

Structural and Functional Characterization of the Drosophila Glycogen Phosphorylase Gene

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We identified a P element insertional mutant of the Drosophila glycogen phosphorylase (DGPH) gene. Glycogen phosphorylase protein concentration and enzyme activity are decreased while glycogen content is increased in flies homozygous for the mutant allele. The DGPH gene has been cloned and sequenced; its open reading frame codes for a protein of 844 amino acids with a predicted molecular mass of 97 kDa. Comparison of the conceptual amino acid sequence of the Drosophila glycogen phosphorylase with glycogen phosphorylase sequences from other organisms shows a high degree of homology to mammalian enzymes. All the residues of the allosteric effector binding sites, the active site, and the site of phosphorylation are exactly conserved, but some of the residues of the glycogen storage site are not. © 1999 Academic Press

Glycogen phosphorylase (1,4 α -D-glucan: ortophosphate α -D glucosyltransferase, E.C.2.4.1.1) is the rate limiting enzyme of glycogen degradation which operates under tight control. Besides its important physiological role, glycogen phosphorylase is also interesting from a theoretical point of view. Rabbit skeletal muscle glycogen phosphorylase was the first example of enzyme regulation by reversible phosphorylation (1). The inactive phosphorylase b is phosphorylated and activated by phosphorylase kinase at a single site (Ser-14) and, in turn, phosphorylase a is dephosphorylated and inactivated by protein phosphatase 1 (2). The effect of covalent modification is modulated by allosteric effec-

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The nucleotide sequence data reported in the paper can be found in the EMBL GenBank under the Accession Nos. AF073117, AF073178, and AF073179.

Abbreviations used: DGPH, Drosophila glycogen phosphorylase; PLP, pyridoxal 5'-phosphate.

tors (2, 3). Both the fine details of the regulation and the catalytic mechanism of the phosphorylase have been resolved at the atomic level (4, 5). X-ray crystallographic analysis revealed that the enzyme is a homodimer of two identical subunits. In each subunit one molecule of the cofactor pyridoxal 5'-phosphate (PLP) is located at the active center nearby the substrate and competitive inhibitor (glucose) binding sites. In addition, separate binding sites for allosteric activators (AMP and IMP), allosteric inhibitors (caffeine and purine nucleosides), and a specific glycogen storage site were identified at the surface of each diade related monomer. The binding site of another allosteric inhibitor, glucose-6-P, overlaps with that of the nucleotide activators. A complicated network of conformational changes connecting the above mentioned sites within the same and in the symmetry related subunit was detected, and the elementary steps of the catalytic cycle were described (4, 6-10).

Since the publication of the complete amino-acid sequence of the rabbit muscle glycogen phosphorylase (11) a number of phosphorylase cDNA sequences have been reported from various organisms. Complete amino-acid sequences were deduced for the glycogen phosphorylase isozymes from rabbit (12), human (13), rat (13) and cattle (14) muscle; rat (15) and human (16, 17) brain; and rat (18) and human (19) liver. Homologues from lower organisms have been cloned as well; e.g., yeast glycogen phosphorylase (20), phosphorylase 1 (21) and phosphorylase 2 (22) from *Dictiostelium* discoideum; glycogen (23, 24) and maltodextrin phosphorylase (25, 26) from E. coli, glycogen phosphorylases from Bacillus subtilis (27), Bacillus stearothermophilus (28) and Haemophylus influenzae (29), type L (30) and type H (31) phosphorylases from potato, phosphorylases from Vicia faba (32) and Ipomea batata (33). The amino acid sequences of the phosphorylases from different organisms have been compared and



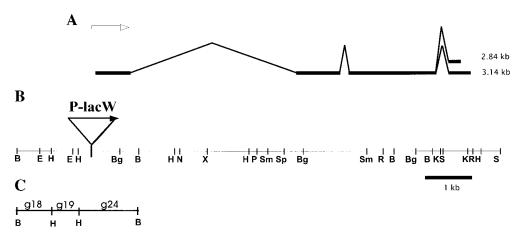


FIG. 1. Molecular map of the region containing the P element insertion in P79/18. Sites for endonucleases BamHI (B), EcoRI (E), HindIII (H), NcoI (N), SaII (S), SphI (Sp), BgIII (Bg), EcoRV (R), KpnI (K), PstI (P), SmaI (Sm) and XhoI (X) are shown. (A) Intron–exon structure of the DGPH transcription unit. Open arrow indicates the direction of transcription. (B) Restriction map of the region. Site of the P-lacW insertion is designated by a triangle. (C) Genomic fragments used for Northern analysis and to screen the Agt cDNA library. (A), (B) and (C) are drawn on the same scale.

their phylogenetic relationship has been determined (34). It turned out that the structure of the catalytic center including the PLP and glucose binding sites was highly conserved in all examples. On the other hand, the sequences of additional ligand binding sites as well as the structural element connecting the two subunits (tower/gate and cap) were more variable. The characteristic site of covalent modification (Ser-14) was found only in the mammalian phosphorylases; in yeast phosphorylase an unrelated Thr residue is phosphorylated by the cAMP- dependent protein kinase and a yeastspecific phosphorylase kinase, while the phosphorylation site is completely absent from the other homologues. These structural data provide a rational explanation for the diverse substrate specificity and regulation of phosphorylases in different organisms.

Glycogen phosphorylase has been purified from *Drosophila melanogaster* (35). The biochemical properties of the inactive *b* and active *a* forms of the enzyme were determined (35, 36) and the regulation by phosphorylation-dephosphorylation in *Drosophila* was demonstrated (37). In the present communication we report the molecular characterization of the gene encoding the glycogen phosphorylase in *Drosophila*. To our knowledge, this is the first report on the molecular genetic dissection of an insect phosphorylase. We also present a comparison of the *Drosophila* glycogen phosphorylase amino acid sequence with that of the published glycogen phosphorylases in order to identify its place in the phylogenetic tree of phosphorylases.

MATERIALS AND METHODS

Drosophila cultures and stocks. Standard fly techniques were carried out as described by Ashburner (38). Genetic symbols used here can be found in Lindsley and Zimm (39). The y^+CyO balancer

chromosome carrying a $P(y^+)$ insertion was kindly provided by Dr. Allen Shearn. The mutant 79/18 was isolated previously (40) by making use of the P-lacWenhancer detector (denoted from here on as $P(w^+)$). Stock $Dp(2;2)dpp^{s7}$, Df(2L)dp-38a, net ed dp cl/CyO (denoted as Df(2L)dp-38a from here on) was kindly provided by Dr. William Gelbart. Stock Df(2L)dp-38a carries a deletion between 22B1-2 and 22F1-2 which uncovers the region of the P element insertion in P79/18.

Generating deletions by imprecise excision of the P element. The $P(w^+)$ element in P79/18 was remobilized to induce imprecise deletions of the inserted transposon. $yw_; P(w^+)/CyO$ females carrying the P element insertion were crossed to $Sb\Delta 2$ -3/TM6 males carrying the $\Delta 2$ -3 transposase source (41). Jumpstarter males carrying both the P element and the transposase source were crossed to $yw_; y^+CyO/Sco$ females and the white-eyed progeny, possibly lacking the P element

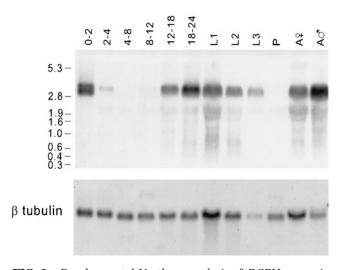


FIG. 2. Developmental Northern analysis of *DGPH* expression. The Northern filter was probed with the g24 DNA fragment (see Fig. 1). To monitor the relative RNA contents of the lanes the same blot was hybridized with a β -tubulin probe. Embryonic stages (0–2, 2–4, 4–8, 8–12, 12–18, and 18–24 h), larval stages (L1, L2, and L3), pupal stage (P) and adult females (A $\mathfrak P$) and males (A $\mathfrak F$) are shown.

-1680 tcagtgtcgtgagaaacgcaaagtatagcgacattccgcaacgagtgttcgaatcaaaatgaaatcgagtacttagaact<u>taccctgcgaactcagcactctgc</u>tatcagtaaaatgcg -1560 tegittitaagaatteeacaaagtattittgagataaateacttgtattataaaaaatattegeageattegitatattattattateacaaegateeaageagtetaattteteeteage -1200 ttgctctcactttcctcctgccaaatgtgctaatcattttcatttgctacgaaatagccgaacaaatggtgagctcacgaaacaagcgggcgctttggtcctttgcaatgttaccccccc -1080 ccctttaccctcctttcagggactttcccgcatgcattcccaagtagttgaacatgggagtgccattatgggaagtaataaacaagaagtggaataactgaattgttcggatagaattgc -960 agatgccctttgtcctgtgattagcatttgttgcacaagtcgagtgaggcacagcagagcacatatattcagtggctttctaaatagatcaattctgtgacaacattttttgaaaacqct -840 tgttttagaatatcagtacgtacatcttaaagctcaagatacgaatgtacccaaagagatatataaaacacaaaatatctgtaagatacaaaagcatcagattttaaaagtatctcttag at a caa a ageta teet taagat tegga a aget teeta gea aget tegea tetee a at a teeta gee ege consideration of the temperature of-560 gtcggttcaaaaagctcaaacccgattcatgggggcgccacccgttctattccaaagcatacaaatgcctcaagatgaaacaaaaacagaaaataaaacaaatacgcatgtatgacct -480-360 ctccggcgagataagaaccgctgcatcagcaacgcacagtgggcgatatggtcaaaatatactgataaaagttattatctgttattgaaatatacaaaattttcattaatcttaatataa -240tata aaa aatcga aaattta attattagttg tatagtta aaaa aacca acctatattg ttg tattatat cocctag atgata attog tag cag ttg at acccag cgc acccactg tg cgc tg garantees and the same continuous account of the same continuous attacks and the same continuous account of the same continuous acc0 -120 gagttgtttacaaaggggagcagcagctgagagaaaagctctggcgcaaaagctatctccgaaagtatgccgaaagtataaagtgagaatcgctccggattttgctgcgaacggtcgtct ${\tt MetSerLysProGlnSerAspAlaAspArgArgLysGlnIleSerValArgGlyIleAlaGluValGlyAsnValThrGluValGlyAsnValGlyAsnValThrGluValGlyAsnValThrGluValGlyAsnValGlyA$ 241 GTGAAGAAGAACTTCAATCGCCACCTGCACTACACCCTGGTCAAGGATCGCAACGTGTCCACCCTGAGGGACTACTACTTCGCCCTGGCCAACACCGTCAAGGACAACATGGTGGGCCGC 28 ValLysLysAsnPheAsnArgHisLeuHisTyrThrLeuValLysAspArgAsnValSerThrLeuArgAspTyrTyrPheAlaLeuAlaAsnThrValLysAspAsnMetValGlyArg 68 TrpIleArgThrGlnGlnHisTyrTyrGluLysAspProLys 721 841 961 taattattegegaaattggeaaaaageaaagactgeaatgggateaatgeeaaagaagtagegeeaaaaaaaggtgtgacacttteaetgeaacaaattgeagaggtgetgeaaaaagee 1081 1201 1321 atacacaatatatgtatatacatacatatgtacatgtatccaaacacgtgcccaaagcgtgagcttttcatttgcaaatttgtcagcatccggctgctaaacatttttgtcctttgctgaa 1441 tgtcagcttaaatcactttcaatttcaagcccaccaaaatgtatctattaattttccattgactcgagcaattttcgcgactagaagttcgagctatatacacttttgggtgataaatat 1681 the test established the test of the state of the $1801\ {\tt tttcagttgtcccccacatgccaattagctcacgcaaccatctaaatagaaatgtatcgaactgacgactgttgtatcttatgggatacttaaacagctgttgttgcgcagcatggattg}$ 1921 qtaqctaqaqttattqaaqacaqttqqtcqtcqatcqqtctctqqaccaatttatqcacaattqaaqaaqttcaqqqaqqtqataqtcacqcaaaaaaqtttattccctqtaqttttc 2161 gcacatetgtcatttatttattgcatatagggaaaaaaacaataaacaaaggcaaacaacactggtcgtttgttggtggggggttctttatcaaaaccgcgaaaaacatatgtctggc 2281 atctaattegtetgtagaaetggeeagaaegaatateaaagaeeetaeacaaacaaetgetgtggaaaacattagegeeggeagttgaatgaeteeaaaagettettatggatetggaag $2521 \ {\tt tttttgccaactcagtatttactgagtgcacctcatatttcataactaagaaaatacctacgttttatagttcatatttttggtatacctatatagatataaacaataatgtgttttatca}$ 2641 cagcatttccattgcccagcacatattgcccgaatcagttcatactccatgttcacctaattctgtatatcttatctgctaaatggactgcaaaatattcctaatcgaaagcccgattct 2761 atataggagcettaaccettgeeetacaaatatgeacacaaaacagetteaacattetgaactaattagetatagtttttttttgeeggaegcatagcateagacatetgaageteatgte 3001 agctgattggtgattgctgatccgtgatccatcatgattagcccagcattggctggtgataataatcgccattccaagccgatatgattcatgcttagccatatgttgcccagcatgcc 3121 aagtgeageetgeaategettgatagegttgetatgaacceaattaatgeeetegtaacategategatagattegeactgaategaaceggtgatagtgaatgeetteetetatttata 3241 82 ArgValTyrTyrLeuSerLe 3721 GGACGCCGGTCTGGGCAATGGTGGTCTGGGTCGTTTTGCCCGCCTGTTTCCTCGACTCGATGGCCACTCTGGGTCTGGCCGCTATGGCTATGGCATCCGTTATGAGTACGGTATCTTCCTC 128 uAspAlaGlyLeuGlyAsnGlyGlyLeuGlyArgLeuAlaAlaCysPheLeuAspSerMetAlaThrLeuGlyLeuAlaAlaTyrGlyTyrGlyIleArgTyrGluTyrGlyIlePheAl 3841 CCAGAAGATCAAGAACGCCAGCAGCTGGAGGAGCCCGATGATTGCTGCCTTATGCCAATCCCTGGGAGAAGGCTCGTCCGGAGTTCATGCTGCCGGTCAACTTCTACGGCCGTGTGAT $168\ a Gln Lys Ile Lys Asn Gly Glu Gln Val Glu Glu Pro Asp Asp Trp Leu Arg Trp Gly Asn Pro Trp Glu Lys Ala Arg Pro Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Met Leu Pro Val Asn Phe Tyr Gly Arg Val II land Glu Phe Tyr Gly Arg Val II land Gly A$ 3961 CGACACGCCCGAGGGCAAGAAGTGGGTGGACACCCAAAGGGTGTTTGCCATGCCCTACGACAACCCCATTCCCGGATACAACAACAACACGCTGCACTCTGTGGTCCGCCAA $208\ eAspThrProGluGlvLvsLvsTrpValAspThrGlnArgValPheAlaMetProTvrAspAsnProIleProGlvTvrAsnAsnAsnAsnHisValAsnThrLeuArgLeuTrpSerAlaLy$ 4081 GTCGCCCATCGACTTCAACCTCAAGTTCTgtaagtatctataaggaaaggacccttgataaggcaagaactaacctatggctatcttcacctatccagTCAACGATGGTGACTACATCC 248 sSerProIleAspPheAsnLeuLysPheP

FIG. 3. Sequence of the DGPH gene and predicted amino acid sequence of its product. Intron and untranscribed sequences are shown in lowercase letters, exons are shown in uppercase letters. The first nucleotide of the longest cDNA isolated in the present work is positioned at +1 and the last nucleotides of the two transcribed variants are indicated by an open circle (o) above the nucleotide sequence. The first nucleotide of the longest EST sequence (EST No. GH08049, Accession No. AI108791) corresponding to DGPH in the clot 485 of the Berkeley

4201 AGGCCGTGCTGGACCGCAATCTGGCTGAGAACATCTCACGTGTCCTGTACCCCAACGACAACTTCTTCGAGGGCAAGGAGCTGCGTCTGAAGCAGGAATACTTCATGTGCGCCGCCACGC
265 lnAlaValLeuAspArgAsnLeuAlaGluAsnIleSerArgValLeuTyrProAsnAspAsnPhePheGluGlyLysAspLeuArgLeuLysGlnGluTyrPheMetCysAlaAlaThrL
4321 TGCAGGATATCATCCGCCGCTACAAGGCCTCGAAGTTCGGATCCCGGGAGGCGGTCCGCAACACCTTCGATCACTTCCCCGACAAGGTGGCCATTCAGCTGAACGATACCCATCCGT

345 euAlaIleProGluLeumetArgIleLeuValAspGluGluHisLeuThrTrpGluLysAlaTrpAspIleThrvalArgSerCysAlaTyrThrAsnHisThrValLeuproGluAlaL 4561 TGGAGCGCTGGCCCGTCTCCCTGCTGGAGTCGATCCTGCCCCGCCATCTGCAAATCATCTATCACATCAACTTCCTGCACATGGAGAATGTGAAGAAGAAGTTCCCCGACGATTTGGACC 385 euGluArgtrpProValSerLeuLeuGluSerIleLeuproArgHisLeuGlnIleIleTyrHisIleAsnPheLeuHisMetGluAsnValLysLysLysPheProAspAspLeuAspA 4686 GCATGCGCCGCATGTCGATGGTGGAGGAGGATGGCGAGAAGCGCATCAACATGGCTCATCTGTCCATCGTCGGCTCCACGCCGTCAACGGTGTGGCCGCCATCCACTCGCAGATCCTAA 425 rgMetArgArgMetSerMetValGluGluAspGlyGluLysArgIleAsnMetAlaHisLeuSerIleValGlySerHisAlaValAsnGlyValAlaAlaIleHisSerGlnIleLeuL 465 ysAspSerLeuPheHisAspPheTyrGluMetGluProGlnLysPheGlnAsnLysThrAsnGlyIleThrProArgArgTrpLeuLeuLeuCysAsnProGlyLeuSerAspleuIleA 4921 CCGAGAAGATCGGCGACGAGTGGCCAGTGCATCTGGACCAACTGGTTGCTCTGAAGAAGTGGGCAAAGGACCCCAACTTCCAGCGCAATGTAGCCCGCGTCAAGCAGGAGAACAAGCTGA 505 laGluLysIleGlyAspGluTrpProValHisleuAspGlnLeuValAlaLeuLysLysTrpAlaLysAspproAsnPheGlnArgAsnValAlaArgValLysGlnGluAsnLysLeuL 5046 AGCTGGCCGCCATTCTGGAGAAGGACTACGGCGTTAAGATCAACCCCTCTTCCATGTTCGACATCCAGGTGAAGCGTATTCACGAGTACAAGCGCCAGCTGCTGAACTGCACATCA 545 ysLeuAlaAlaIleLeuGluLysAspTyrGlyValLysIleAsnProSerSerMetPheAspIleGlnValLysArgIleHisGluTyrLysArgGlnLeuLeuAsnCysLeuHisIleI 5161 TCACCCTGTACAACAGGATCAAGAAGGATCCCACAGCCAACTTCACCCCGAGGACAATCATGATCGGAGGCAAGGCTGCTCCGGGCTACTATGTGGCCAAGCAGATCATCAAGCTCATCT 605 leThrLeuTyrAsnArgIleLysLysAspProThrAlaAsnPheThrProArgThrIleMetIleGlyGlyLysAlaAlaProGlyTyrTyrValAlaLysGlnIleIleLysLeuIleC 5281 GCGCCGTGGGCAACGTTGTGAACAACGATCCCATTGTGGGCGATAAGCTCAACGTTATCTTCCTGGAGAACTACCGTGTGACCCTGGCCGAGAAGATTATGCCCGCCGCCGACTCTGTCCG 645 ysAlaValGlyAsnValValAsnAsnAspProIleValGlyAspLysLeuAsnValIlePheLeuGluAsnTyrArgValThrLeuAlaGluLysIleMetProAlaAlaAspLeuSerG 5401 AGCAGATCTCAACCGCCGGCACAGAGGCCTCTGGTACCGGCAACATGAAGTTCCAGCTGAACGGCGCCCCTCACCATCGGCACCCTGGACGGTGCCAACGTTGAGATGGCCGAGGAGATGG 685 luGlnIleSerThrAlaGlyThrGluAlaSerGlyThrGlyAsnMetLysPheGlnLeuAsnGlyAlaLeuThrIleGlyThrLeuAspGlyAlaAsnvalGluMetAlaGluGluMetG 5521 GTCTGGACAACATCTTTATCTTCGGCATGACCGTCGACGAGGTGGAGGCGCTCAAGAAGAGGGCTACAATGCCTACGACTACTACAACGCCAACCCCGAGGTCAAGCAGGTGATTGACC 725 lyLeuAspAsnIlePheIlePheGlyMetThrValAspGluValGluAlaLeuLysLysLysGlyTyrAsnAlaTyrAspTyrTyrAsnAlaAsnProGluValLysGlnValIleAspG 5641 AAATCCAGGGCGGATTCTTCAGCCCCGGCAATCCCAACGAGTTCAAGAACATTGCCGACATTCTGCTTAAGTACGACCACTACTACTTGCTGGCCGACATCCAACGATGCGTACATCAAGGCCC 765 lnIleGlnGlyGlyPhePheSerProGlyAsnProAsnGluPheLysAsnIleAlaGluIleLeuLeuLysTyrAspHisTyrTyrLeuLeuAlaAspTyrGluAlaTyrIleLysAlaG 805 lnAspLeuValSerLysThrTyrGln 5881 AAGTGGCTGGAGATGTCCATCAACAACATTGCGTCCAGCGGCAAATTCTCGTCGGATCGCACCATCGCCGAGGATTACGCCCGGGAGATTTTGGGGAGTGGAGCCCACCTGGGAGAAGCTCCCA 817 LysTrpLeuGluMetSerIleAsnAsnIleAlaSerSerGlyLysPheSerSerAspArgThrIleAlaGluTyrAlaArgGluIleTrpGlyValGluProThrTrpGluLysLeuPro 857 AlaProGluAspGlnProGlnAsnEnd

6121 CCAAGTGAACACGGGCCACAGCACCGAATTGTTAATATATTTTATTAATAAAAATCAAGATAAACAATTCGCCTAGTTCTCTCATCACTGTGGAATCGAATCGAATCGAATCAACAACGGT
6241 ACCTACCTAAAAAAGACCTCAGGAACACTTTCAAAGTAAAAGAAAAGTCTATTAAAAATAATGGAAAGAGTTGTAAAAAAACGTTAGTGGCAAGCTTGTTTAGATATCTGATATCGGTGTTT

A2-3 o

6961 cggtgacttctcctatggattggcaaccacattagttgtaattccagtacgcaattccagaacttgggaatgttgtcgac 7040

FIG. 3—Continued

was selected. Such white eyed males were crossed to yw; y^+CyO/Sco females in one male crosses. Resulting yw; $P(w^+)/y^+CyO$ males and females were crossed to each other and the lack of Cy^+ flies (flies without the y^+CyO balancer) indicated possible imprecise deletion resulting in lethality.

Nucleic acid procedures. Standard molecular biology techniques were performed as described by Sambrook et al. (42), unless otherwise indicated. DNA sequences adjacent to the insertion were isolated by plasmid rescue: genomic DNA from P79/18/CyO flies was digested with EcoRI, ligated and transformed into XL-1 Blue competent cells. The rescued clones were analyzed by Southern blotting. A genomic fragment near the P element insertion was used as a probe to screen a genomic EMBL3 λ phage library. The phage clones were analyzed by restriction mapping, and a clone overlapping the P element insertion on both sides was chosen for further subcloning. A fragment overlapping (g24) and two adjacent (g18 and g19) to the insertion site (Fig. 1) were subcloned and used to screen a λ gt11 cDNA library, kindly provided by Dr. L. Kauvar, (43) made of 0- to 16-h-old embryos. The inserts of the cDNA clones were subcloned into Bluescript SK+ vector and sequenced. Dideoxy chain termination method was used with a USB Sequenase Ver. 2.0 kit to sequence cDNA clones and most of the subclones of the genomic λ -phage. To help sequencing, some internal primers were synthesized. In the

homozygous strain *DGPH*^{Ins31}/*DGPH*^{Ins31}, the fragment carrying the imprecise P element excision was PCR amplified with primers E4FD: 5′-TACCCTGCGAACTCAGCACTCTGC-3′ and B4UP: 5′-GCGCGG-AACTCACCTTGGG-3′ (Fig. 3) using Takara ExTaq DNA polymerase and the PCR product was sequenced. Some regions were sequenced on an ABI 50 DNA sequencer. Sequence analysis was done using the DNA Star software. Oligonucleotides were synthesized on a Pharmacia Gene Assembler by phosphoramidite chemistry.

For a developmental Northern analysis, polyA $^+$ RNA was isolated, electrophoresed and transferred to nitrocellulose filters as described in Török *et al.* (44). PolyA $^+$ RNA was isolated from embryos, larvae and adults, and the RNA blot was probed with fragment g24 which overlaps the P element insertion.

The homozygous nonviable lines derived from the remobilization experiment of *P79/18* were screened by Southern analysis for deletions or remaining P element sequences.

Western blot analysis. Proteins from third instar larval homogenates were separated in 8% SDS gel and transferred to nitrocellulose membrane (42). Following incubation with affinity-purified primary rabbit anti-DGPH antibody (kindly provided by Dr. Andor Udvardy) in a 1:15000 dilution, the filter was washed and incubated in secondary peroxidase-conjugated goat anti-rabbit antibody (Dako) in a

1:200 dilution. ECL Western blot detection reagents (Amersham Life Science) were used to visualize the DGPH protein.

Assay of glycogen phosphorylase activity. Phosphorylase activity was determined in the direction of glycogen synthesis as described earlier (35) with the exception that 20 mM NaF was also added to the extraction buffer in order to inhibit phosphorylase phosphatase activity. Assays were carried out in triplicates and in two different dilutions of the extracts in the presence and in the absence of 1 mM AMP. The amount of total phosphorylase was calculated on the basis of the assumptions that the specific activity of phosphorylase b was 1.9 times less than that of phosphorylase a and phosphorylase a was activated by AMP 1.1-fold (36).

Assay of glycogen concentration. Glycogen was assayed by the iodine (I_2 -KI) staining in the presence of saturating $CaCl_2$ in a 10% TCA extract using rabbit liver glycogen as standard (45).

RESULTS

Isolation of P element insertions in the Drosophila glycogen phosphorylase (DGPH) gene. The stock carrying the P element insertion in the Drosophila glycogen phosphorylase gene was generated in a large scale P element insertional mutagenesis and designated as 79/18 (40). It was isolated as a potential tumour suppressor mutation displaying lethal overgrowth of the brain and the imaginal discs, but later analysis revealed that the overgrowth phenotype and the lethality was the result of a second-site mutation which had no relevance to the P element insertion. The second-site mutation was removed via genetic recombination and the resulting stock was named P79/18.

The P element insertion in P79/18 maps to chromosome 2 at 22B. P79/18 homozygous animals are fully viable with no phenotypic defect visible. Stock Df(2L)dp-38a/CyO carries a deletion which uncovers the region of P element insertion in P79/18. P79/18 proved to be fully viable over the above deletion and no visible phenotypic defects were detectable either. To generate loss of function mutations in the *DGPH* gene, we remobilized the P element insert of P79/18 and established 100 mobilized lines that had lost their eye color due to the excision of all or part of the P element. These lines were analyzed by Southern blotting (data not shown) to identify imprecise excisions that could disrupt the *Drosophila* glycogen phosphorylase gene. Out of the 100 excision lines none proved to contain deletions in the DGPH gene. In one of the excision lines ($DGPH^{lns31}$) a 209-bp sequence of the 5' end of the P element was retained but no part of the genomic sequence was deleted, as revealed by sequencing of the allele. DGPH^{Ins31} in combination with Df(2L)dp-38a/CyO displays weak viability with only 39% of the *DGPH*^{Ins31}/*Df(2L)dp-38a* animals surviving to adulthood.

Cloning of the Drosophila glycogen phosphorylase gene. To identify the gene responsible for the mutant phenotype, the genomic sequence flanking the P element insertion was cloned via the plasmid rescue method. Screening a Drosophila genomic DNA library with a genomic fragment adjacent to the P element insertion yielded a phage clone which contained the

insertion site and flanking sequences on both sides (Fig. 1). Northern blot analysis of wild type polyA⁺ RNA performed with fragment g24 overlapping the P-element insertion site revealed the presence of two relatively abundant transcripts of 2.9 and 3.2 kb in 0-2 hours old embryos (Fig. 2) and the 3.2-kb transcript in the further stages of development. Fragment g24 was also used to screen an embryonic cDNA library and three cDNAs were isolated. Determination of the nucleotide sequence of the genomic DNA and the cDNAs showed that both classes of transcripts belong to the same transcription unit with identical 5' ends but exhibiting different lengths in their 3' ends, as indicated in Fig. 1A. The 3.2-kb transcripts were found to contain a 3' extension of 278 nucleotides by comparison to the 2.9 kb transcripts (Fig. 3). Alignment of the sequences of the three cDNAs with the genomic DNA sequence revealed four exons of 402 (or larger), 529, 1607, and 601 nucleotides separated by three introns of 3178, 70 and 84 nucleotides, respectively. The last exon of the 3.2 kb class of cDNAs is 278 nucleotides longer. The cDNA sequence displays an open reading frame of 844 codons initiated by an ATG present in the first exon 160 nucleotides downstream from the 5' end of the longest cDNA. This open reading frame encodes a protein with a predicted molecular mass of 97,010 Daltons and is preceded by a sequence which conforms to the Drosophila translation start consensus sequence ANN (C/A) A (A/C) (A/C) ATGN (46). A canonical polyA addition site AATAAA is located 30 or 21 nucleotides from the start of the polyA tract in the 2.9 and 3.2 kb classes of transcripts, respectively. The P element insert in 79/18 is inserted 51 nucleotides upstream from the 5' end of the cDNA or 29 nucleotides upstream from the beginning of the longest EST sequence GH08049 (accession number AI108791) available in the Berkeley Drosophila Genome Project. Search in databases revealed a high degree of homology between the cDNA nucleotide sequence and sequences encoding glycogen phosphorylases of various organisms.

In situ hybridization of fragment g24 to salivary gland chromosomes and to a Southern blot of wild type genomic DNA (data not shown) indicates that there is only one glycogen phosphorylase gene in *Drosophila*.

Functional analysis of the DGPH gene. To determine the expression and the abundance of the DGPH transcript, fragment g24 was used to probe a developmental Northern blot of wild type polyA⁺ RNA. The probe revealed two major transcripts of 3.2 and 2.9 kb and several minor ones (Fig. 2). The size of the two major transcripts is in accordance with the size of the two cDNAs composed from sequencing data. The DGPH messages are abundant in preblastoderm embryos (0–2 h) but there is a sudden drop in the amount of the 3.2-kb transcript and the 2.9-kb transcript disappears during blastoderm formation and gastrulation

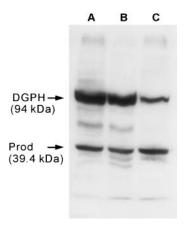


FIG. 4. Western blot of DGPH protein expression in (A) wild type, (B) *P79/18* homozygous and (C) *DGPH*^{Ins31} homozygous L3 larvae. To monitor the amount of protein loaded in the different lanes, the same blot was treated with anti-Prod antibody.

 $(2-4\ h)$. The 3.2-kb transcript also disappears almost completely during germ band extension and at the beginning of germ band shortening $(4-8\ h)$ followed by its progressive increase during the later embryonic stages. The amount of the 3.2 kb transcript remains more-or-less constant all through the larval life but decreases suddenly in the pupal stage. Again, the level of the 3.2 kb transcript increases in the adult stage and becomes abundant, especially in males.

The Drosophila glycogen phosphorylase protein was visualized using anti-DGPH antibody (Fig. 4). As a control for the amount of proteins loaded in the different lanes, anti-Prod antibody (47) was used. The anti-DGPH antibody detected a major protein of 94 kDa and several smaller ones which are most probably degradation products. While the amount of DGPH protein in P79/18 homozygotes seems to be unaffected, it is radically reduced in DGPH^{Ins31} homozygotes. To examine whether this decrease in the level of the protein correlates with the enzyme activity, we measured the glycogen phosphorylase activity both in third instar larvae and in adults in *P79/18* and *DGPH*^{Ins31} homozygotes (Table 1). As expected, enzyme activity is greatly reduced in DGPH^{Ins31} homozygous larvae and adults compared to that of wild type animals (thirteen-fold and six-fold, respectively). The increased difference in the enzyme activity between the larvae and the adults of DGPH^{Ins31} homozygotes can be explained by the fact that the lethal phase of the mutation is in the third larval instar. If we suppose that the low *DGPH* level is responsible for the lethality then those mutants which survive to adulthood are expected to have a higher enzyme level than those dying as larvae, and the overall enzyme activity in a larval sample should be lower than that in the adults. There is no significant reduction in the glycogen phosphorylase enzyme activity of P79/18 homozygotes.

Glycogen content of third instar larvae of both P79/18 and $DGPH^{Ins31}$ homozygotes was also mea-

TABLE 1

Calculated Glycogen Phosphorylase Activities of Wild-Type and DGPH Mutant Adults and Larvae

Stock Oregon-R (wild type)	Stage A	Specific activity $(U/mg) \pm SD$	
		0.402 ± 0.059	$(n = 8)^*$
	L3	0.233 ± 0.027	(n = 3)
P79/18/P79/18	Α	0.374 ± 0.085	(n = 8)
	L3	0.191 ± 0.057	(n = 3)
DGPH ^{Ins31} /+	Α	0.308 ± 0.052	(n = 8)
	L3	0.154 ± 0.009	(n = 3)
DGPH ^{Ins31} /DGPH ^{Ins31}	Α	0.069 ± 0.018	(n = 8)
	L3	0.017 ± 0.004	(n = 3)

Note. Glycogen phosphorylase activity was measured as described under Experimental. $(n)^*$ is the number of independent extractions. In each extract the activities were measured in triplicates and in two different dilutions. A, adults; L3, third-instar larvae.

sured. As expected, $DGPH^{lns31}$ third instar larvae, which are seriously deficient for glycogen phosphorylase enzyme activity, have a higher glycogen content, while the glycogen content of P79/18 homozygotes is the same as that of wild type third instar larvae (Table 2).

Comparison of the DGPH protein with glycogen phosphorylases from other organisms. Alignment of the DGPH conceptual amino acid sequence with that of other known mammalian sequences is displayed in Fig. 5. The active site, the pyridoxal phosphate binding site, the purine nucleoside inhibitor site, the phosphorylation and AMP binding sites and the glucose-6-p binding site are all highly conserved in the *Drosophila* enzyme; they are identical to those of the rabbit muscle glycogen phosphorylase. Changes of amino acids only occur in the glycogen storage site, all at nonconservative positions.

Phylogenetic relationships of the *Drosophila* glycogen phosphorylase to 21 phosphorylases from other organisms is presented in Fig. 6. The phylogenetic tree indicates that *DGPH* is more closely related to mammalian phosphorylases than to phosphorylases from the other species tested.

TABLE 2
Glycogen Content of Wild-Type and DGPH Mutant
Third-Instar Larvae

Stocks	μ g glycogen/mg body wt \pm SD (n)*
Oregon-R	$5.95 \pm 0.41 (11)$
P79/18/P79/18	$3.79 \pm 0.40 (10)$
DGPH ^{Ins31} /+	$4.91 \pm 0.70 (10)$
DGPH ^{Ins31} /DGPH ^{Ins31}	$8.11 \pm 1.20 (13)$

Note. Glycogen content of third-instar larvae was measured as described under Experimental. $(n)^*$ is the number of independent extractions.

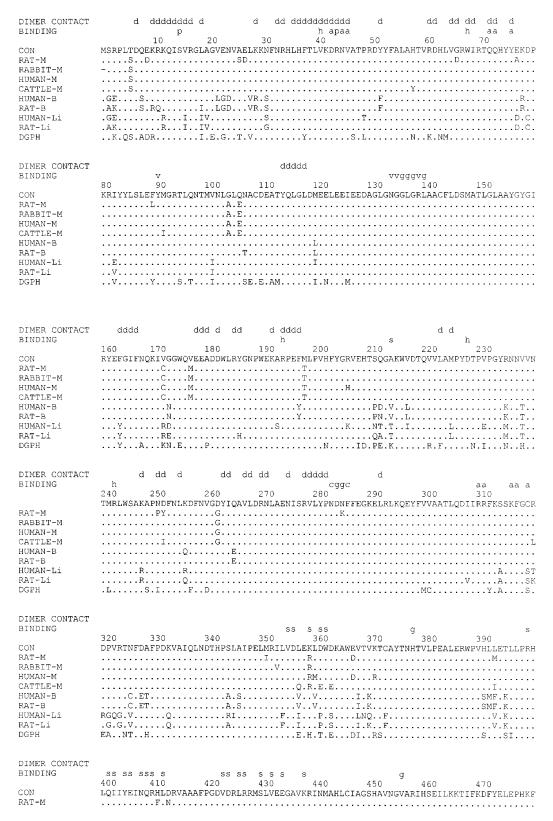


FIG. 5. Multiple alignment of the DGPH protein with the available mammalian glycogen phosphorylases. Alignment was done using the DNA Star software with the identity weight table of the Clustal method. Dot indicates amino acid identity. M stands for muscle, B for brain and Li for liver type glycogen phosphorylases. Residues forming contacts with the other subunit are indicated with d in the Dimer Contact row. Different ligand binding residues are indicated in the Binding row: p Ser-14 phosphorylation site; p glucose-6-P binding site; p active site; p glycogen storage site; and p purine nucleoside inhibitor site. The consensus sequence, indicated as p con, was generated by the DNA Star MegAlign program. Accession numbers of the aligned sequences are the same as in Fig. 6.

```
.....F.N....
PARRIT-M
HUMAN-M
            .....F.N....
            CATTLE-M
HUMAN-B
            E. A. H. L. VI DC. VI V.QSV E. E. E. K. IV.L.K. I EGS. V. K. D.V.TKV. S. D.
RAT-B
HUMAN-Li
            .....K...IV.L.K.I..M...I..EGG....V.C....K..D.V.TQV...S...D..
RAT-Li
DGPH
            .....H..FL.MEN.KKK.D.L..M...M..DGE......V.....A..Q..DSL.H...M..Q..
DIMER CONTACT
            480
BINDING
                   490
                           500
                                   510
                                          520
                                                  530
                                                          540
                                                                  550
            QNKTNGITPRRWLLLCNPGLAELIAERIGEEYISDLDQLRKLLSFVDDEAFIRDVAKVKQENKLKFSAYLEKEYKVKINP
RAT-M
            .....V....V....YL.Q....T...H...
.....V....I.....Y.....A...R...H...
RABBIT-M
           V. I. Y. A. R. H.
VV. VV. DF. A. R. H.

VVM. I. A. Y. S. H.

DT.V.K. FLT. S. K. PL.S. V. F.

I.V. GFLT. S. K. L. Q.

K. D.VK. S. T. H. LG. DV. L. EL. Q. T.

D K. D. VK. S. T. H. G. DI L. EI. Q.

SD. K. D. WPVH. VA. KKWAK. PN. Q. N. R. LA. I. D. G.
HUMAN-M
CATTLE-M
HUMAN-B
RAT-B
HUMAN-Li
RAT-Li
DGPH
DIMER CONTACT
                                                    d
BINDING
                 vvv c gg
                           580
                                   590
                                                  610
            560
                   570
                                           600
                                                          620
            SSMFDVQVKRIHEYKRQLLNCLHVITLYNRIKKDPNKFFVPRTVMIGGKAAPGYHMAKMIIKLITAIGDVVNHDPVVGDR
           RAT-M
RABBIT-M
HUMAN-M
CATTLE-M
HUMAN-B

      C.
      H.
      I.
      T.T.
      V.S.
      V.SVA.
      N.M. SK

      .
      M.
      K.L.
      I.
      SVA.
      N.M. SK

      .
      H.
      M.
      K.
      I.
      V.SVAE.
      N.M. SK

      .
      I.
      TAN.T.
      I.
      YV.Q.
      C.V.N.
      N.I. K

RAT-B
HUMAN-Li
RAT-Li
DGPH
DIMER CONTACT
                  vvv v
BINDING
                                     gggggvv v
                                  670
            640 650 660 670 680 690 700 710 LRVIFLENYRVSLAEKVIPAADLSEQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGEENLFIFGMRVEDVE
           RAT-M
RABBIT-M
HUMAN-M
CATTLE-M
HUMAN-B
RAT-B
            HUMAN-Li
            ......T....D..A
.N.....T...IM....Q...L...M.LD.I...T.DE..
RAT-Li
DGPH
DIMER CONTACT
BINDING
                                  750
                                          760
            ALDQKGYNAQEYYDRLPELRQVIEQISSGFFSPKQPDLFKDIVNMLMHHDRFKVFADYEAYIKCQEKVSALYKNPREWTR
RAT-M
            R...R.....I...I.E.L......V.....E...D..E....
           RABBIT-M
HUMAN-M
           CATTLE-M
HUMAN-B
RAT-B
HUMAN-Li
RAT-Li
DGPH
DIMER CONTACT
BINDING
                          820
                                  830
                                          840
            MVIRNIATSGKFSSDRTIAEYAREIWGVEPSRORLPAPDEKI
            .....L....
RAT-M
RABBIT-M
            HUMAN-M
            CATTLE-M
            .....M.....
HUMAN-B
            K.....DLQI.P.NIPRD
           RAT-B
HUMAN-Li
RAT-Li
DGPH
```

FIG. 5—Continued

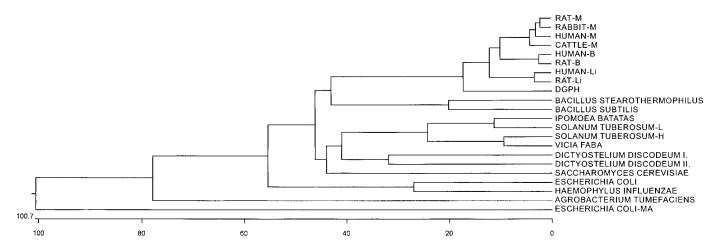


FIG. 6. Phylogeny of glycogen phosphorylases based on their complete amino acid sequences. Multiple alignment of complete protein sequences was done by the Clustal Method with PAM 250 value to obtain the phylogenetic tree using DNAStar software. The abscissa shows the number of amino acid substitution events. M indicates muscle type, Li liver type and B brain type glycogen phosphorylases, MA stands for maltodextrin phosphorylase. Accession numbers of the glycogen phosphorylase sequences are the following: Rat-M P09812; Rabbit-M P00489; Human-M B23093; Cattle-M S82859; Human-B P11216; Rat-B P53534; Human-Li P06737; Rat-Li P09811; *Drosophila* glycogen phosphorylase AF073178, AF073179; *Bacillus stearotermophilus* D87026; *Bacillus subtilis* P39123; *Ipomea batata* L25626; *Solanum tuberosum*-H M69038; *Vicia faba* Z36880; *Dictiostelium discoideum* I X62142; *Dictiostelium discoideum* II P34114; Yeast P06738; *Escherichia coli* P13031; *Escherichia coli*-MA V00304; *Haemophylus influenzae* P45180; *Agrobacterium tumefaciens* AF033856.

DISCUSSION

In this paper, we describe the *Drosophila* glycogen phosphorylase gene and the effect of its decreased activity. The P element insertion in P79/18 did not result in a significant decrease of either the amount of protein or the enzyme activity. The glycogen content of third instar larvae did not increase either. However, $DGPH^{Ins31}$ homozygotes display a marked reduction in both the amount of protein and the enzyme activity and consequently have an increased glycogen content.

The *DGPH* gene has been found to encode a protein of 844 amino acids with a predicted molecular mass of 97,000 Da. This prediction is in correlation with the estimated molecular mass of the purified protein (35) and that of the protein detected by the antiphosphorylase antibody in a crude Drosophila extract (Fig. 4). The DGPH protein shows a high degree of homology to mammalian glycogen phosphorylases. The homology to the rabbit muscle glycogen phosphorylase begins close to the N terminus, at aa 3, and extends nearly to the C terminus, till aa 840. The rabbit and the Drosophila glycogen phosphorylase amino acid sequences can be aligned without insertions or deletions; DGPH is only longer due to the two amino acids added at the C terminus. All the residues comprising the active site, the pyridoxal phosphate binding site, the purine nucleoside inhibitor site, the phosphorylation and AMP binding sites and the glucose-6-P binding site in *DGPH* are identical to those of the rabbit muscle glycogen phosphorylase. Changes of amino acids only occur in the glycogen storage site, all at nonconservative positions. Here, Ala214, Glu406, Gln409, Arg410, and Leu412 are changed to Lys, His, Phe, Leu, and Met, respectively, compared to the rabbit muscle glycogen phosphorylase (Fig. 5). Obviously, these replacements at the glycogen storage site do not affect the kinetics of glycogen utilization at the active site, since the $K_{\rm M}$ glycogen is nearly the same with both the rabbit and Drosophila phosphorylases (35). Residues that form close contact with residues in the other subunit are also highly conserved in the DGPH; 89% of the dimer contact residues are identical to those of the muscle enzyme.

The Ser-14 residue, the site of covalent phosphorylation is well conserved in the *Drosophila* glycogen phosphorylase which reflects that the enzyme is more closely related to the mammalian glycogen phosphorylases than to the enzymes of lower species described before. Except for *DGPH*, Ser-14 has only been conserved in mammalian glycogen phosphorylases (34). The presence of the conserved Ser-14 and its environment is in agreement with the observation that the enzyme can be phosphorylated *in vitro* and *in vivo* (37).

DGPH shows the same high degree of homology both to the rabbit muscle and the human liver glycogen phosphorylases (72.4 and 72.7%, respectively) at the amino acid level. Newgard *et al.* (19) have shown that there is a distinct difference in codon usage pattern between the rabbit muscle and the human liver glycogen phosphorylases in particular, and in liver and muscle coding sequences in general. The difference in codon usage can be ascribed to the third codon position,

where 60% of the nucleotides are either deoxyguanosine or deoxycytidine residues in the liver glycogen phosphorylase compared with 85.8% in the muscle enzyme. The Drosophila glycogen phosphorylase gene displays a codon usage similar to that of the rabbit muscle glycogen phosphorylase gene with 83% G+C at the third codon position. Thus, we can conclude that though DGPH shows the same level of homology to the rabbit muscle and the human liver glycogen phosphorylases at the amino acid level it is phylogenetically more closely related to the rabbit muscle glycogen phosphorylase if we consider codon usage.

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