylation between hepatocyte and hepatoma cells have been depicted in the figure. The remaining CpG sites do not show any differences in their methylation pattern between rat hepatocyte and hepatoma cells (data not shown). The open circles represent unmethylated CpGs, and the black circles represent methylated CpGs. The degree of methylation is indicated by a + number (−, 0%; +, 1–25%; ++, 26–50%; ++++, 51–75%; ++++, 76–100%). Each row represents a single clone. The methylated CpG dinucleotide mapping of the rat HKII CpG island: −350 to +1 bp (A) and +1 to +781 bp (B).

CpG island of the HKII gene showed no methylation in hepatocytes or hepatoma cells.

In addition to the above, two other observations of potential interest emerged from these analyses. First, in the hepatocyte CpG island, the CpG sites downstream to the transcription initiation site (CpG−385, CpG−540, CpG−560, CpG−572, and CpG−717) showed higher degrees of DNA methylation (28–75%) as compared with the CpG sites upstream of the transcription initiation site, except for CpG−229. Second, the methylated CpG sites fell in some of the confirmed and putative sites for binding of different transcription factors, e.g. CpG−294 in cAMP-response element; CpG−54, CpG−70, and CpG−417 in OC boxes; CpG−415 in the activator protein-2-binding site; CpG−548 in the E-box, and CpG−566 in the NF-1 binding site.

In summary, these data show that those CpG sites indicated above that lie within a CpG island are methylated in hepatocytes where the expression of HKII is nearly silent and are unmethylated in the model hepatoma as-30d where this enzyme is markedly elevated.

In hepatocytes, DNMT inhibitors increase the basal level of HKII expression at both the mRNA and protein level—To determine to what extent methylation may be involved in silencing HKII expression in hepatocytes, we treated the hepatocyte cell line “clone 9” with different concentrations of DNMT inhibitors (5′azaC or 5′azadC) and then monitored the expression of HKII mRNA and protein (see “Experimental Procedures”). Clone 9 cells, as liver hepatocytes, express predominantly glucokinase (27) and down-regulate other hexokinase types.

Results presented in Fig. 5A show, relative to the untreated control (lane 1), that HKII mRNA expression in the hepatocyte cell line (clone 9) is activated both by 5′azaC and 5′azadC treatment (lanes 2–9 versus lane 1). Here mRNA from as-30d hepatoma cells was used as a positive control (lane 10). Quantitation of HKII mRNA expression in Fig. 5A by densitometric scanning showed that maximal activation was about 5-fold with 5′azaC (5 μM) and 5.8-fold with 5′azadC (2.5 μM) (Fig. 5B).

In order to determine whether the increase in HKII mRNA expression was reflected also by an increase in protein expression, cell lysates from the hepatocytes (clone 9 cells) were treated for 120 h with 5′azaC and 5′azadC and then subjected to SDS-PAGE (Fig. 5C, lower panel) followed by Western analysis (Fig. 5C, upper panel). The resultant immunoblot obtained with a polyclonal HKII antibody revealed that both 5 μM 5′azaC (lane 3) and 2.5 μM 5′azadC (lane 4) showed significant induction of HKII protein compared with the untreated control (lane 1). This was a consistent finding in a number of different experiments.

In experiments not reported here, we tested lysates of 5′azaC-treated clone 9 cells also for the induction of hexokinase activity using a spectrophotometric glucose-6-phosphate dehydrogenase-coupled assay (12). The treated cells exhibited a maximal specific hexokinase activity of about 3 mmol of glucose 6-phosphate formed per min/mg of protein, whereas untreated cells exhibited no detectable activity. This result was very dependent both on obtaining fresh cells from the supplier and assaying for hexokinase activity in the exponential growth phase.

Hepatocytes Stably Transfected with dMTase Also Exhibit Increased Basal Levels of HKII mRNA and Protein—In order to test more directly whether DNA methylation plays a role in silencing HKII expression in hepatocytes, these cells (clone 9) were stably transfected with dMTase (see “Experimental Procedures”) and monitored for induction of HKII mRNA and protein. As shown in Fig. 6A, cells stably transfected with dMTase showed several-fold higher expression of HKII mRNA (2nd lane) than untreated cells (1st lane). The total cell lysate prepared from the same cells and subjected to SDS-PAGE (Fig. 6B, lower panel) followed by Western analysis (Fig. 6B, upper panel) showed also an increased level of hexokinase protein (2nd lane) relative to the control (1st lane). (It will be noted that two bands are observed here for HKII expression at the protein level consistent with an earlier report of a slightly larger precursor form of HKII in the as-30d hepatoma (28).)

Taken together, the last two experiments described here are
consistent with a role for methylation events in down-regulating the expression of HKII in hepatocytes.

**DISCUSSION**

The study reported here was undertaken to test the hypothesis that methylation events may be involved in down-regulating HKII gene expression in normal hepatocytes, whereas demethylation events may be contributing in part to its activation during tumor formation or progression. Considering that the high glycolytic/high hexokinase phenotype is one of the most commonly observed among highly malignant tumors (1–3, 9), and that it is used worldwide via positron emission tomography scanning to detect many human cancers (29), the hypothesis tested here takes on added significance. Specifically, as it relates to the rat hepatocyte/AS-30D hepatoma experimental system studied, the data obtained provide substantial support for the hypothesis tested. Thus, we have identified within the HKII gene a single CpG island that encompasses the transcription start site, first exon, and part of the first intron (Fig. 1), and we have gone on to show that this island is significantly methylated in hepatocytes (Figs. 2–4) but completely devoid of methylation in the AS-30D hepatoma (Fig. 4). Finally, in other experiments we have shown that demethylation agents like 5’azaC, 5’azadC, and dMTase activate the basal level of expression of HKII mRNA and protein in hepatocytes (Figs. 5 and 6).

It will be noted that demethylation agents cause modest increases in HKII mRNA and protein expression levels in hepa-
help promote the high glycolytic tumor phenotype by binding their respective transcription factors, thus enhancing transcription of the HKII gene.

Although much attention has focused on the role of hypermethylation of certain genes in cancer, particularly tumor suppressor genes (22, 31, 32), less attention has been given to those that are hypomethylated like the glycolytic related gene described here. Nevertheless, there is now a rapidly growing list of cancer-related genes where hypomethylation has been observed. These include those for v-globin in breast and colon adenocarcinomas (33), parathyroid hormone and catalase in colon adenocarcinoma (33), MUC1 in breast carcinoma (34), α-chorionic gonadotropin in benign and malignant colon polyps (35), β-chorionic gonadotropin in chorionicarcinoma (36), BCL-2 in human B cell chronic lymphocytic leukemia (37), H-Ras and metallothionein (MT-1) in mouse lymphosarcoma (38), and c-myc in colorectal carcinoma (39). How these genes become hypomethylated is of considerable interest with some recent studies implicating dMTase. Thus, the levels of dMTase correlate with the demethylation of CpG sites in the c-erbB-2, survivin, and lung resistance protein genes in ovarian cancers (40, 41).

On the basis of the above examples and results presented in this report, it is conceivable that during transformation of hepatocytes to highly malignant hepatoma cells, the levels of dMTase may be elevated or the enzyme may be activated, e.g. by phosphorylation or dephosphorylation. This in turn would lead to a progressive demethylation of the HKII gene and perhaps ultimately to the formation of a more accessible open conformation of the chromatin, thus allowing various transcription factors to bind and activate transcription.

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Hexokinase Gene Activation by DNA Demethylation in Cancer
