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Biochimica et Biophysica Acta 1368 (1998) 129–136

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Quantitative determinations of the steady state transcript levels of hexokinase isozymes and glucose transporter isoforms in normal rat tissues and the malignant tumor cell line AH130

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Received 19 March 1997; revised 29 July 1997; accepted 31 July 1997

Abstract

The steady state transcript levels of the four hexokinase (HK) isozymes and four glucose transporter (GLUT) isoforms were determined quantitatively by Northern analysis of RNA samples from rat tissues using synthetic fragments of the RNAs encoding the HK isozymes and GLUT isoforms. Results showed that the levels of HK isozyme transcripts were low in rat tissues, the level of that most highly expressed, the type I isozyme (HKI), in the brain being 0.025% of the total poly(A)$^+$ RNA. A good correlation was found between the reported HK activities and the total amounts of transcripts encoding all HK isozymes in various tissues, showing that the HK activities in tissues can be estimated from the total amount of transcripts encoding HK isozymes. The proposed associated expressions of HK isozymes and GLUT isoforms in particular tissues were confirmed at their transcript levels. The steady state transcript levels of type II HK and the type 1 GLUT isoform in the malignant tumor cell line AH130 were also determined quantitatively. © 1998 Elsevier Science B.V.

Keywords: Hexokinase; Glucose transporter; Energy metabolism; Northern blotting; Quantitative evaluation of transcript levels

1. Introduction

The uptake of glucose and its phosphorylation are major processes in cellular glucose metabolism. In mammalian cells, four isoforms of the transporter (GLUT1 to GLUT4) are known to facilitate the uptake of glucose through the plasma membrane (for recent reviews, see [1–5]). GLUT1 is expressed in a variety of tissues, and so is thought to be responsible for ''basal'' glucose uptake in various cells. On the other hand, the expressions of the other isoforms are tissue specific: GLUT2 is predominantly expressed in the liver, pancreatic β-cells, small intestine and kidney, GLUT3 in the brain, and GLUT4 in muscles and adipose tissue [1–5].

The first step in the sequential reactions in glucose metabolism is catalyzed by the four (type I to IV) isozymes of hexokinase (HK) (for reviews, see [6,7]). As type I HK (HKI) is expressed predominantly in all tissues except skeletal muscle and liver, it is considered as the ''basic'' HK, like GLUT1 of the GLUT isoforms. Expression of the type II isozyme (HKII) is highest in skeletal muscle and adipose tissue, that of the type III isozyme (HKIII) at very low levels in all tissues, and that of the type IV isozyme (HKIV),

Abbreviations: GLUT, glucose transporter; HK, hexokinase; SSC, saline sodium citrate; RT-PCR, reverse transcription followed by the polymerase chain reaction; BAT, brown adipose tissue; WAT, white adipose tissue

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PII S0005-2736(97)00189-2

usually known as glucokinase (GK), at high levels in the liver and pancreatic β-cells (for reviews, see [8–10]).

Based on these tissue specific distributions of the GLUT isoforms and HK isozymes, their associated expressions have been postulated [9,10]: GLUT1 with HKI, GLUT4 with HKII, and GLUT2 with HKIV. However, these combinations were mainly concluded from the qualitative transcript levels of the GLUT isoforms and enzymic activities of the HK isozymes. For understanding the mechanisms controlling energy metabolism in various tissues, quantitative comparison of their transcript levels in various tissues is of importance. Therefore, in this study, we analyzed the steady state transcript levels of GLUT isoforms and HK isozymes quantitatively using synthesized RNA fragments. We also determined the transcript levels of HKII and GLUT1 in the malignant tumor cell line AH130.

2. Materials and methods

2.1. Materials

Isogen, used in purification of RNA, and an in vitro RNA synthesis kit were purchased from Nippon Gene (Tokyo). *Taq* DNA polymerase, a *Bca*BEST DNA labeling kit and Oligotex dT < super > were from TaKaRa Shuzo (Kyoto). [α-^{32}P]dCTP (specific radioactivity, 111 TBq/mmol) was from Amersham (Bucks., U.K.). The multifunctional phagemids pTZ18R and pTZ19R were obtained from Toyobo (Osaka) and from Riken DNA Bank (Wako), respectively.

2.2. Construction of plasmids and preparation of cDNA probes

All cDNA fragments except that of HKI were prepared by RT-PCR as described previously [11,12] using first strand cDNAs prepared by reverse transcription of mRNA as templates. The cDNA fragments of GLUT isoforms with nucleotide sequences 414–929 for GLUT1 [13], 507–951 for GLUT2 [14], 278–771 for GLUT3 [15] and 242–799 for GLUT4 [16] and those of HK isozymes with nucleotide sequences of 1615–2133 for type II [17], 2250–2677 for type III [18] and 116–696 for type IV [19] were amplified. A cDNA clone of rat HKI was obtained from a cDNA library of rat heart (Clontech, code RL1006b). The inserted cDNA fragment of HKI was subcloned into pUC19 vector using restriction sites of *Eco*RI that were added artificially during preparation of the library. From this plasmid, a cDNA fragment of about 510 bp corresponding to nucleotides 937–1452 [20], prepared by digestion with *Eco*RI and *Nco*I, was used for construction of the expression vector. These cDNA fragments were subcloned into the multifunctional phagemid pTZ18R or pTZ19R, and after confirmation of their nucleotide sequences, the plasmids obtained were digested with the restriction endonucleases at the multicloning sites. The inserted cDNA fragments were gel-purified and used as probes in Northern blotting. As we used different regions of the entire cDNAs encoding GLUT isoforms and HK isozymes as probes, the cross-hybridizations between probes and RNA samples could not be examined by Northern blot analysis. However, on use of high stringency hybridization conditions, the degrees of cross-hybridizations of all probes used in this study were estimated to be less than 5% by Southern blot analysis (data not shown).

2.3. Preparations poly(A)$^+$ RNA samples from rat various tissues

RNA samples of various tissues were obtained from 4-week old male Wistar rats fed on standard laboratory chow with water ad libitum at 24°C. Tissues from five rats were pooled and total RNAs were purified with Isogen by the method recommended by the supplier, which was essentially based on the report of Chomczynski and Sacchi [21]. Poly(A)$^+$ RNA was purified from total RNA using oligotex dT < super > .

2.4. Preparation of synthetic RNA fragments

Fragments of the RNAs encoding GLUT isoforms and HK isozymes were prepared as follows. First, for obtaining double stranded linear DNA, the expression vectors prepared as described above were digested at the 3′-terminus of the inserted cDNA fragment and digested plasmids were precipitated with ethanol. With 1.0 μg samples of these linearized DNAs as templates, the RNA fragments encoding GLUT iso-

forms and HK isozymes were transcribed using T7 RNA polymerase. After complete digestion of template DNA with DNaseI, synthesized RNA fragments were purified by phenol/chloroform extraction and then repeated ethanol precipitation. The concentrations of RNA samples were determined spectorophotometrically in a Shimadzu spectrophotometer, model UV-160.

2.5. Northern blotting and quantitative determination of transcript levels

Northern blotting was carried out essentially as described previously [22,23]. Briefly, RNA was subjected to electrophoresis in 1.0% agarose containing formaldehyde, transferred to a nitrocellulose membrane, and hybridized by the standard procedure. Unless otherwise noted, samples of 1.0 μg of poly(A)$^+$ RNA obtained from various normal tissues of Wistar rats and 10–100 pg of synthesized RNA fragments were used for analyses. After hybridization with probes, the membranes were washed three times with 2 × saline sodium citrate (SSC) containing 0.1% SDS at room temperature for 10 min and twice with 1 × SSC containing 0.1% SDS at 60°C for 30 min each time and exposed to X-ray film with an intensifying screen at −80°C. The amount of each transcript was determined from its intensity in the autoradiogram with an ATTO image analyzer model AE-6900 connected to a Macintosh model Quadra 650. The amount of each transcript was normalized based on the intensity of the hybridization signal observed with the corresponding synthesized RNA fragment.

For staining of the transferred RNA, the membrane was first soaked in 5% acetic acid for 15 min, then immersed in solution containing 0.04% methylene blue and 500 mM sodium acetate (pH 5.2) for 10 min and washed with water for 10 min.

3. Results

3.1. Purity of synthesized RNA samples

For quantitative evaluation of transcript levels, it is important to determine the exact concentrations of synthesized RNA samples and their high purities. Contaminating free nucleotides in the RNA samples

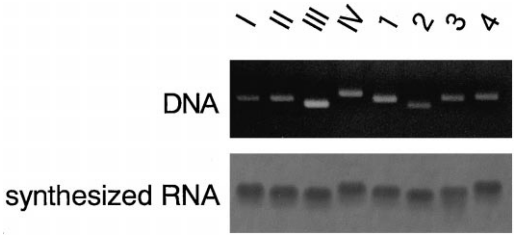

Fig. 1. Denaturation agarose gel electrophoresis of synthesized RNA samples of HK isozymes and GLUT isoforms. After synthesis of RNAs, template DNAs were completely digested with DNaseI. Then after elimination of the DNaseI by phenol/chloroform extraction, RNA samples were purified by repeated ethanol precipitation. For confirmation of their purities and concentrations, a fixed amount of each RNA sample (2.5 μg) was subjected to electrophoresis in denaturing agarose gel. The RNA samples were transferred to a nitrocellulose membrane and stained by methylene blue. I to IV and 1–4 represent RNA samples of HKI to HKIV and GLUT1 to GLUT4, respectively. The panel labeled ''DNA'' shows results on electrophoresis of the cDNA fragments used as probes in non-denaturing agarose gel.

used as substrates in RNA synthesis would increase the ''apparent'' concentration of RNA samples. Thus, we first purified synthesized RNA samples by repeated ethanol precipitation to eliminate contaminating free nucleotides completely. The extent of elimination of free nucleotides from the synthesized RNA samples was examined by measuring the optical absorbance of the filtrate obtained by centrifugation of RNA samples in Ultrafree C3-LGS (Millipore, cut-off M.W. = 10 000) at 4500 × *g* for 20 min and was concluded to be negligible in all RNA samples (data not shown). Then, after determination of the concentrations of synthesized RNA samples from their absorbances, we further examined the purities and amounts of RNA by denaturing agarose gel electrophoresis. As shown in Fig. 1, results showed that all the synthesized RNA samples gave a single band of the same intensity at the expected site of migration. Therefore, their purities were concluded to be high enough for further quantitative analyses.

3.2. Steady state transcript levels of HK isozymes in rat tissues

Next, we analyzed the steady state transcript levels of the four HK isozymes in rat normal tissues. For this, samples of 1.0 μg of poly(A)$^+$ RNA obtained from rat brain, heart, kidney, liver, skeletal muscle,

and white and brown adipose tissues (WAT and BAT) were subjected to electrophoresis and transferred on nitrocellulose membranes. Four membranes with each of these samples prepared in this way were used for hybridization with probes. As shown in Fig. 2, the transcript of HKI was observed in all tissues except liver; the signal was strongest in the brain, and the intensities in the heart, kidney and WAT were almost the same, while those in skeletal muscle and BAT were very weak. The signal of HKII was weak in heart, skeletal muscle and adipose tissues. HKIII gave no significant signal except a weak one in brain. Expression of HKIV was only observed in the liver.

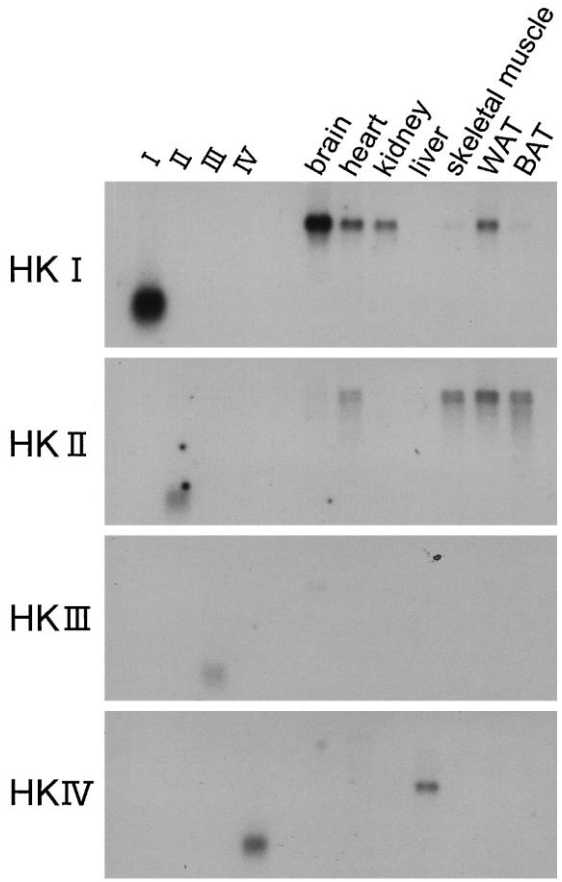

Fig. 2. Steady state transcript levels of the four HK isozymes in various rat tissues. Samples of 1 μg of poly(A)$^+$ RNA obtained from various tissues were subjected to denaturing agarose gel electrophoresis, transferred to nitrocellulose membranes and hybridized with probes. I to IV show results for synthesized RNA samples encoding HKI to HKIV, respectively.

3.3. Quantitative determinations of the steady state transcript levels of HK isozymes

In general, the steady state transcript levels of particular proteins in various tissues can be compared by the intensities of their hybridization bands, as described above. However, it is difficult to compare the amounts of the transcripts of different isoforms or isozymes exactly due to the different specific radioactivities and the affinities of the probes with transcripts. Therefore, we measured the intensities of hybridization bands with known amounts of synthesized RNA fragments used as standards. As the steady state transcript levels of these four HK isozymes were markedly different in various tissues, we used various amounts of RNA fragments of the four isozymes to determine the amount of each transcript exactly; 100 pg for HKI, 20 pg for HKII, 10 pg for HKIII, and 20 pg for HKIV. As the hybridization intensity of the synthesized RNA fragment of HKII used as external standard was not different from that in the presence of poly(A)$^+$ RNAs of WAT, it was concluded that presence of irrelevant RNAs did not cause any changes in the hybridization intensities of synthesized RNA fragments. In addition, we examined the purity of poly(A)$^+$ RNA from the steady state transcript levels of β-actin in these RNA samples. Results showed that the transcript levels of β-actin were not markedly different in RNA samples from various tissues (data not shown). Therefore, the transcript levels of these HK isozymes in different tissues could be determined quantitatively based on the assumption that the probes hybridize with the synthesized RNA fragments and transcripts in various tissues equally and gave the same signal intensity.

Table 1 shows normalized values for the steady state transcript levels of HK isozymes in various tissues. HKI, the most abundantly expressed HK isozyme, was found in the brain at a level of 0.382 fmol/μg of poly(A)$^+$ RNA. Of the four isozymes, the level of HKIII was the lowest, its highest level being only 0.006 fmol/μg of poly(A)$^+$ in the brain. Assuming that the average length of transcripts was 2.0 kb, the total amount of transcripts in 1.0 μg of poly(A)$^+$ RNA was calculated to be 1.52 pmol. Therefore, even in the brain, the amount of transcripts encoding HKI was estimated to be only 0.025% of the total poly(A)$^+$ RNA, indicating a very

Table 1
Amounts of transcripts encoding the four isozymes of hexokinase in normal rat tissues

Tissue	HKI	HKII	HKIII	HKIV	Total (relative amount in %)
Brain	0.382	0.010	0.006	ND	0.398 (100)
Heart	0.130	0.074	ND	ND	0.204 (51.3)
Kidney	0.079	ND	ND	ND	0.079 (19.8)
Liver	ND	ND	ND	0.058	0.058 (14.6)
Muscle	0.009	0.112	ND	ND	0.121 (30.4)
WAT	0.101	0.170	ND	ND	0.271 (68.1)
BAT	0.007	0.156	ND	ND	0.163 (41.0)

Values are shown in fmol/μg poly(A)$^+$ RNA. Numbers in parentheses in the column labeled ''total'' represent the sum of each transcript relative to that in brain. WAT and BAT: white adipose tissue and brown adipose tissue, respectively. ND: no hybridized band, or too low signal intensity to be analyzed.

low level of transcripts of HK isozymes. The total amounts of the transcripts encoding these HK isozymes in various tissues were determined from the sum of amounts of the transcripts of all the HK isozymes. Results indicated that HKI and HKII are the major determinants of the steady state transcript level of the HK isozymes.

3.4. Quantitative analysis of steady state transcript levels of GLUT isoforms in various tissues

Next, we determined the amounts of the transcripts encoding the four GLUT isoforms in RNA samples obtained from various rat tissues in the same way as for HK isozymes. As shown in Fig. 3, the tissue distributions of these four GLUT isoforms were essentially the same as those reported previously [13–16,24–26]. For quantification of the transcript levels, Northern blot analysis was performed with fixed amounts of synthesized RNA fragments; 50 pg for GLUT1, 100 pg for GLUT2, 20 pg for GLUT3 and 100 pg for GLUT4. From the hybridization intensities of these RNA fragments, the amounts of transcripts were determined quantitatively as summarized in Table 2.

The transcribed levels of HK isozymes in various tissues were markedly different, but those of the GLUT isoforms were less, the maximum difference being about 5-fold between the highest level of GLUT4 in muscle (0.699 fmol/μg of poly(A)$^+$) and the lowest level of GLUT3 in the brain (0.139 fmol/μg of poly(A)$^+$ RNA). In addition, the results in Tables 1 and 2 showed the specific association of the expressions of certain HK isozymes and GLUT isoforms, as suggested previously [9,10]: HKI with GLUT1, HKII with GLUT4 and HKIV with GLUT2.

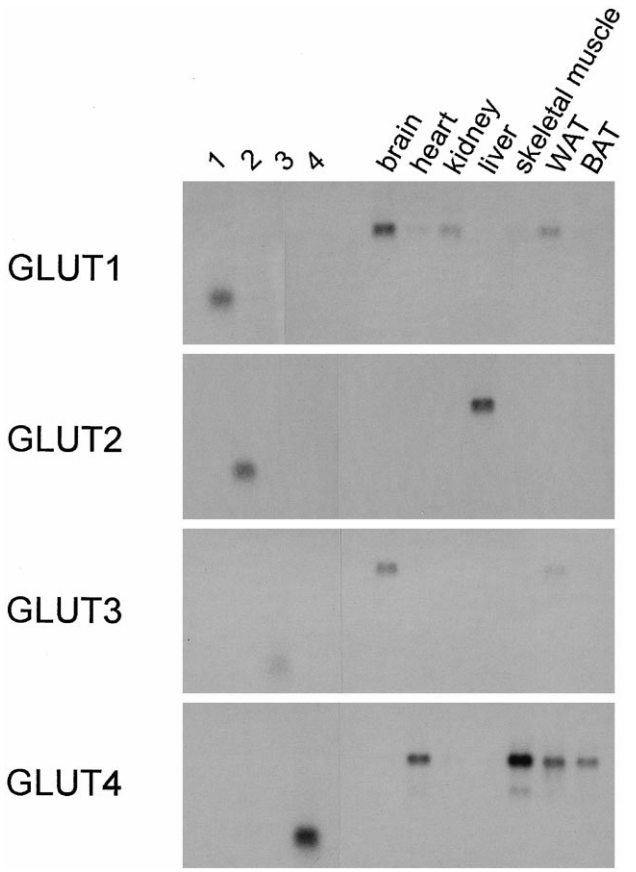

Fig. 3. Steady state transcript levels of the four GLUT isoforms in various rat tissues. Samples of 1 μg of poly(A)$^+$ RNA from the indicated tissues were analyzed as described in the legend of Fig. 2. Results for synthetic RNA samples encoding GLUT1 to GLUT4 are shown in columns 1–4.

Table 2
Amounts of transcripts encoding the four GLUT isoforms in normal rat tissues

Tissue	GLUT1	GLUT2	GLUT3	GLUT4	Total (relative amount in %)
Brain	0.297	ND	0.139	ND	0.436 (100)
Heart	0.019	ND	ND	0.288	0.307 (70.4)
Kidney	0.076	ND	ND	0.018	0.094 (21.6)
Liver	ND	0.616	ND	ND	0.616 (141.3)
Muscle	0.011	ND	ND	0.699	0.710 (162.8)
WAT	0.090	ND	0.045	0.464	0.599 (137.4)
BAT	ND	ND	ND	0.239	0.239 (54.8)

Values are shown in fmol/μg poly(A)$^+$ RNA. For details, see legend to Table 1.

3.5. Quantitative analysis of steady state transcript levels of GLUT1 and HKII in the malignant tumor cell line AH130

We found previously that the steady state transcript level of HKII was specifically and remarkably elevated in a synchronized manner with that of GLUT1, in rapidly growing poorly differentiated malignant tumor cell lines such as rat AH130 [12,23]. Therefore, we next determined their absolute amounts in AH130 cells by the method described above. The amounts of the transcripts encoding HKII and GLUT1 expressed in AH130 cells were determined to be 2.5 and 18.5 fmol/μg of poly(A)$^+$ RNA, respectively. These values are significantly greater than those observed in normal tissues. As described above, the highest transcript level of HKII was observed in white adipose tissue (0.170 fmol/μg of poly(A)$^+$ RNA) and that of GLUT1 in the brain (0.297 fmol/μg of poly(A)$^+$ RNA). Namely, the transcript levels of HKII and GLUT1 in AH130 cells were approximately 15 and 62 times those in normal tissues, respectively, corresponding to about 0.16% and 1.22% of the total poly(A)$^+$ RNA species, respectively.

4. Discussion

The transcript levels of particular proteins have usually been compared by northern blot analysis using the same amounts of mRNAs. However, this is not possible for quantitative determination of the transcript levels of different mRNA species due to the differences in the specific radio activities and affinities of the probes. For quantitative determination of the transcript levels of proteins in various tissues by Northern analysis, the intensities of hybridization bands of mRNA samples with probes must be normalized by the hybridization intensities with exact amounts of synthesized RNA fragments. By this procedure, the amount of a steady state transcript encoding certain protein can be determined exactly and compared with those of other proteins. To our knowledge, this method was first carried out by Stepien et al. [27] for determination of the transcript levels of three isoforms of the human mitochondrial adenine nucleotide translocator (ANT1-3) in various tissues.

In this study, based on the method of Stepien et al. [27], we determined the ''absolute'' amounts of the HK isozymes and GLUT isoforms transcribed. For this, we first synthesized RNAs of HK isozymes and GLUT isoforms and determined their concentrations exactly. Then, we determined their steady state transcript levels quantitatively in normal rat tissues and the transcript levels of HKII and GLUT1 in the malignant tumor cell line AH130. Results showed that the transcript level of HKI in the kidney was as low as 0.0052% of the total poly(A)$^+$ RNA (Table 1, 0.079 fmol/μg of poly(A)$^+$ RNA (= 1.52 pmol)). This value was in accordance with the results of cDNA screening. Namely, Nishi et al. [29] reported that on molecular cloning of cDNA clones encoding HKI from a cDNA library of human kidney, 79 clones of 1×10^6 phages gave positive signal in the first screening. This finding indicates that the amount of HKI mRNA is 0.0079% of the total mRNA. Similarly, from the yield of glucokinase from the liver, the content of glucokinase in total liver proteins was estimated to be as low as 0.005% by Andreone et al. [19]. The present study indicated a similar value of

0.0038% (Table 1). Thus, the results of the present study are in accord with reported results. In this study, we expressed the amount of a particular mRNA relative to total poly(A)$^+$ RNAs in the tissue cells. When the total amount of poly(A)$^+$ RNAs in cells changes markedly, description of the amount of transcript relative to the total amount of cellular DNA might be useful.

The total amounts of transcripts encoding the four HK isozymes in various tissues were determined from the sums of the amounts of each transcript as shown in Table 1. The relative transcript levels in various tissues were in the order, brain (100), white adipose tissue (68), heart (51), brown adipose tissue (41), skeletal muscle (30), kidney (20) and liver (15). It is interesting that the same order was observed in the HK activity in various tissues except skeletal muscle, although the specific enzyme activities of HK isozymes were different, being in the order: brain (100), heart (62), kidney (49), liver (15) and skeletal muscle (10) [30]. Therefore, the sum of the amounts of transcripts could be a useful parameter of the total HK activities in various tissues. The exceptional low HK activity in skeletal muscle from that expected by the steady state transcript level could be because expression of the most abundant type II HK in skeletal muscle is known to depend greatly on the contractile activity (more than 30-fold) [31]. Therefore, the activity and transcript level of HKII in skeletal muscle may vary remarkably in different samples.

We confirmed in this study that a certain HK isozyme is expressed in association with a particular GLUT isoform in various tissues: HKI with GLUT1, HKII with GLUT4, and HKIV(GK) with GLUT2 [9,10]. Therefore, the expressions of these HK isozymes and GLUT isoforms could be regulated by the same mechanisms. The molecular mechanisms of the transcriptional regulations of GLUT isoforms and HK isozymes in various tissues has been studied extensively [8–10,32,33], but it is still unknown whether the expressions of HK isozymes in a particular tissue are regulated by the same mechanism as those of the GLUT isoforms. To understand the mechanisms regulating energy metabolism in various tissues, it is important to examine how the associated expressions of HK isozymes and GLUT isoforms in tissues are regulated.

According to the ''general association'' concept [9,10], expression of HKI is associated with that of GLUT1 in normal tissues. Recently, we found a novel association of HKII with GLUT1 in various malignant tumor cells (submitted for publication and also Ref. [12]). This finding was confirmed by quantitative determinations of the transcript levels of HKII and GLUT1 in AH130 cells, which showed that the transcriptional regulations of HKII and GLUT1 in malignant tumor cells are different from those in normal tissues. Recently, two enhancer elements that are responsive to serum, growth factor and oncogenes were found in the gene encoding the GLUT1 isoform [33]. The gene encoding HKII has been studied extensively [11,12,28,34–40], but the mechanism of its transcriptional regulation during carcinogenesis is not yet understood. Studies on the molecular mechanism of its transcriptional regulation in relation to that of the GLUT1 gene in tumor cells are in progress.

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