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Evidence for multiple genes coding for the isozymes of hexokinase in the highly glycolytic AS-30D rat hepatoma

W. Christian Wigley* and Richard A. Nakashima

Department of Chemistry and Biochemistry, Texas Tech University, P.O. Box 4260, Lubbock, TX 79409, USA

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We have compared Southern blots of rat hepatoma DNA probed with Types I, II and III hexokinase cDNAs isolated from normal rat tissues. Hybridization patterns show several fragments recognized by both the Type I and II clones while no resemblance is observed between the Type III probe and the other two isozymes. It therefore appears that the Type I-like and Type II-like hepatoma isozymes are coded for by similar yet separate genes, while a dissimilar third gene codes for the Type III-like isozyme. In addition, a loss of heterozygosity was detected at the Type III locus in the AS-30D hepatoma when compared to normal tissue. As only the Type II-like isozyme is highly expressed in highly glycolytic tumors, these data have implications for differential gene regulation between the tumor isozymes.

Hexokinase; Isozyme; Polymorphism; AS-30D hepatoma

1. INTRODUCTION

Increased glucose catabolism is characteristic of rapidly growing tumor cells [1]. An estimated 60% of cellular ATP is generated via anaerobic glycolysis in the highly glycolytic AS-30D rat hepatoma cell line [2]. It has been reported that the activities of the 'rate-limiting' glycolytic enzymes are elevated in AS-30D cells over normal rat liver, with the greatest increase (over 100-fold) being seen in hexokinase activity [3].

The conversion of glucose to glucose-6-phosphate in mammalian tissues is catalyzed by three isozymes of hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), Types I, II, and III. These isozymes each consist of a single polypeptide chain with a molecular weight of approximately 100 kDa. They are all characterized by a low K_m for glucose and sensitivity to inhibition by the product glucose-6-P. A fourth enzyme, glucokinase (EC 2.7.1.2, sometimes called Type IV hexokinase) has a high K_m for glucose, is not sensitive to inhibition by glucose-6-P, has a molecular weight of roughly 50 kDa, and resembles the hexokinases of yeast [4].

Since it is not known whether the isozymes of tumor and normal cells are identical, the tumor isozymes will be referred to as Type I-like, II-like, and III-like. When the enzyme composition of AS-30D cells was compared with normal liver tissue, a shift from primarily glucokinase in liver to a Type II-like hexokinase in the hepatoma was observed [3]. Recently, Shinohara et al. have shown that the expression patterns of hexokinase isozymes differ between the AH130 hepatoma and normal rat liver, with Type II-like being the predominant tumor isozyme [5]. Very little Type I-like and no Type III-like expression was reported in the tumor [5].

It has been proposed that the low K_m hexokinases arose from the duplication and fusion of a 50 kDa ancestral hexokinase similar to yeast hexokinase and mammalian glucokinase [6,7]. Comparisons of cDNA sequences from the C- and N-terminal halves of the Types I, II and III hexokinases with those from glucokinase and yeast hexokinase have shown extensive sequence similarity, especially within the proposed glucose and nucleotide binding domains [7–9]. This homology lends support for the evolutionary relationship proposed above.

While it has been suggested that the Type I, II and III isozymes in normal rat tissues are coded for by separate genes [10], no conclusive data has been presented, and this question has yet to be addressed in rat tumor cells. The only full-length gene from this family of enzymes characterized to date codes for rat liver glucokinase [11]. In the present study, we provide evidence from Southern blotting with cDNA probes coding for the three low K_m hexokinases for the existence of a separate gene for each isozyme. The Type III isozyme appears to be highly polymorphic in tumor versus normal tissues. This further supports the existence of distinct genes coding for the hexokinase isozymes in rats. This is the first report of a distinct allelic

*Current address: Department of Molecular Genetics, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235, USA.

Correspondence address: R.A. Nakashima, Department of Chemistry and Biochemistry, Texas Tech University, P.O. Box 4260, Lubbock, TX 79409, USA. Fax: (1) (806) 742 1289.

polymorphism in tumor versus normal cells at the Type III hexokinase locus.

2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

Restriction enzymes and the 'Prime-It' labeling kit were obtained from Stratagene. Hybridization transfer membranes (Hybond-N$^+$) were from Amersham Corp. [α-^{32}P]dCTP was from New England Nuclear. Autoradiography was carried out at −70°C using Kodak XAR-5 film with intensifying screens. All chemicals were of the highest grade commercially available.

2.2. *Animals and tumor cells*

The AS-30D hepatoma cell line was obtained from Dr. Peter L. Pedersen of the Johns Hopkins University, School of Medicine (Baltimore, MD). The cells were grown in the abdominal cavities of female Sprague–Dawley rats from Sasco Inc. (Omaha, NE). Cells were collected and purified according to Parry and Pedersen [12].

2.3. *cDNA probes*

The cDNA clones coding for the types I, II, and III isozymes of rat hexokinase were the generous gift of Drs. John E. Wilson, David A. Schwab, and Annette P. Thelen of Michigan State University (East Lansing, MI).

2.4. *DNA purification and Southern hybridization*

Total cell DNA was isolated by a modification [13] of the method of Enrietto et al. [14]. Southern blotting and hybridization with radiolabeled probes was carried out according to Sambrook et al. [15]. Radiolabeling was done by the random priming method [16].

3. RESTULTS

In order to determine the number and similarity of genes coding for the hexokinase isozymes, total cell DNA from the AS-30D hepatoma cell line was isolated and Southern blotted. As shown in Fig. 1, hepatoma DNA digested with several restriction endonucleases was probed with cDNAs coding for the three isozymes of hexokinase. Comparisons of hybridization patterns for the Type I and II cDNAs revealed several fragments recognized by both probes. However, since a majority of the bands were unique to one of the two probes (Fig. 1), it would seem that the Type I-like and II-like hexokinases are coded for by separate genes. The banding pattern generated by the Type III cDNA probe appears very divergent from either of the other two hexokinases (Fig. 1). This lack of resemblance supports the conclusion that a third distinct gene codes for the Type III-like hexokinase.

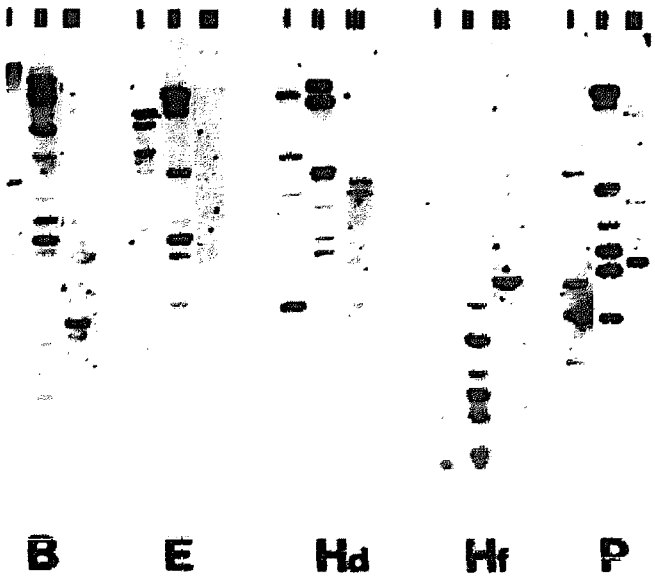

Fig. 1. Southern hybridization of AS-30D DNA with Types I, II, and III hexokinase. Following digestion with the corresponding enzymes (*Bam*HI, B; *Eco*RI, E; *Hind*III, Hd; *Hin*fI, Hf; and *Pst*I, P), AS-30D DNA was analyzed by Southern blotting as described in section 2 with the Types I, II, and III cDNA probes.

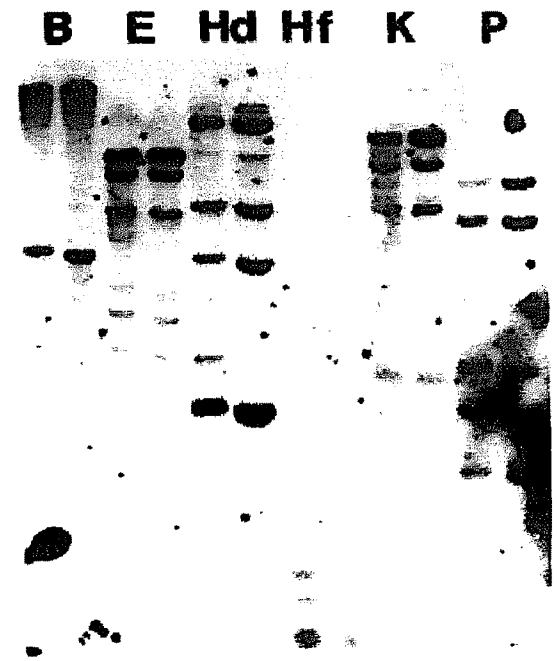

Fig. 2. Southern blot of AS-30D versus normal rat liver DNA probed with the Type I hexokinase cDNA. DNA from the AS-30D hepatoma and one normal rat liver (left and right lanes respectively in each set of digests) was digested with *Bam*HI, B; *Eco*RI, E; *Hind*III, Hd; *Hin*fI, Hf; *Kpn*I, K; and *Pst*I, P and then hybridized with the Type I probe as in Fig. 1.

The Types I, II and III probes were also hybridized to Southern blots of normal rat liver DNA, and the banding patterns generated were compared with those of the AS-30D hepatoma. All restriction fragments recognized by the Type I (Fig. 2) and II (Fig. 3) probes were identical between tumor and normal tissues. In addition, the Type II probe hybridized with much greater intensity to the hepatoma DNA when compared with normal rat liver (Fig. 3). However, when AS-30D hepatoma and normal rat liver DNA were probed with the Type III hexokinase cDNA, an allelic loss was consistently seen in the tumor (Fig. 4). The polymorphic nature of the Type III locus as well as the distinct restriction patterns of this gene when compared with the other two isozymes lends additional support for a uni-

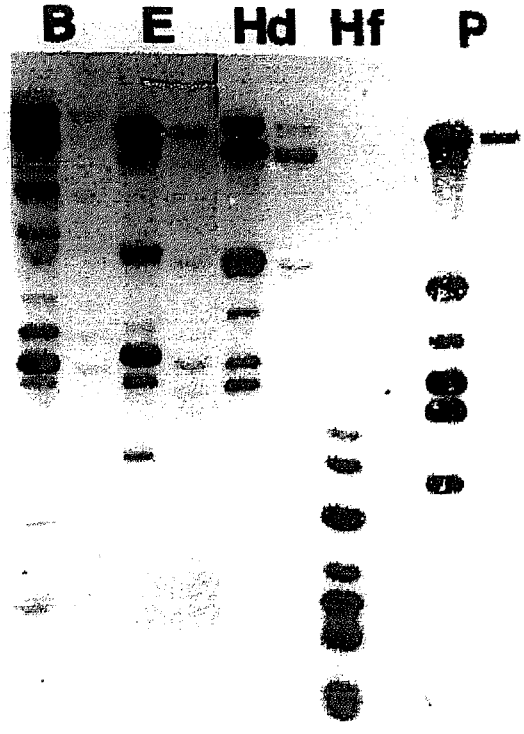

Fig. 3. Southern analysis of AS-30D vers[illegible] ormal rat liver DNA probed with the Type II hexokinase cDNA. AS-30D and normal liver DNA (left and right lanes respectively in each set of digests) was probed with the Type II cDNA. (The hybridization conditions and enzymes used are as in Fig. 1).

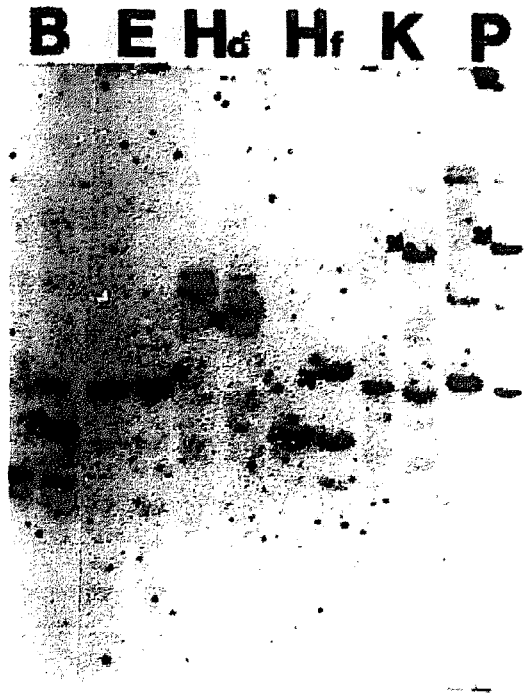

Fig. 4. Type III hexokinase comparison of AS-30D and normal liver DNA. AS-30D and normal liver DNA (left and right lanes respectively in each set of digests) was probed with the Type III cDNA as in Fig. 2. The arrows indicate the allele in each of the normal rat liver DNA digests which is absent in the corresponding hepatoma digest. (The enzymes used are *Bam*HI, B; *Eco*RV, E; *Hin*dIII, Hd; *Hin*fI, Hf; *Kpn*I, K; and *Pst*I; P).

que gene coding for this isozyme of hexokinase in tumors.

4. DISCUSSION

The availability of cDNAs for the Types I, II and III isozymes of hexokinase recently cloned from normal rat tissues has made possible, for the first time, direct observation of the number and relationships of their corresponding genes in tumor cells. In the present study, we provide evidence for three separate genes coding for the individual tumor isozymes. The Type I-like and II-like genes appear to be closely related, since they share several restriction fragments and the relative numbers of bands recognized by the two probes are comparable. The Type III-like hexokinase banding patterns are comprised of fewer fragments and are quite simple in contrast to the Type I-like and II-like genes. The Type III-like hexokinase did not share any restriction fragments with either the Type I-like or II-like isozymes, consistent with its lower degree of sequence homology compared with the latter two isozymes [7–9]. It is worth noting that both the Type I and III probes hybridized equally well with tumor and normal DNA while the Type II cDNA recognized the hepatoma DNA much more intensely than the normal DNA. This is an interesting observation since the Type II-like gene is highly expressed [5] and the specific activity of the isozyme is greatly increased in highly glycolytic hepatoma cells when compared with normal tissues [3].

The cDNA sequences have been compared for both the C- and N-terminal halves of the three isozymes [7–9]. The C-terminal half of the Type III clone shows 73 and 75% homology to the Type I and II clones, respectively. However, the Type I and II cDNAs are about 87% homologous over the same region. Similarly, the N- terminal half of the Type III clone is 55 and 58% homologous with the Type I and II isozymes which, in turn, share 82% homology. These sequence comparisons parallel the similarities we have observed between the restriction patterns of the Type I-like and II-like isozymes as well as the distinct nature of the Type III-like gene.

Finally, we report for the first time a distinct Type III hexokinase allelic polymorphism between normal and tumor tissues. Since the cloning and characterization of genes for the hexokinases from transformed cells would seem the logical next step in understanding the regulation of their expression, analysis of the number of genes as well as their relation would seem beneficial to the completion of this task.

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