Characterization of the Rat Type III Hexokinase Gene Promoter
A FUNCTIONAL OCTAMER 1 MOTIF IS CRITICAL FOR BASAL PROMOTER ACTIVITY*

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A 1532-base pair 5'-flanking region of the gene encoding rat type III hexokinase has been cloned and sequenced. The total sequence includes positions −1548 to −17 (A of the translational start ATG as position +1). Using luciferase reporter constructs transfected into PC12 (rat pheochromocytoma) and L2 (rat lung) cells, basal promoter activity has been associated with sequence between −182 and −89. This includes a single transcriptional start site, an adenine at position −134 identified by primer extension. Together with previously cloned cDNA sequence, this accounts for an mRNA of approximately 3.9 kilobases, found by Northern blotting of RNA from rat lung and kidney. Sequence upstream of the transcriptional start site was devoid of canonical TATA and CAAT elements. An octamer 1 (Oct-1) binding site, located between positions −166 and −159 was shown by deletion analysis and site-directed mutation to be critical for promoter activity. Nuclear extracts from PC12 cells contained a protein (or proteins) specifically binding the octamer sequence, and supershift experiments with anti-Oct-1 indicated involvement of this ubiquitously expressed transcription factor in the complex. Sequence including the Oct-1 site and immediately adjacent regions was protected from DNase I digestion in footprinting experiments with nuclear extracts from PC12 cells. Reverse transcription polymerase chain reaction indicated that levels of type III hexokinase mRNA in rat tissues increased in the order brain < liver < lung ~ kidney; immunoblotting indicated that type III hexokinase protein in these tissues increased in a similar manner.

Hexokinase (ATP:α-hexose 6-phosphotransferase, EC 2.7.1.1) catalyzes the phosphorylation of Glc. While further metabolism of the product, Glc-6-P, via the glycolytic pathway is of the most general importance, Glc-6-P may also be directed to alternative metabolic fates such as glycogen synthesis or the hexose monophosphate pathway. Thus, the hexokinase reaction may be considered the initial step in metabolism of Glc by any of several alternative routes.

In mammals, there are four isozymes of hexokinase (reviewed in Ref. 1), generally designated as types I, II, III, and IV. Based on sequence comparisons, these are clearly homologous proteins, but they each exhibit unique characteristics that are reasonably presumed to adapt them for distinct physiological roles. The type IV isozyme (2), more commonly known as "glucokinase," is a 50-kDa protein expressed primarily in liver and pancreatic β-cells. Glucokinase is not susceptible to feedback inhibition by physiologically relevant levels of Glc-6-P and has a relatively low affinity for the substrate Glc, with half-saturation at approximately 6 mM Glc. These kinetic properties admirably suit the type IV isozyme for its role as a "glucose sensor," with the rate of Glc phosphorylation being responsive to changes in plasma [Glc] and leading to corresponding changes in insulin secretion from β-cells or incorporation of Glc into storage forms (glycogen and fatty acids) in liver.

In contrast, the type I, II, and III isozymes are 100-kDa proteins, thought to have evolved by duplication and fusion of a gene encoding an ancestral 50-kDa hexokinase (1, 3) (which also represents a predecessor of the 50-kDa type IV isozyme). In addition to their virtual identity in molecular mass and extensive sequence similarity, these isozymes are also similar in exhibiting a high affinity for substrate Glc, with $K_m$ values in the submillimolar range, and in their susceptibility to feedback inhibition by physiological levels of Glc-6-P, with $K_i$ values in the micromolar range. However, they differ in other aspects of their regulatory kinetics, in their levels of expression in various tissues, and in their subcellular distribution (1). Thus, while the type I isozyme is ubiquitously expressed in mammalian tissues, the type II isozyme is expressed primarily in insulin-sensitive tissues such as heart, skeletal muscle, and adipose tissue. The type III isozyme is found at low levels in virtually all tissues but at high levels in none (1, 4) and, within tissues, exhibits a highly selective expression in only limited cell types (5). Moreover, while the type I and type II isozymes are known to associate with mitochondria, an interaction mediated by a hydrophobic N-terminal sequence of these isozymes (6–9), the type III isozyme is associated with the nuclear periphery (5, 10). It is notable that the mitochondrially bound type I isozyme and perinuclear type III isozyme, where expressed, coexist in the same cells. Such observations are consistent with the view that these isozymes are adapted to play distinct roles in mammalian Glc metabolism.

A full understanding of the metabolic roles that may be associated with the various isozymes must include knowledge of factors governing the expression of the genes encoding these isozymes. Previous studies have provided considerable information about the promoters and associated regulatory cis-elements for the type I (11, 12), type II (13–15), and type IV (2) isozymes. It is surely premature to say that the transcriptional regulation of these genes has been well defined, but it is clear that, not unexpectedly, marked differences between the isozymes also exist at this level. Thus, the promoter for type I hexokinase (11, 12) has the characteristics associated with genes for ubiquitously expressed "housekeeping enzymes," i.e. lacking a classical TATA element and being located within a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF 168710.‡ J. A. W. was involved in the initial cloning of the promoter region. All subsequent experimental work was conducted by S. S.

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"CpG island" (16, 17). In contrast, expression of the type II isozyme is more restricted and regulated, at least in part, by insulin. The promoter for the type II isozyme (13–15) contains classical TATA and CAAT elements frequently found in genes expressed in a tissue-specific pattern. These studies have provided a foundation for further understanding of transcriptional regulation of the genes encoding the type I, II, and IV isozymes. In contrast, there have been no previous studies on the promoter and regulatory cis-elements governing the expression of the type III isozyme. Thus, we initiated the present work, which has resulted in cloning and characterization of the promoter region for the gene encoding the rat type III hexokinase.

**EXPERIMENTAL PROCEDURES**

**Materials—**PC12 (rat adrenal pheochromocytoma) and L2 (rat lung) cell lines were obtained from American Type Culture Collection (Manassas, VA). Cosmids, calf serum, horse serum, and medium for cell culture were products of Hyclone Laboratories (Logan, UT). Oligonucleotides were synthesized by the Macromolecular Structure Facility at Michigan State University. Various kits and reagents used for molecular biological methods were obtained from sources indicated in appropriate context below. Oligonucleotides containing consensus binding sites for the transcription factors octamer 1 (Oct-1) and AP2 were purchased from Promega (Madison, WI). Other standard chemicals and biochemicals were products of Sigma or Roche Molecular Biochemicals.

**Cloning of the 5'-Flanking Region of the Rat Type III Hexokinase Gene—**The 5'-upstream region of the rat type III HK gene was obtained using the rat GenomeWalker Kit from CLONTECH ( Palo Alto, CA), following the protocol recommended by the manufacturer. Briefly, the GenomeWalker Kit contains five rat genomic DNA libraries, each containing products from digestion of rat genomic DNA with one of five restriction enzymes (EcoRI, ScaI, DraI, PvuII, or SphI) ligated to a 5'-“adapter.” Sequences of interest are obtained by PCR using an “adapter primer” (AP1) supplied by CLONTECH and a gene-specific primer (GSP1) recognizing known sequence from the gene of interest. A second round of “nested” PCR, using a second adaptor primer (AP2) and gene-specific primer (GSP2), is then conducted, with resulting products of interest cloned by standard methods. To facilitate cloning, AP2, GSP2, and GSP4 (see below) were designed to include MluI sites at their 5'-ends.

GSP1 had the sequence CTGGGGCCAGCCTTGAATCTT, which corresponds to positions 39–60 (all positions are numbered with A of the translational start codon, ATG, as +1) of the previously published rat hexokinase type III DNA sequence (18). GSP2 had the sequence AACGCCGTACTACCCGAGTTCAGA, corresponding to positions −38 to −17 in the 5'-untranslated region (18), with an additional six nucleotides at its 5'-end introducing an MluI site for cloning purposes. In all PCRs, the Expand Long Template PCR System (Roche Molecular Biochemicals) was used. For the primary PCR (AP1 and GSP1), one cycle of denaturation at 95 °C for 15 s, followed by seven cycles of 2 s at 94 °C and 4 min at 70 °C and another 36 cycles of 2 s at 94 °C and 4 min at 65 °C, with a final extension at 68 °C for 10 min. The primary PCR products were diluted 1:100, and 1 μl was used for the second amplification reaction with primers AP2 and GSP2. Cycling parameters in the secondary PCR were the same as that used during the primary PCR except that the first and second steps consisted of five and 32 cycles, respectively.

Additional 5' sequence upstream of the type III hexokinase gene was obtained in a similar manner using gene-specific primers, GSP3 and GSP4. The latter were based on sequence of a 274-bp clone (see below) obtained after the initial nested PCR with GSP1 and GSP2 and representing positions −290 to −17 of the 5'-flanking sequence. The sequence in GSP3 and GSP4 primers were CCGAAGAGGACCCA-GAATGG and GACAGGCTTCCAGATGGGACGACC, respectively, with GSP4 again including 5' sequence to introduce an MluI site for cloning purposes.

The gel-purified PCR products were digested with MluI and cloned into a similarly digested promoterless luciferase reporter vector, pGL2-Basic (Promega, Madison, WI). Cloned fragments were sequenced by the Michigan State University DNA Sequencing Facility.

**Generation of Luciferase Reporter Constructs—**Deletion of specific regions of the cloned 5' sequence was accomplished through judicious use of available restriction sites and subcloning into compatible sites. In some cases, PCR was used to create flanking restriction sites for cloning purposes. Methods were essentially as in similar previous work from this laboratory (11, 12).

Mutations were done using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA), following the protocol from the manufacturer. The 274-bp fragment corresponding to the −290 to −17 region (see above) was isolated from the pGL2-Basic plasmid after digestion with HindIII and KpnI. The gel-purified fragment was subcloned into the HindIII–KpnI cloning site of the pBluescript KS + plasmid (Stratagene). Mutations were introduced into putative Oct-1 binding site and transcription initiation (Inr) elements (see below). For the Oct-1 site, the sequence was changed to TAGGATAT to TAGGCGG, and for the Inr TTCACCTT was changed to TGAGATCT; the underlined nucleotides indicate the mutations. The mutations were confirmed by direct sequencing, and the 274-bp fragment containing the desired mutations was isolated from the pBluescript vector and cloned back into the HindIII–KpnI-digested pGL2-Basic vector.

Plasmids used in transfection experiments were purified using an EndoFree Plasmid Isolation Kit (Qiagen, Valencia, CA), and purity was verified by %26Ag26/2620 and agarose gel electrophoresis. In all constructs, orientation of insert was verified by direct sequencing.

**Cells with Luciferase Reporter Constructs—**PC12 cells were grown as described earlier (11, 12). L2 cells were grown at 37 °C under 5% CO2, with Ham's F-12 medium containing 10% Cosmic Calf serum. The day before transfection, cells were plated into six-well tissue culture dishes at a density such that the cells reached 70–80% confluence by the time of transfection; for PC12 cells, the plates were coated with rat tail type I collagen (Collaborative Biomedical Products, Bedford, MA).

Transfections were done with LipofectAMINE PLUS reagent (Life Technologies, Inc.), following the protocol provided by the manufacturer. Each transfection was done using 2 μg of luciferase reporter construct DNA and 100 ng of an internal control plasmid pRL-CMV (Promega). Four hours after transfection, the transfection medium was removed by aspiration, 3 ml of complete medium (containing serum and antibiotics) was added, and the plates were returned to the incubator.

At 48 h post-transfection, medium was removed and wells were rinsed with phosphate-buffered saline to remove detached cells and residual growth medium. Then 250 μl of 1× passive lysis buffer, provided in the Dual-Luciferase Reporter Assay System (Promega), was added per well. Cells were dispersed by scraping with a disposable plastic cell lifter. The samples were transferred to 1.5-ml microcentrifuge tubes and subjected to two cycles of freeze (−80 °C) thaw (room temperature) to ensure complete cell lysis and then centrifuged at 12,000 × g for 3 min in a refrigerated microcentrifuge. Supernatants were used for assay of luciferase activities.

Firefly and Renilla luciferase activities were sequentially measured using the Dual-Luciferase Reporter Assay System (Promega) and following manufacturer's instructions. Luciferase activities were determined using a Turner model 20 Luminometer, with a 3-s predelay followed by a 20-s measuring period. The Renilla luciferase activity, expressed from the CMV promoter, provided an internal control to monitor transfection efficiency. Firefly luciferase activities were normalized based on the Renilla luciferase activity in each well. Statistical analysis of the results was done with GraphPAD Instat, version 1.13 (Graph Pad Software, San Diego, CA).

**RNA Isolation and Northern Blots—**The levels of the mRNA for type III hexokinase are elevated in the lungs of rats exposed to hyperoxia (19). We are grateful to Dr. Carl White (Department of Pediatrics, National Jewish Medical and Research Center, Denver, CO) for providing mRNA isolated from hyperoxic rat lung. Total RNA was isolated from other tissues of normal (i.e. nonhyperoxic) rats using the TRIZOL reagent (Life Technologies), following the protocol suggested by the manufacturer. The Poly(A)Tract mRNA Isolation System (Promega) was used to purify mRNA from the total RNA preparations. The integrity of the RNA was analyzed by formaldehyde-agarose gel electrophoresis. For Northern blotting, poly(A)+ RNA, isolated from normal rat kidney (5 μg) or hyperoxic rat lung (1 μg), was fractionated on a 6% formaldehyde, 1.2% agarose gel and blotted onto a Hybond-N+ membrane (Amersham Pharmacia Biotech) using a VacuGene vacuum blotting apparatus (Amersham Pharmacia Biotech). The blot was prehybridized for 4 h at 42 °C in a solution containing 5× SSPE, 5× Denhardt’s solution, 0.1% SDS, 50% formamide, and 100 μg/ml denatured salmon sperm DNA. Hybridization was conducted for 20 h at
42 °C in the same solution except that 1× Denhardt’s was used instead of 5×. The probe used was the previously described (18) cDNA clone, L 7.1–1, for type III hexokinase, labeled with [32P]dCTP by random priming with the high Prime DNA labeling kit (Roche Molecular Biochemicals). The DNA was digested twice at room temperature, 10 min each time, with 2 × SSPE, 0.1% SDS, followed by twice at 42 °C for 3 min each time, in 0.2 × SSPE, 0.1% SDS. Hybridization and washing were done in a rotating glass tube within a hybridization oven (HybriStrip HB-1D, Techne, Princeton, NJ). The hybridization signal was detected by exposure (3 days, −70 °C) on Kodak Biomax MR film with two intensifying screens.

**RT/PCR—** For RT/PCR analysis of type III hexokinase mRNA in different rat tissues, the SuperScript Preamplification System (Life Technologies) was used to synthesize the first strand cDNA. Five μg of total RNA isolated from various rat tissues was treated (15 min, room temperature) with DNase I in 10 μl of reaction mix containing 20 μg Tris-Cl, pH 8.3, 50 mM KCl, 1 μM dithiothreitol, 10 mM MgCl₂, each dNTP, 40 units of RNasin (Promega), and 50 μg/ml actinomycin D. Primer extension was initiated by adding 1 μl (200 units) of Moloney murine leukemia virus reverse transcriptase (Promega) and incubating at 42 °C for 1 h. Thereafter, 2 μl (18 units) of RNase ONE (Promega) was added, and the reaction mix was further incubated at 37 °C for 30 min. The reverse transcriptase reaction product was purified using a QIAquick PCR purification column (Qiagen) and eluted in 60 μl of water. The eluted product was vacuum-concentrated to near dryness and resuspended in 5 μl of sequencing stop solution (95%/v/v formamide, 10 mM EDTA, pH 5.0, 0.1% (v/v) bromophenol blue, and 0.1% (v/v) xylene cyanol), denatured at 65 °C for 5 min, and analyzed on a 6% sequencing gel. To permit identification of the transcriptional start site, adjacent lanes received the products of a dideoxy sequencing reaction, generated with the same oligonucleotide primer used in the primer extension reaction as the sequencing primer. In this case, sequencing was done using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) with [32P]dATP as the labeling reagent.

**RESULTS**

Cloning the 5'-Flanking Region of the Gene for Rat Type III Hexokinase—The overall strategy and relevant experimental results are shown in Fig. 1. In the initial nested PCR with GSP1 and GSP2 as gene-specific primers, a 300-bp fragment was amplified from the SspI library provided with the GenomeWalker Kit (Fig. 1B). This was cloned and designated as pHK3. Sequencing of pHK3 disclosed a 274-bp genomic sequence, which included a 64-bp segment at its 3'-end that exactly matched sequence in the 5'-untranslated region of the previously described cDNA (18), confirming that pHK3 did indeed represent the 5'-flanking region of the type III hexokinase gene.

Using sequence from pHK3, additional gene-specific primers (GSP5 and GSP4) were designed. These were used to again amplify sequences of interest in the genomic libraries provided with the GenomeWalker Kit. PCR products were cloned and sequenced after amplification of the EcoRV, DraI, PvuI, and SspI libraries (Fig. 1C). From these, the −1.4-kilobase pair fragment amplified from the PvuI library was cloned and designated p2. Sequencing of p2 provided a 1342-bp sequence that included 84 bp at its 3'-end that exactly matched sequence at the 5'-end of pHK3. Thus, p2 includes further upstream regions of the 5'-flanking...
Sequence. Together, pHK3 and p2 define sequence from −1548 to −17 in the 5′-flanking region of the type III hexokinase gene (Fig. 2).

**Sequence Analysis of the 5′-Flanking Region**—The 5′-flanking sequence was analyzed using GCG (Genetics Computer Group, Madison, WI) and TRANSFAC (23). This disclosed the existence of few consensus transcriptional factor binding sites or other common cis-elements within the region (Fig. 2). The proximal sequence of the 5′-flanking region was devoid of canonical cis-acting elements such as TATA, CAAT, or GC box, although some TATA- or CAAT-like elements were noted in both clones pHK3, C, products from a second nested PCR amplification using AP2 and GSP4, an upstream primer designed from pHK3 sequence information. A −1.4-kilobase pair (kb) PCR product amplified from the PurII library was cloned as p2. B and C, molecular weight markers; E, EcoRV; Sc, ScaI; Dr, DraI; Po, PvuII, Ss, SspI.

**Functional Characterization of the Rat Type III Hexokinase Promoter Region**—To identify the critical element(s) required for promoter activity, firefly luciferase reporter constructs containing various regions of the 5′-flanking region were transfected into PC12 and L2 cells. As noted under “Experimental Procedures,” cells were co-transfected with the pRL-CMV plasmid provided the minimal promoter for the gene encoding rat type III hexokinase. The adenine identified as the transcriptional start site marked with an asterisk. All positions are numbered with A of the translational start codon, ATG, as position +1.

Results of transfection experiments with L2 cells (Fig. 3B) were similar to those obtained with PC12 cells, with the −182 to −89 region again being critically important for promoter activity. One notable difference between the results obtained with PC12 and L2 cells was seen with plasmids pHK3 and p2+. With PC12 cells, the promoter activity of these two constructs was similar, while with L2 cells, p2+ had substantially more activity than pHK3. This suggests that one or more enhancer elements, functional in L2 cells but not PC12 cells, may exist upstream from position −290. However, this was not further pursued in the present study.

**Effects of Mutations in the Oct-1 Motif and Inr on Basal Promoter Activity**—The results of transfection experiments described above indicated that sequence from −182 to −146 and from −121 to −89 is critical for promoter activity. Sequence analysis had shown the existence of a reverse consensus Oct-1 binding site between positions −166 and −159 but did not.
A

![Graph A]

B

![Graph B]

**Fig. 3. Functional analysis of the promoter elements of the rat type III hexokinase gene.** The various deletion constructs included sequence shown at the left, inserted into the luciferase reporter vector, pGL2-Basic. The constructs pΔ4 and pINRD contained site-directed mutations at Oct-1 and Inr sites, respectively (see text). Firefly luciferase activities expressed in PC12 (Fig. 3A) or L2 (Fig. 3B) cells transfected with the reporter constructs have been normalized on the basis of Renilla luciferase activities expressed in PC12 (Fig. 3A) or L2 (Fig. 3B) cells, and luciferase activities were compared with those seen after transfection with pRD2 itself. Normalized luciferase activities were 198 ± 9 for pRD2 and 177 ± 12 for pCA2 (mean ± S.D. for two experiments, four transfections with each construct per experiment). While not precluding the possibility that CA repeats may have a negative effect in other sequence contexts, these results do not indicate a general negative effect of CA repeats on activity of the type III hexokinase promoter.

**Fig. 4. DNase I footprint analysis of the promoter region.** An end-labeled 274-bp MluI fragment (positions -290 to -17) of the rat type III hexokinase promoter was incubated with indicated amounts of nuclear extract from PC12 cells and subsequently digested with DNase I. A single protected region, including the Oct-1 site (see text), is evident. Other lanes are as follows: Probe, the undigested probe; M, end-labeled FX174 DNA/HinII markers (Promega); G+A, Maxam-Gilbert sequencing ladder of the probe DNA.

indicate the presence of any known potential cis-elements in the region between -121 and -89 except for a classical Inr between positions -112 and -104. To investigate the possible importance of these sequences in governing the basal promoter activity, site-directed mutations were introduced into these putative sites, and functional consequences of the mutations were then examined with luciferase reporter constructs transfected into PC12 and L2 cells.

With PC12 cells, mutation of the Oct-1 site from the consensus sequence of TAGCATAT to TAGCCGCG (mutated bases shown in boldface type) resulted in an approximately 5-fold reduction in luciferase activity when the mutated plasmid, pΔ4, was compared with the corresponding wild type plasmid, pHK3 (Fig. 3A). The reduction was somewhat less, approximately 3.5-fold, when these plasmids were transfected into L2 cells (Fig. 3B), but clearly the mutation had a substantial effect on promoter activity in both cell types.

In contrast, mutation of the Inr from TTCACTTCT to TGAGAATCT caused only a slight decrease in promoter activity when the mutant vector, pINRD, was transfected into either PC12 or L2 cells (Fig. 3, A and B).

**Effect of CA Repeats on Basal Promoter Activity—**Previous studies (26, 27) have shown that CA repeats in the upstream promoter region may exert a negative effect on promoter activity. To examine this possibility for the type III hexokinase promoter, the region from -657 to -506, which harbors an extended stretch of CA repeats, was cloned 5′ to the minimal promoter elements (-182 to -89) in the pRD2 construct. The resulting reporter construct, pCA2, was transfected into PC12 cells, and luciferase activities were compared with those seen after transfection with pRD2 itself. Normalized luciferase activities were 198 ± 9 for pRD2 and 177 ± 12 for pCA2 (mean ± S.D. for two experiments, four transfections with each construct per experiment). While not precluding the possibility that CA repeats may have a negative effect in other sequence contexts, these results do not indicate a general negative effect of CA repeats on activity of the type III hexokinase promoter.

The Consensus Oct-1 Binding Site Is Protected by PC12 Nuclear Protein(s) in DNase I Footprinting.—Footprinting revealed that a nuclear extract from PC12 cells protected a region between -173 and -158 from digestion by DNase I (Fig. 4). This region includes the reverse consensus Oct-1 site.

**EMSA and Supershift Experiments Demonstrate Presence of Oct-I-like Protein in the DNA-Protein Complex—**Incubation of a labeled probe representing sequence from -290 to -17 with a nuclear extract from PC12 cells resulted in the appearance of one major shifted band (Fig. 5) in EMSA experiments. Excess unlabeled probe competed with the labeled DNA, as expected. However, the corresponding region from plasmid pΔ4, which also represents sequence from -290 to -17 but with a mutation in the Oct-1 binding site (see above), did not compete with the wild type probe. Moreover, a 22-mer oligonucleotide containing a consensus Oct-1 binding site effectively competed with the radiolabeled probe, while a 26-mer oligonucleotide including the consensus binding site for the noncandidate transcription factor, AP2, did not compete.

In supershift experiments, a polyclonal anti-Oct-1 antibody (kindly provided by Dr. Winship Herr, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) completely shifted the protein-DNA complex to higher regions in the gel (Fig. 6). In contrast, the addition of a control polyclonal antibody, anti-rat type I hexokinase, had no effect, confirming the specificity of
The mRNA for Type III Hexokinase—The previously isolated cDNA (18) for rat type III hexokinase included the 2772-bp coding region (924 amino acid residues) plus approximately 850 bp of 5'-untranslated sequence. Based on additional 5'-untranslated sequence determined in the present study, and with identification of the transcriptional start site, the total 5'-untranslated sequence is composed of about 135 bp. Thus, together with a polyadenylate tail likely to be approximately 200 nucleotides (28), the expected size of the mRNA for type III hexokinase is ~3.9 kilobase pairs. A single mRNA of the expected size was seen after Northern blotting of mRNA isolated from normal rat kidney or hyperoxic rat lung (Fig. 7B). The mRNA for type III hexokinase is present in rat tissues (1, 4). However, Allen et al. (19) reported low levels of the type III isozyme and presumably its mRNA, lung or PC12 cells were unsuccessful. This was probably due to primer extension and using mRNA isolated from normal rat kidney or hyperoxic rat lung (Fig. 7B).

Expression of Type III Hexokinase mRNA and Protein in Rat Tissues—RT-PCR results (Fig. 8A) indicated similar levels of the internal control mRNA, for β-actin (20), in rat brain, kidney, lung, and liver, and in PC12 cells. In contrast, the levels of type III hexokinase mRNA varied considerably, being low in brain and somewhat higher in liver, while lung and kidney had the highest levels of the message. Relatively low levels were also present in PC12 cells.

The levels of type III hexokinase protein in these rat tissues, detected by immunoblotting (Fig. 8B), varied in a similar manner, with the relative intensity of the immunoreactive band decreasing in the following order: lung > kidney > liver > brain.

**DISCUSSION**

We have cloned and sequenced a 1532-bp rat genomic DNA fragment representing the ~1548 to ~17 region of the gene encoding rat type III hexokinase. Basal promoter activity has
been associated with sequence between −182 and −89, and the transcriptional start site has been identified as an adenine at −134. Unlike the promoter for the type II isozyme (13–15), the proximal promoter region was devoid of canonical TATA and CAAT elements. This is also the case with the type I isozyme (11, 12), but, in contrast to the TATA-less promoter for the type I isozyme, the promoter for the type III isozyme is not located within a CpG island (16, 17). Thus, the present study complements earlier work on the promoters for the type I (11, 12) and type II (13–15) and clearly, and not unexpectedly, indicates that these isozymes vary greatly in the character of their promoter regions and hence in their transcriptional regulation.

A consensus Oct-1 binding site, in reverse orientation and located at positions −166 to −159, was identified as being critical for activity of the type III hexokinase promoter. The ability of the octamer motif to function in both orientations in other promoters has previously been noted (29–31). Oct-1 is a ubiquitously expressed (24) member of the POU domain transcription factor family (32, 33). Despite its own ubiquitous nature, Oct-1 has been shown to be involved in transcriptional regulation of genes expressed in a tissue/cell-specific manner (34–35), as is the case with the type III isozyme of hexokinase.

Isomers of Oct-1, generated by differential splicing, have been described (33–35), as is the case with the type III isozyme of hexokinase. These typically function as transcriptional initiation sites (25), which seems unlikely here, since the identified transcriptional start site was further upstream, an adenine at position −134. Moreover, mutations in the Inr sequence had only modest effect on promoter activity. Thus, it seems more likely that the diminished promoter activity resulting from deletion of the −121 to −89 region reflects the detrimental effect of a truncated 5′-untranslated region on subsequent translation of the mRNA. Consistent with this, more limited deletion from the 5′-untranslated region by deletion of sequence between −89 and −17 also resulted in significant, but less marked, reduction of luciferase expression in both PC12 and L2 cells (Fig. 3, A and B; compare plasmids pR2 and pRD2).

The cloning and characterization of the promoter region for the type III isozyme will facilitate elucidation of the molecular mechanism(s) by which the cell-specific (5) expression of this gene is regulated, in normal tissues as well as under imposed stresses such as hyperoxia (19).

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