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Human Brain Glycogen Phosphorylase

CLONING, SEQUENCE ANALYSIS, CHROMOSOMAL MAPPING, TISSUE EXPRESSION, AND COMPARISON WITH THE HUMAN LIVER AND MUSCLE ISOZYMES*

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We have cloned the cDNA encoding a new isozyme of glycogen phosphorylase (1,4-D-glucan:orthophosphate D-glucosyltransferase, EC 2.4.1.1) from a cDNA library prepared from a human brain astrocytoma cell line. Blot-hybridization analysis reveals that this message is preferentially expressed in human brain, but is also found at a low level in human fetal liver and adult liver and muscle tissues. Although previous studies have suggested that the major isozyme of phosphorylase found in all fetal tissues is the brain type, our data show that the predominant mRNA in fetal liver (24-week gestation) is the adult liver form.

The protein sequence deduced from the nucleotide sequence of the brain phosphorylase cDNA is 862 amino acids long compared with 846 and 841 amino acids for the liver and muscle isozymes, respectively; the greater length of brain phosphorylase is entirely due to an extension at the far C-terminal portion of the protein. The muscle and brain isozymes share greater identity with regard to nucleotide and deduced amino acid sequences, codon usage, and nucleotide composition than either do with the liver sequence, suggesting a closer evolutionary relationship between them. Spot blot hybridization of the brain phosphorylase cDNA to laser-sorted human chromosome fractions, and Southern blot analysis of hamster/human hybrid cell line DNA reveals that the exact homolog of the newly cloned cDNA maps to chromosome 20, but that a slightly less homologous gene is found on chromosome 10 as well. The liver and muscle genes have previously been localized to chromosomes 14 and 11, respectively. This suggests that the phosphorylase genes evolved by duplication and translocation of a common ancestral gene, leading to divergence of elements controlling gene expression and of structural features of the phosphorylase proteins that confer tissue-specific functional properties.

Mammalian glycogen phosphorylases are found in at least three isozymic forms that can be distinguished by functional and structural properties as well as by the tissues in which they are preferentially expressed (1-5). A brain form has been described previously as the predominant isozyme in adult brain and most fetal tissues, whereas adult liver and muscle tissues are known to express primarily the liver and muscle isozymes, respectively. Each phosphorylase isozyme fulfills different physiological requirements even though all forms of the enzyme catalyze the same reaction, the phosphorolysis of glycogen to yield glucose 1-phosphate. The muscle enzyme provides metabolic fuel for muscular contraction, the liver form plays a central role in the maintenance of blood glucose homeostasis, and the brain type is associated primarily with provision of an emergency glucose supply during periods of anoxia or hypoglycemia. Phosphorylase isozymes exhibit differences in regulatory properties that may be related to their distinct physiological roles, especially with regard to their response to the allosteric ligand AMP.

The cDNAs encoding rabbit muscle and human liver glycogen phosphorylases, as well as the gene for the human muscle form, have been cloned and sequenced (5-8). In addition, the genes for muscle and liver phosphorylase have been localized to human chromosomes 11 and 14, respectively (9, 10). The crystal structure of rabbit muscle phosphorylase has allowed us to identify and characterize the active site and five allosteric sites of this enzyme (11, 12). Using this structure, we have identified amino acid substitutions predicted by the liver phosphorylase cDNA sequence that may be involved in specifying functional differences between the liver and muscle isozymes (13).

In this report we describe the cloning and sequence analysis of the cDNA encoding an isozyme of glycogen phosphorylase that is preferentially expressed in adult human brain tissues (hereafter referred to as the "brain" isozyme). The cloning of this cDNA has allowed us to 1) analyze and compare the expression of the brain and liver phosphorylase messenger RNAs in human adult brain, liver, and muscle tissues, as well as human fetal liver tissues, 2) map the brain phosphorylase gene to chromosome 20 and a homologous gene to chromosome 10, thus demonstrating that the human phosphorylase genes are unlinked, and 3) compare the human brain phosphorylase cDNA and predicted protein sequences with their human muscle and liver counterparts, thereby gaining insight into structural and evolutionary relationships among the three isozymes.

MATERIALS AND METHODS

*Construction of a Human Brain Astrocytoma cDNA Library—*Poly(A⁺) RNA isolated from the human astrocytoma cell line U251

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03544.

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was used to synthesize double-stranded cDNA using oligo(dT) as the first strand primer. Actinomycin D (40 $\mu\text{g}/\text{ml}$) was included during first-strand synthesis to reduce hairpin formation. The second strand was synthesized by the procedure of Okayama and Berg (14) using RNase H and *Escherichia coli* DNA polymerase and DNA ligase. The cDNAs were treated with *EcoRI* methylase and *S*-adenosylmethionine. T4 DNA polymerase was used to generate blunt ends. After ligating *EcoRI* linkers to the ends, the cDNAs were digested with *EcoRI* and separated on a 1.5% agarose gel. cDNAs greater than 1.5 kb¹ in length were inserted into the *EcoRI* site of $\lambda\text{gt}10$ (15).

Cloning and Sequence Analysis of the Human Brain Phosphorylase cDNA—The human brain astrocytoma library described above was screened with radiolabeled rabbit muscle and human liver glycogen phosphorylase cDNA fragments encoding amino acids 301–842 + 20 bases of 3'-untranslated region and amino acids 1–195 + 115 bases of 5'-untranslated region, respectively. These probes were chosen because they had been previously shown to cross-react with a rabbit brain mRNA of larger size than the rabbit liver or muscle phosphorylase mRNAs (5). Approximately 200,000 plaques were screened with a 1:1 mixture of the two probes under conditions of low stringency to allow maximal cross-reactivity among the tissue-specific sequences (35% formamide, containing $5 \times \text{SSC}$ ($1 \times = 0.15 \text{ M NaCl}$, 0.015 M sodium citrate), 5 mM NaH_2PO_4 , 0.2 mg denatured salmon sperm DNA/ml, and $1 \times \text{Denhardt's}$ solution at 42 °C). A total of 8 clones were obtained, with insert sizes ranging from 2.0 to 3.8 kb. Restriction enzyme digestion and dideoxynucleotide sequence analysis (16) revealed that three of the clones were identical to the human liver glycogen phosphorylase cDNA characterized previously in this laboratory (5). The five remaining clones (all greater than 3.4 kb in length) encoded a form of glycogen phosphorylase which was homologous to, but clearly distinguishable from, both the human liver and muscle phosphorylase sequences. The two longest clones of the latter class were sequenced in their entirety, using the strategy outlined in Fig. 1.

Northern Blot Analysis—Human adult brain and liver tissues were obtained at postmortem examination and stored at -80°C until use. Human fetal liver tissue was obtained from a 24-week-old abortus. Human skeletal muscle tissue (calf) was obtained from an amputated limb minutes after surgery. Poly(A)⁺ RNA was prepared from these tissues by the method of Ashley and MacDonald (17), electrophoresed through a formaldehyde/agarose gel, and transferred onto a MSI magna nylon 66 membrane filter. Identical panels containing human brain, skeletal muscle, fetal liver, and adult liver poly(A)⁺ mRNA, as well as RNA isolated from the human astrocytoma cell line U251, were hybridized to cDNA fragments encoding either the human liver or newly isolated isozyme of glycogen phosphorylase. The liver probe is a 2.0-kb *EcoRI/Xba* fragment that encodes amino acids 195–845 + 20 bases of 3'-untranslated region. The new probe is a 3.47-kb *EcoRI* fragment (see Fig. 1), encoding amino acids 89–862 + 1.15 kb of 3'-untranslated region. The two blots were hybridized with similar concentrations of probe (6×10^7 cpm in 10 ml of hybridization fluid; both probes had specific activities of approximately 5.5×10^8 cpm/ μg DNA), at high stringency (50% formamide, containing $5 \times \text{SSC}$, 5 mM NaH_2PO_4 , 0.2 mg denatured salmon sperm DNA/ml, and $1 \times \text{Denhardt's}$ solution at 42 °C), and washed identically (2×45 min, $2 \times \text{SSC}$, 0.1% SDS at 55 °C, followed by 2×30 min $0.1 \times \text{SSC}$, 0.1% SDS at 55 °C). Both panels were autoradiographed for 14 h at -80°C .

Chromosomal Mapping—The human brain cDNA clone was nick translated to a specific activity of approximately 1×10^9 cpm/ μg and shipped to the Los Alamos National Laboratory. There the probe was hybridized to nitrocellulose filter panels containing denatured total DNA from individual laser-sorted human chromosomes, as described previously (9, 10). The filters were then washed and exposed to autoradiography for 14 h. Since chromosomes 9, 10, 11, and 12 sort to the same spot, analysis of hamster/human cell line DNA was undertaken to resolve them. Southern blot analysis was performed by loading 20 μg of genomic DNA from individual hybrid lines, prepared as described (18), in each lane. The filter was probed with the 1.1-kb nick-translated *PstI/EcoRI* fragment (3×10^7 cpm, specific activity 6.5×10^8 cpm/ μg) encompassing the majority of the 3'-untranslated region of the brain phosphorylase cDNA (see Fig. 1) using the high stringency hybridization conditions of Church and Gilbert (19) (15% formamide, 0.2 M Na_2HPO_4 , pH 7.2, 1 mM EDTA, 1% bovine serum albumin, and 7% SDS at 58 °C). The blot was

washed first with $2 \times \text{SSC}$, 1% SDS at 50 °C for 30 min, and then with $0.1 \times \text{SSC}$, 0.1% SDS at 70 °C for 1 h.

RESULTS AND DISCUSSION

A brain astrocytoma $\lambda\text{gt}10$ cDNA library was screened with liver and muscle phosphorylase cDNA fragments (as described under "Materials and Methods"), resulting in the identification of eight hybridizing clones. *EcoRI* restriction analysis of the inserts revealed that the hybridizing clones could be divided into two classes. Three of the clones had an internal *EcoRI* site, resulting in the generation of 2.0- and 0.8-kb bands. The remaining five clones exhibited *EcoRI* bands of 3.47 kb alone or 3.47 and 0.35 kb (data not shown). The *EcoRI* restriction pattern obtained for the former class of clones is consistent with that described previously for the full-length liver phosphorylase cDNA (5, 10). Sequence analysis of these clones confirmed that they are identical to the liver phosphorylase cDNA. The size of the latter class of clones (3.47 kb + 0.35 kb = 3.82 kb) seemed to preclude the possibility that they represent liver or muscle phosphorylase mRNAs, since previous Northern blot analysis has sized these messages at 3.2 and 3.4 kb, respectively, in rabbit tissues (5). The 0.35-kb *EcoRI* fragment was ligated into the sequencing vector M13, and the larger fragment was subcloned according to the strategy depicted in Fig. 1. Dideoxynucleotide sequencing of these cloned fragments shows that they correspond to a phosphorylase isozyme with a nucleotide and deduced amino acid sequence distinct from the human liver and muscle phosphorylase sequences (see below).

Northern blot hybridization analysis was undertaken in order to characterize the tissue expression pattern of the newly isolated phosphorylase clone and to compare it with the pattern obtained with the liver phosphorylase cDNA. Poly(A)⁺ mRNA was prepared from human adult brain, muscle, and liver and 24-week-old fetal liver tissues, as well as the astrocytoma cell line U251. Panel A of Fig. 2 shows the hybridization pattern obtained when the liver phosphorylase cDNA was used to probe filters containing these types of RNA. Strong hybridization to a 3.2-kb mRNA is observed in both adult and fetal liver tissues (lanes L and FL). The liver probe also detects an identically sized message in U251 RNA and a slightly larger (approximately 3.4 kb) message in adult muscle. The latter likely represents cross-hybridization to the muscle-specific phosphorylase mRNA since this message is identical in size with that seen in rabbit muscle tissues when probing with the rabbit muscle phosphorylase cDNA; hybrid-

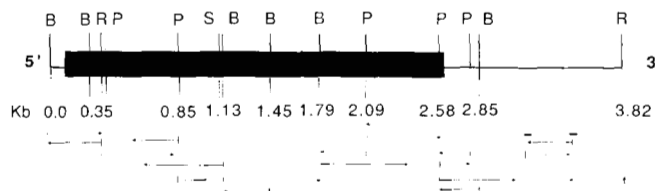


FIG. 1. Subcloning and nucleotide sequencing strategy for the human brain glycogen phosphorylase cDNA. The restriction enzymes *EcoRI* (R), *SacI* (S), *BamHI* (B), and *PstI* (P) were used to subclone fragments of the brain phosphorylase cDNA into M13 (strains MP 18 and MP 19) as shown. The protein-encoding region of the cDNA is depicted by the dark bar, untranslated regions are shown as lines. The distance of each restriction site from the 5' end of the clone is given in kilobases (kb) directly under the map, and below that, arrows indicate the direction and distance over which each fragment was sequenced. The two small boxes centered under the 3'-untranslated region of the cDNA indicate two oligonucleotides used as sequencing primers due to the absence of any convenient restriction sites in this area.

¹ The abbreviations used are: kb, kilobase(s); SDS, sodium dodecyl sulfate.

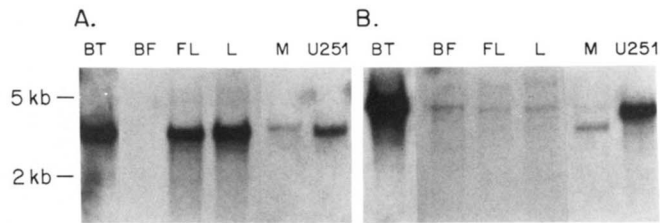


FIG. 2. Tissue distribution of glycogen phosphorylase mRNA in human tissues, assessed with liver and brain phosphorylase cDNA probes. Filters containing poly(A⁺) RNA were hybridized with radiolabeled liver phosphorylase cDNA (panel A) or brain phosphorylase cDNA (panel B) probes, as described under "Materials and Methods." Lanes: *BT*, 10 μ g of poly(A⁺) RNA isolated from the temporal lobe of adult brain; *BF*, 2 μ g of poly(A⁺) RNA isolated from the frontal lobe of adult brain; *FL*, 5 μ g of poly(A⁺) RNA isolated from 24-week-old fetal liver; *L*, 5 μ g of poly(A⁺) RNA isolated from adult liver; *M*, 2 μ g of poly(A⁺) RNA isolated from adult skeletal muscle (calf); *U251*, 5 μ g of poly(A⁺) RNA isolated from the human brain astrocytoma cell line from which the cDNA library was derived.

ization of the liver cDNA to this message occurs even at high stringency because of the high level of expression of the muscle phosphorylase message in adult muscle tissues (5). The distribution of liver-type mRNA in brain appears to be region-specific; no hybridization is seen to frontal lobe RNA (*BF*), whereas a strong signal is observed in temporal lobe (*BT*). In contrast, as shown in panel B of Fig. 2, the newly isolated phosphorylase cDNA hybridizes preferentially to a 4.2-kb mRNA found in both of the adult brain samples; low levels of expression of a 4.1-kb message are revealed by this probe in human adult muscle tissue, and adult and fetal liver tissues, as well (note that the amount of poly(A)⁺ RNA loaded was as follows: 2 μ g in *M* and *BF*, 5 μ g in *L*, *FL*, and *U251*, and 10 μ g in *BT*). There is also significant hybridization to a 3.4-kb message found in muscle mRNA that again likely represents cross-hybridization to the muscle-specific phosphorylase message. Finally, the newly isolated cDNA hybridizes to a band of approximately 4.1 kb in *U251* RNA.

These data indicate that the newly cloned phosphorylase corresponds to a mRNA that is larger and that has a tissue expression profile distinct from the mRNA encoding liver phosphorylase. Previous Northern blot analysis of rabbit tissues at low stringency also showed the existence of a 4.2-kb band in whole brain RNA as the major species capable of cross-hybridizing with the liver and muscle cDNA probes (5). Thus, we conclude that the new clone encodes the "brain" or "brain-specific" isozyme of phosphorylase.

Protein gel electrophoresis has demonstrated that glycogen phosphorylase isozyme expression is developmentally regulated (4, 20, 21). In fetal rat tissues, the predominant isozyme is the brain type (4, 20). At birth, liver and muscle expression switches exclusively to the liver and muscle isozymes, respectively; adult brain contains the brain form predominantly, with a small amount of the muscle isozyme (4, 20). Other adult tissues express a mixture of all three types of phosphorylase in proportions that vary depending on the species (4, 20, 21). Our results seem to contradict this scenario at several levels. First, we were able to clone cDNAs encoding the liver isozyme of phosphorylase from a library prepared from a cell line derived from human brain, a tissue usually described as expressing only the brain and muscle isozymes. The solution to this apparent paradox probably lies in the fact that a tumor cell line was used. Several studies have shown a loss of normal tissue-specific expression in transformed cell lines for enzymes of carbohydrate metabolism such as pyruvate kinase and phosphofructokinase (22, 23). Second, our data also reveal

that most human tissues express more than one type of mRNA. In particular, the temporal lobe of human brain expresses significant amounts of liver phosphorylase mRNA, in contrast to the rabbit, where no liver phosphorylase mRNA is detectable in whole brain extracts (5). The reasons for having more than one phosphorylase mRNA expressed in a given tissue are unclear, especially since the current results do not conform well to earlier protein electrophoresis studies that showed no liver phosphorylase activity in human brain extracts or brain phosphorylase activity in liver or muscle tissues (21). The final and perhaps most interesting observation is that the predominant mRNA present in 24-week-old (midgestation) fetal liver is the adult liver form; the brain-type RNA is present at the same low constitutive level in both fetal and adult liver and in adult human muscle tissues. There are several possible explanations for the discrepancies between the current data and those pertaining to the developmental expression profile of phosphorylase isozymes in rat: 1) human fetal liver, unlike rat fetal liver, contains primarily the adult liver isozyme of phosphorylase (a possibility that has also been implied by previous investigators (21)); 2) in human fetal liver, the liver gene is actively transcribed but inefficiently translated, whereas the small amount of brain phosphorylase message is rapidly translated; or 3) the human brain isozyme only predominates in early fetal liver development and is replaced by the adult liver isozyme well before birth. The current data cannot discriminate among these possibilities, nor do they suggest that the brain isozyme is not predominantly expressed in other human fetal tissues. With three cDNA clones in hand, it will now be possible to address developmental expression of phosphorylase isozymes in more detail.

The nucleotide and deduced amino acid sequence of the three human phosphorylase isozymes, brain (this work), liver (5), and muscle (8) are shown in Fig. 3. The protein predicted by the brain phosphorylase cDNA sequence is 862 amino acids long with a molecular mass of 112,917 daltons, a value in close agreement with that previously determined by gel electrophoresis for the rat brain phosphorylase monomer (24). In contrast, human muscle and liver phosphorylase contain 841 and 846 amino acids, respectively. The increased size of the brain phosphorylase protein is entirely due to insertion of amino acids at the C terminus; the three human phosphorylase isozymes can be aligned without insertions or deletions so that amino acids 1–830 match. Previous workers have often resolved phosphorylase isozymes by using slab or disc polyacrylamide electrophoresis in the absence of SDS; under these conditions, the brain isozyme is usually described as a "faster migrating band" than the liver or muscle types (1–4, 20, 21). We have determined that the isoelectric point predicted by the primary sequence of brain phosphorylase is 6.15, as compared to 6.24 and 6.42 for the liver and muscle phosphorylase isozymes, respectively, values consistent with the previously observed electrophoretic patterns.

The brain phosphorylase amino acid sequence is 83% identical to the muscle sequence and 80% identical to the liver sequence. The liver and muscle sequences are also 80% identical. Thus, the sequences of the human muscle and brain phosphorylase isozymes are more closely related than either are to the liver isozyme. The same is true at the nucleotide level where the identities are 79% for the brain/muscle comparison, versus 74% (brain/liver) and 73% (muscle/liver). Fig. 3 also provides the sequence of 80 bases of 5'-untranslated region and all of the 3'-untranslated region of the brain phosphorylase cDNA. Whereas the liver and muscle 5'-untranslated sequences have been shown to be approximately

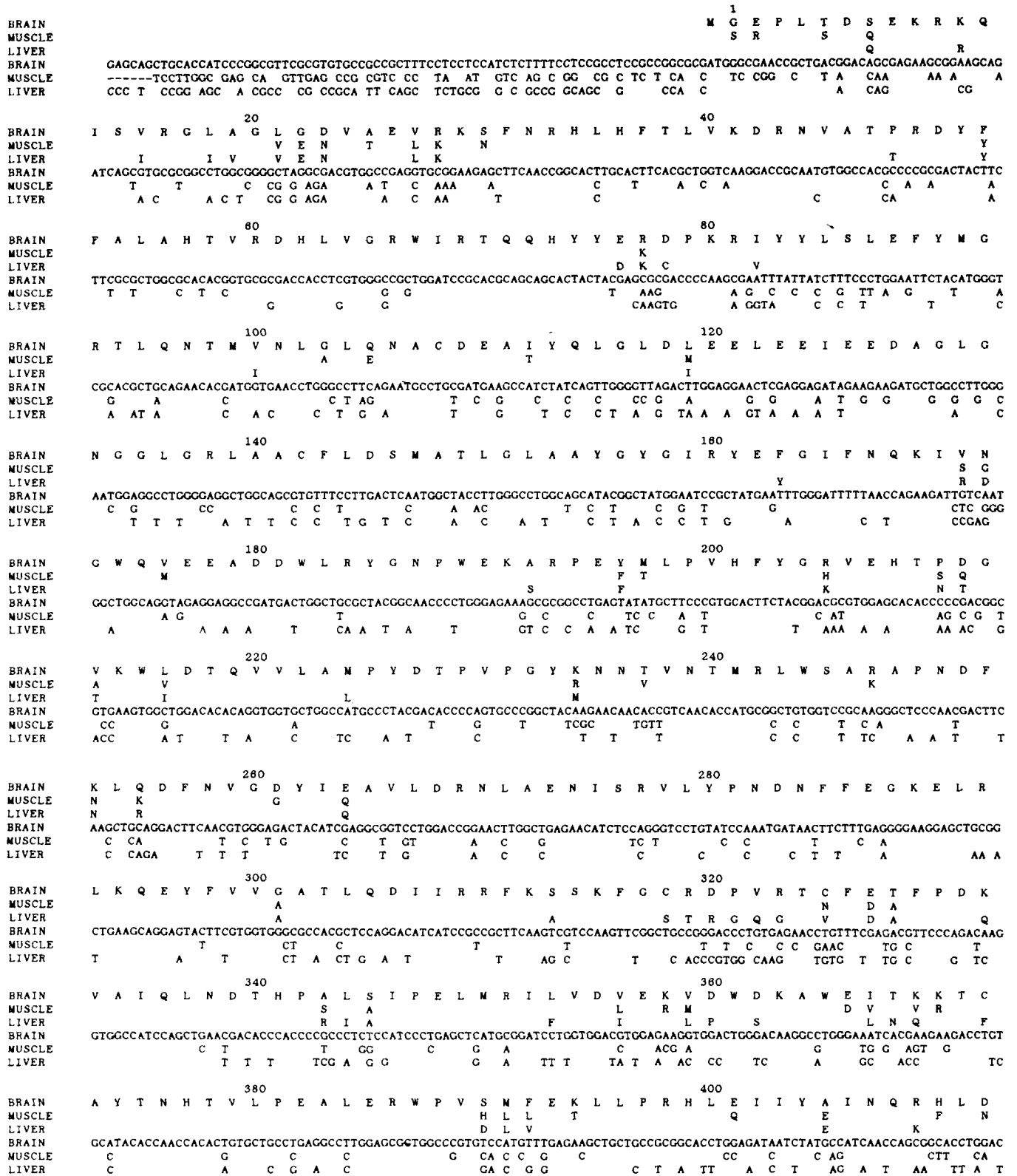


FIG. 3. DNA and deduced amino acid sequences of human brain (this work), liver (5), and muscle (8) isozymes of glycogen phosphorylase. The numbers above the brain sequence indicate the amino acid number, with the residues immediately after the initiator methionine (glycine in brain and liver phosphorylase, serine in muscle) designated number 1. The brain phosphorylase amino acid and nucleotide sequences are presented in their entirety in the first and fourth lines of each row, respectively, except for the 5'- and 3'-untranslated regions, where the brain nucleotide sequence is found in the first line of the row. The brain sequence serves as the reference sequence; only nucleotide and amino acid residues that are nonidentical in the liver and muscle phosphorylases are given in the remaining, labeled lines. Stop codons are indicated by an asterisk and the polyadenylation signals AATAAA (liver and muscle) or ATTAATA (brain) are underlined. Note the correction of two nucleotides in the liver sequence relative to Ref. 5, resulting in an E to V change at amino acid residue 82 and E to V at residue 232.


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BRAIN      CCCCTGCCCTCTGCTCGGCTCTGCACCTGGTATATGGGTCATGGACCAGATGGGGCTTCCCTTTGTAGCCATCCAATGGGCATTGTGTGGGTGCTTGGAAACCCGGGATGACTGAGGGG
BRAIN      GACACTGGAGTGGGTGCTTGTGTCTGCTGTCTCAGAGGCCCTGGTCAGGATGAAGTGGCTGACACAGCTTAGCTTGGTTTTGCTTATTCAAAGAGAAAATAACTACACATGAAATGA
BRAIN      AACTAGTGAAGCCTTTTCTGTTTTAGAAATGAAAATGTACTTGGTCACTTTTGTGCTTGGAGAGGCCCATTTTCTAGCCTGGCAGGGGAGGTCCTGTGCCCTCCCGCTTACTCTCTGC
BRAIN      TGTGTCTGAGGTGCATTCTCTGTTGTTACACACAAGGGCCAGGCTCCATTCTCCCTCCCTTCCACCACTGCCACAGCCTCGTCTGAAAAAGGACCAGGGGTCCTGGAGGAACCCAT
BRAIN      TTGTGCTCTGCTTGGACAGCAGGCTGGCACTGGGAGGTGGGGTGGAGCCCTCACAGCCTTGGCCCTCCCAAGGCTCGAACTGCCTCCCATGCCCCAAGAGAGAGGGGACGGAAACAG
BRAIN      GCTACTGTCTTCCCTGTGGAATGCCGGAGAAATCTAGACCTTGCATGCTGGATCTGGGCTCGGGGGAGGCTCTTTTCTCCCTGGCCTCCAGTGCCCCACAGGAGGATCTGGCCACGG
BRAIN      TGCACAGCCACCAGGCACTACAGCCTTTATTGAGTGGGGCAAGTGTGGGCTGTGGTCTGGCCCTGACAGCATCTTCCCAGGCAGGGGCTCTGTGGAGGAGGGCCATACTCCCTAG
BRAIN      TTGGCCACTGGGGCCACCACCTGACCACCACTGTGCCCTCATTGTTACTGCCTTGTGAGATAAAAACTGATIAAACCTTTGTGGCTTTGTTGG-TTAAAAA...

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FIG. 3—continued

63% identical (5), the brain sequence appears to be unrelated to the other two. The greater length of the brain phosphorylase mRNA detected on Northern blots is largely attributable to the much longer 3'-untranslated region of the brain phosphorylase cDNA (1147 base pairs) as compared to the liver (170 base pairs) and muscle (263 base pairs) sequences.² The longer 3'-untranslated region of the brain phosphorylase cDNA is a feature shared with the brain isoforms of two other isozyme families, Na⁺K⁺-ATPase and protein kinase C (25, 26). The cDNA encoding brain aldolase, in contrast, has a 3'-untranslated region of similar length to that found for the liver and muscle forms.³ The 3'-untranslated region of the brain cDNA contains 57% G + C compared with 55% for the muscle and 26% for the liver messages. In addition to this similarity in nucleotide composition, the brain and muscle messages have a nearly identical 11-nucleotide sequence, CTGCCAGCC_AC. This sequence was previously shown to be located at amino acid 833 of the muscle phosphorylase sequence, exactly conserved among rat, rabbit, and human muscle phosphorylase cDNAs (6), leading to the suggestion that it may play a role in regulation of muscle phosphorylase gene expression. The sequence appears in the brain message 90 nucleotides into the 3'-untranslated region, but is not found in any region of the liver phosphorylase cDNA. No other regions of significant sequence identity are observed when comparing the 3' ends of the muscle and brain phosphorylase cDNAs. This finding, coupled with the abrupt divergence of the three phosphorylase sequences around amino acid 830, suggests that the muscle and brain genes evolved in part via a deletion or insertion of DNA near the codon for amino acid 830 resulting in relocation of the 11-nucleotide element.

We have compared previously the human liver and rabbit muscle phosphorylase cDNA and predicted protein sequences (5). That study demonstrated that despite an 80% amino acid identity, two distinct patterns of codon usage were used to

² The difference in size between the brain phosphorylase mRNA depicted in Fig. 2 (~4.2 kb) and the brain phosphorylase cDNA clone described in Fig. 3 (3.82 kb) is presumably due to the lack of a poly(A) tail in the latter. Some of the difference may also be due to unsequenced regions of the 5'-untranslated region since no primer extension or S1 mapping was done to define the 5' end. Note that we have assumed that translation initiates at the methionine codon in the brain phosphorylase cDNA corresponding to the start site in the other two sequences. This seems likely since 1) no other ATG codons are found in the 80 nucleotides upstream of this site in the brain sequence, 2) the protein predicted by the open reading frame that begins at this site is 112,917 daltons, in exact agreement with the estimated molecular mass of the brain phosphorylase monomer (24), and 3) the amino acid residues 1-14 have been shown to be important in the covalent modification of muscle phosphorylase by phosphorylation and dephosphorylation (12). Since brain phosphorylase is recognized by muscle phosphorylase kinase, it is unlikely that any modification (elongation) has occurred at the N terminus.

³ D. Tolan, personal communication.

encode these proteins, *i.e.* the rabbit muscle phosphorylase cDNA was shown to have a significantly higher proportion of deoxyguanosine (G) and deoxycytosine (C) residues at third codon positions (85%) than its human liver counterpart (60%). Based on the data in Fig. 3, we can now calculate a third position G + C content of 77% for the human muscle and 82% for the human brain coding sequences. Furthermore, the overall G + C content of the coding sequences of the three messages is 49% for liver, 57% for muscle, and 59% for brain. This analysis provides a further indication that the brain and muscle genes have a closer evolutionary relationship to each other than to the liver gene.

Some gene families (such as those for the globins or some of the serine proteases) are found grouped together in tandem array, implicating an evolutionary mechanism involving gene duplication and retention of the gene copies on a single chromosome. This strategy does not apply to the phosphorylase gene family. We have shown previously that the genes for human muscle and liver phosphorylase map to chromosomes 11 and 14, respectively, by hybridizing the appropriate DNA probes to laser-sorted human chromosomal fractions spotted onto nitrocellulose (9, 10). Fig. 4 depicts the results of this kind of chromosome mapping experiment performed with the human brain phosphorylase cDNA probe. Strong hybridization is seen to the spot containing chromosome 20. A lesser

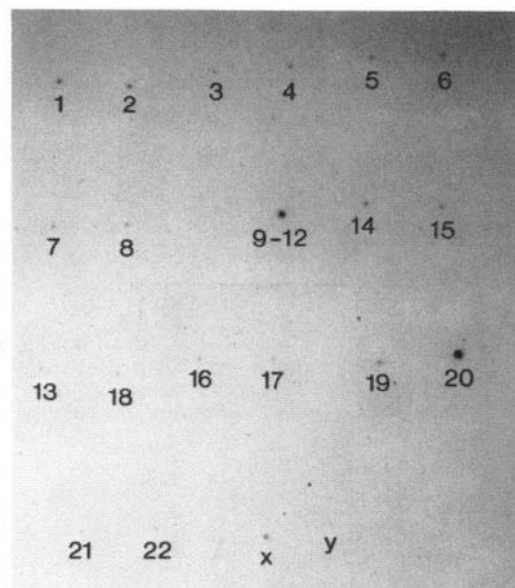


FIG. 4. Assignment of the brain phosphorylase gene to human chromosome 20. Autoradiograph of a panel of nitrocellulose filters, each with two fractions of flow-sorted human chromosomes as numbered. The chromosomal DNA was denatured and hybridized to a radiolabeled 3.47 *Eco*RI fragment of brain phosphorylase cDNA (see Fig. 1) as described in Ref. 10.

signal is seen on the spot containing a mixture of chromosomes 9, 10, 11, and 12. The latter spot can be interpreted in one of two ways. It is either due to the larger amount of DNA on this spot than on any other and/or a small amount of cross-hybridization with the muscle phosphorylase gene on chromosome 11, or it represents a separate gene with significant homology to the brain phosphorylase cDNA. In order to distinguish among these possibilities, we carried out further mapping studies using DNA purified from hamster/human cell lines. The lines were chosen so as to be able to unequivocally distinguish between chromosomes 9, 10, 11, and 12; a line containing chromosome 20 was also included. To further clarify the issue, the hybridization was performed using the 1.1-kb *Pst*/*Eco*RI fragment encompassing the majority of the 3'-untranslated region of the brain-specific cDNA (see Fig. 1), since this region has no homolog in the muscle- or liver-specific phosphorylase cDNAs. The results, shown in Fig. 5 confirm the strong hybridization to a gene found on chromosome 20, but also reveal the presence of a somewhat less homologous fragment on chromosome 10. Thus, we conclude that the gene with exact homology to the human brain phosphorylase cDNA cloned in this study maps to chromosome 20, but that a gene encoding a very similar sequence is found on chromosome 10. It is unclear from the current study whether the gene on chromosome 10 is an expressed or pseudogene. There is no evidence for multiple mRNA bands in Fig. 3B; given the high homology of the two genes, however, it cannot be ruled out that the "brain like" transcripts found in different tissues are derived from one or the other of the brain like genes. The issue will be resolved in future studies in which the transcripts in the cell lines containing either chromosome 10 or 20 will be analyzed.

Clearly, the genes encoding the tissue-specific isozymes of phosphorylase are unlinked. It has been suggested that the genes encoding tissue-specific forms of aldolase are found on separate chromosomes because of tetraploidization, since re-

lated aldolase genes are found on morphologically similar chromosomes (aldolases A and C on chromosomes 16 and 17, aldolase B and an aldolase pseudogene on chromosomes 9 and 10) (27). The localization of the phosphorylase genes to three morphologically unrelated chromosomes thus would appear to involve a more specific translocation mechanism.

Our data suggest that the liver phosphorylase gene has evolved by a route distinct from that taken by the muscle and brain genes, since the latter have retained greater similarities in primary sequence, codon usage, and nucleotide composition. Interestingly, the muscle and brain phosphorylase gene products are also more responsive to the allosteric effector AMP than the liver protein, although AMP activation does not appear to be cooperative in brain phosphorylase as it is in the muscle form (28, 29). It is reasonable to assume that the phosphorylase genes were duplicated and dispersed in the genome, leading to the evolution of regulatory elements controlling tissue-specific and developmental patterns of gene expression, as well as allosteric regulatory properties tailored to fit each isozyme's physiological role. Sequence analysis of phosphorylase isozymes has led to insights into the evolution of allosteric regulatory sites in this protein, as described elsewhere (13, 30, 31). Cloning of the brain isozyme of phosphorylase now permits a careful assessment of developmental regulation of phosphorylase gene expression and "isozyme switching." The recent observation that muscle cells in culture, which normally produce only the brain (fetal) isozyme of phosphorylase, can be stimulated by innervation to produce the mature muscle isozyme (32) suggests that cell culture techniques or the heterokaryon approach of Blau and associates (33) will be useful for defining the regions of the various phosphorylase genes that are involved in gene regulation. To this end, we are currently isolating the liver and brain phosphorylase genes, using our cDNA clones as probes.

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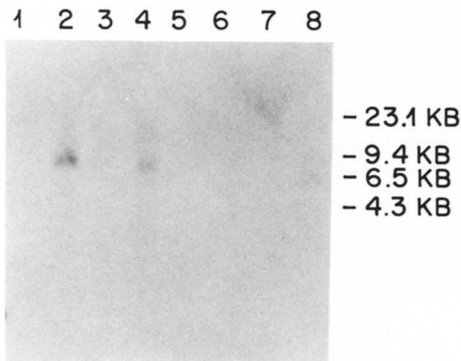


FIG. 5. Resolution of chromosomes 9, 10, 11, and 12 by southern blot analysis of hamster/human hybrid cell line DNA. The filter contains 10 μ g of hamster genomic DNA (lane 1) or 20 μ g of DNA isolated from various hamster/human hybrid cell lines (lanes 2-8) digested to completion with *Eco*RI. It was probed with a 1.1-kb *Pst*/*Eco*RI fragment derived from the 3'-untranslated region of the human brain phosphorylase cDNA as described under "Materials and Methods." The hybrid lines contain the following human chromosomes: lane 2: line CF26/20 contains 4p, 15, and 20; lane 3: line CF 26/18 contains 12, 15, and 17; lane 4: line SiF4D2A contains 4, 10, 11, 14, and X; lane 5: line Q3D2 contains 1, 2, 3, 12, 13, 14, 16, 21, and 22; lane 6: line Rag Stuart A9 is a mouse/human hybrid line and contains 3, 5, 11, 14, 15, 16, 18, and 21; lane 7: line 5067-20 contains 3, 5, 6, 9, 11, 19, 21, and X; lane 8: line 5067-13 contains 9 and 11 but is otherwise incompletely characterized. Assignment of the less homologous gene to chromosome 10 is made because hybridization occurs to a band in lane 4 (contains 10 and 11) but not lanes 6 or 7 (contain 11 only).

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