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Use of two transcription starts in the *G6PD* gene of the bark beetle *Ips typographus*

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Abstract

The enzyme glucose-6-phosphate dehydrogenase (G6PD) of the bark beetle *Ips typographus* is derived from a gene that includes eight exons and spans over 7100 nucleotides (nt). By means of two transcription starts, the gene generates two mRNA isoforms that are present in similar amounts in the larvae, pupae and adults. The A isoform includes exon IA of 115 nt, which is followed by intron 1a extending to position 3457 of the gene. The B mRNA isoform begins with exon IB (100 nt) that occupies positions 3291–3390 within the 1a intron. Exons II to VII are included in both mRNA isoforms. The gene contains 31.6% (36.5% in the translated region) of the GC nucleotides. Two transcription starts and the exon/intron organization distinguish bark beetle *G6PD* from the homologous genes known in other insects. Two enzyme variants were detected in the protein extracts of individual bark beetles but their relationship to the A and B mRNA isoforms is uncertain.

Keywords: G6PD, *Ips typographus*, beetle genomics, transcription start, insect genes.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) is the initiating, rate-limiting enzyme of the pentose phosphate pathway that generates ribose-5-phosphate and NADPH. Most of the core enzyme sequence spanning about 500 amino acids is conserved in all organisms (Mason *et al*., 1995). On the other hand, single amino acid replacements associated with altered electrophoretic behaviour of the enzyme occur relatively frequently at the species level and render G6PD a suitable marker for population studies (Young *et al*., 1964). For example, the major A and B allozymes of *Drosophila melanogaster* G6PD, which differ by having either Leu or Pro in position 382, migrate in native electrophoresis as a dimer or a tetramer and are easily distinguished from one another (Eanes *et al*., 1993).

The eight-toothed spruce bark beetle, *Ips typographus*, is a feared pest that causes devastating outbreaks in the spruce forests throughout most of Eurasia (Stenseth & Kirkendall, 1989). The importance of genotype in pheromone communication (Miller *et al*., 1996) and the reproduction efficiency (Führer & Klipstein, 1980; Cognato *et al*., 1999) in related bark beetle species indicates that the frequency and magnitude of the outbreaks may be affected by the genotype. Allozymes derived from a number of loci, including G6PD, were examined in an attempt to verify the existence of a relationship between beetle performance and the genotype (Stauffer *et al*., 1992; Gruppe, 1997; Pavlíček *et al*., 1997) but this goal was not reached because no unequivocal genotype markers could be identified. Stimulated by investigations performed on other insects, we decided to search for suitable markers at the gene level. In *Drosophila melanogaster* it has been shown that the coding region of the *G6PD* gene harbours ten times more nucleotide mutations than could be recognized by enzyme assays (Eanes *et al*., 1993). Similarly, five alleles differing in the recognition sites of two restrictases were detected in two introns that had been identified in the *G6PD* gene of the Mediterranean fruit fly, *Ceratitis capitata* (He & Haymer, 1999). We chose to analyse the *G6PD* gene in *I. typographus*, taking advantage of the partial knowledge of this gene in several flies and a moth (Fouts *et al*., 1988; Scott *et al*., 1993; Soto-Adames *et al*., 1994). In the course of our research we discovered that the structure of the *G6PD* gene in *I. typographus* is very different from that in *D. melanogaster*, the only insect whose *G6PD* gene had been described in detail.

Results

Isolation of the conserved G6PD *region*

Based on the known *G6PD* genes we expected the total length of the *I. typographus G6PD* transcript to be around 1.7 kb. PCR was chosen to isolate the central conserved

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Table 1. Positions of the forward and reverse primers in the *G6PD* gene (Fig. 2). With the exception of the degenerate primers DF and DR, the sequences of primers can be read from the gene sequence. Primers on the same line were usually used as a pair

Forward		Reverse	
Primer	Nucleotide positions	Primer	Nucleotide positions
DF	5283→5303	DR	5874→5854
FA	37→62	RA	3322→3298
FB	3357→3382	RC	5484→5464
IF1	3728→3751	R0	5406→5382
IF2	4321→4338	RT	5541→5527
InF	513→533	InR	2925→2907
F1	5382→5406	R1	5627→5601
F2	5604→5630	R2	5854→5828
F3	5833→5856	R3	6803→6783
F4	6292→6311	R4	6951→6932

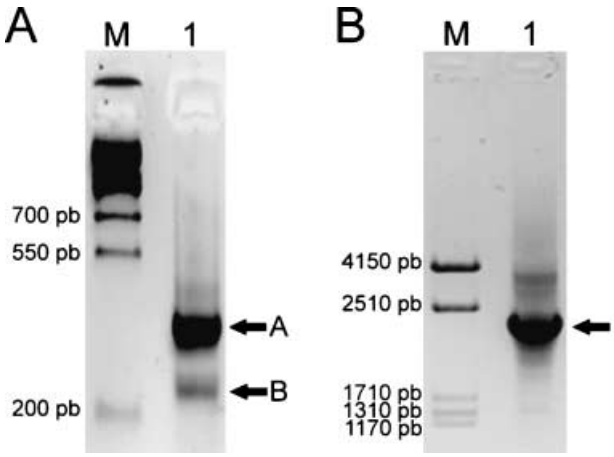

Figure 1. Isolation of the *G6PD* gene fragments in *Ips typographus*. (A) Amplification of two different 5′ cDNA ends with 5′ RACE (M, markers; 1, amplified products A and B (arrows)). (B) Amplification of a gene region spanning exon IB, intron 1b, exon II, intron 2 and part of exon III from the genomic DNA (M, markers; 1, amplified 2.2 kb sequence (arrow)).

part of the gene. The sequences of G6PD proteins from seven animal species, ranging from *D. melanogaster* to man, were aligned and the central regions of full amino acid conservation were used to design the degenerate oligonucleotide primers DF and DR (Table 1). No preferential codon usage was considered. Total RNA from the bark beetle larvae was employed in the synthesis of the first strand cDNA that served as the PCR template. A product of about 500 nt was amplified, gel-purified, cloned into a plasmid vector and sequenced. The result revealed a long ORF that encoded a protein highly homologous to the G6PD of other organisms, particularly insects (Soto-Adames *et al*., 1994).

Identification of complementary cDNA sequences

The missing terminal regions of the cDNA were amplified by RACE PCR with specific primers based on the 500 nt fragment. The 3′ RACE with primers F1 and F2 (Table 1) combined with the oligo(dT) anchor yielded three products of which the largest one complied with the expected cDNA length and was subcloned and sequenced. The result revealed that its 5′ end overlapped with the previously isolated 500 nt *G6PD* fragment. The ORF of this fragment continued through most of the 3′ RACE product and was closed with a stop codon located 129 and 135 nt upstream from a proximal and a distal putative polyadenylation signal, respectively. A stretch of 11 nt separated the second polyadenylation signal from the poly(A) tail attachment.

The 5′ RACE based on the specific primers R0 and R1 (Table 1) in combination with the commercial anchored primer (see Experimental procedures) generated two unequally long DNA fragments (Fig. 1A) that were designated A (360 nt) and B (341 nt). At their 5′ ends, the A form contained a unique sequence of 115 nt and the B form a different sequence of 100 nt. The remaining 3′ regions were identical and overlapped by more than 200 nucleotides with the 500 nt *G6PD* cDNA fragment described above. The 5′ regions of the 5′ RACE products included a translation initiation codon followed by an ORF continuous with that of the 500 nt segment and the sequence amplified with the 3′ RACE. A stretch of thirty-six nt upstream of the initiation codon in the A form and sixty-six nt in the B form probably represented complete or nearly complete leader sequences.

The existence of two cDNAs differing from one another only by a short 5′ region was verified in independent RT-PCR analyses. Primers matching specific sequences beginning from the putative translation initiation codon of the two 5′ RACE products were combined with the R0 reverse primer (Table 1). Sequencing of the amplified products confirmed the existence of two cDNAs with long, and for the most part identical, ORFs.

The genomic sequence of G6PD

The cDNA analysis described above revealed the presence of two *G6PD* transcripts that were identical except for the 115th (A type) or 100th nt (B type) at the 5′ terminus. These two cDNAs could be derived from two independent genes, from two alleles of a single gene and/or from a gene generating two different mRNAs. Genomic DNA was analysed to distinguish between these possibilities. To increase the probability of the disclosure of different alleles or multiple genes, genomic DNA was isolated from three bark beetle populations. The A and B type 5′ ends were specifically amplified with the type-specific forward primers FA and FB in combination with the reverse primer RC (Table 1). A product about 2.2 kb long was obtained (Fig. 1B) with the FB/RC primer pair in all three DNA samples and was analysed in detail in our standard bark beetle stock. As expected, the product included a coding region sequence (thirty-four nt) specific for the B cDNA type. It was followed

by a short intron (sixty-seven nt), short second exon (119 nt), long second intron (1738 nt), and part of a third exon (seventy-seven nt) that contained sequence matching the R0 primer. No product could be amplified from the genomic DNA with the FA/R0 primer combination, possibly because of the very long target sequence (see below).

Complete sequence of the G6PD *gene*

A succession of specific primers was used to identify the whole *G6PD* gene. The presence of three introns was envisaged by analogy with the *G6PD* gene of *D. melanogaster* (Fouts *et al*., 1988). Primers flanking the presumed intron borders were designed for PCR with the genomic DNA. The primer pair FA and RA (Table 1) was used to amplify the region extending from the A type cDNA sequence, which was expected to lie at the gene's 5′-terminus, to the sequence common to both A and B cDNA types and later found to represent exon II. The product of 3285 nt consisted mostly of a long intron whose sequence was verified with the additional primers InF and InR (Table 1). Close to the 3′ end of this A type-specific intron we identified a sequence identical to the B type cDNA. Both the first exon (IB) and the first intron (1b) of the B type lay within the first intron of the A type, while the second exon was shared by both types. The A and B cDNA types were apparently derived from a single gene by the mechanism of alternative transcription start.

The *G6PD* gene of *I. typographus* includes exon IA consisting of 115 nt (seventy-nine of them constitute an ORF) that is used in the A mRNA isoform (Fig. 2). The following intron 1a begins at position 116 with a noncanonical splicing sequence GCAGG and ends at 3457 with a splice donor consensus CGTAG. The sequence spanning nucleotides 3291–3390 within intron 1a is used as the first exon (IB) of the B mRNA isoform; in this case, the first 3290 nucleotides terminated with TTCGC are not transcribed and the first intron 1b delimited by canonical donor sites is only sixty-seven nt long. Splicing of the remaining part of the gene is identical in both mRNA isoforms and all intron/exon boundaries conform to the GT-AG rule. The sequence of the second intron, which extends from nucleotides 3577 to 5314, was verified with primers IF1 and IF2 combined with the IR primer (Table 1). The existence and sequences of additional introns were established with primer pairs F1 and R2 (analysis of intron 3 extending from positions 5680 to 5730), F3 and R3 (intron 4, positions 6110–6162), and F4 and R4 (introns 5 and 6 in positions 6380–6433 and 6640–6693, respectively). The last exon contains a stop codon in position 6949 and terminates at position 7112 where a poly(A) tail is attached in both mRNA isoforms.

Southern blot analysis

Numerous analyses of the two cDNA isoforms from the same bark beetle population invariably revealed identical sequences corresponding to exons II–VII, suggesting strongly that the two cDNAs are derived from a single gene. The number of *G6PD* gene copies was verified by Southern analysis. Several isolates of the genomic DNA were digested with the restrictases *Eco*RI, *Pst*I and *Xba*I, respectively, and probed with the central 500 nt cDNA region (corresponding to exons IV and V). A single hybridizing fragment was

*TATTTTAAAGCCAAAGCAGGAGAGTACTCTCTTACA***ATG**AAATCCGCGTGTGTGATATCGTTTTTACTTATTTTATTTAT 80
TGTTTGGTGTGACGGTGTTGGGAACAACACTAAAGgcagg...3165 nt...ttcgc*TTCATTCCGGTAACACAAAGCA* 3312
*ATAAAGTAACTACTGTTTTCATTCCATTAGTACGTAATACAAAC***ATG**TCTTCTCGTCCATTTTTAGAGTGTACTGATGgt 3392
ata...57 nt...cgtagAAAACTCGGAAGTGTGTCTGGCTTTATACAGAAAGTCTCTAAAAGCTATTGACTTGGATCA 3518
TGAAGGGACCCATTTTGATGGACAGTATCCCCATATATTTGTCACTCTGGGGGCATCGgtaag...1728 nt...tcaa 5312
gGGTGACTTGGCGCGCAAGAAAATCTACCCAACCCTTTGGTGGCTTTACAGAGATAATCTAATACCATCAAATACTCACT 5392
TTATTGGATATGCTAGAAGTAACACGACAATACAAGACATTAAATCCAAATGTGAACCTTACATGAAAGTTAAGAAAGGC 5472
GAAGAGAAGCTTTATGAAGAGTTTTGGAAGTTGAATTATTACATTTCCGGAAAATACGATGTCAGAAGTGATTTTGAAAA 5552
GCTAAACCAAGAAATAAGTCAATATGAGAAGGGACCTATTGCAAACAGGTTGTTTTATCTGGCTTTACCTCCTTCCGTAT 5632
TTGAAGACGTTACAGTTTTGATTAAAAACACTTGTATGGCTGAAAAgtatg...41nt...tttagAGGTTGGACTAGAA 5744
TTATTATTGAAAAACCATTCGGAAGAGATTCCGCTTCGTCTCAAAAATTATCTGACCATTTATCTTCTTTGTTTGACGAG 5824
CATCAATTGTATCGTATTGATCATTATTTGGGGAAAGAAATGGTTCAAAATCTGATGACGCTTAGATTTGGAAATCGAAT 5904
CTTTGGACCAGTGTGGAATCGCGATAATATTGCTTCAATACAAATTACTTTTAAAGAACCATTTGGAACCCAAGGAAGAG 5984
GCGGTTATTTTGACGAATTTGGAATTATAAGGGATATCATGCAGAACCACTTGTTGCAAATTTTGACTTTGGCTGCTATG 6064
GAAAAACCTGCTACGATTCACCCTGATGATATTCGTGATGAAAAAgtaag...43nt...tttagGTAAAAGTTTTAAAA 6177
AGTGTTAAAACTTTGACTCTCAATGATGTAGTACTTGGACAATACGTTGGCAATCCAGAAGGAGAGGGTGAAGCTAAAAT 6257
TGGATATCTAGATGATCCTACAGTGCCCGCAGGTTCTGTTACTCCTACCTATGCTGCTGCGGTACTTAGAATAGATAATG 6337
AAAGGTGGGATGGTGTTCCCTTCATTTTAAAATGTGGCAAAGgtacg...44 nt...ttcagCTTTAAATGAAAGGAAA 6450
GCCGAAGTTCGAATCCAATTTGAAGACGTTCCTGGAGATATATTTGATGGAAAAGCTAAAAGAAACGAACTTGTTATTAG 6530
AGTTCAACCTGGAGAAGCTTTGTATGTTAAGCTTATGGTTAAGACTCCCGGAATGGCTTTTGATATGGAAGAAACTGAAC 6610
TTGACCTAACTTATGGACATCGTTACGAGgtatt...44 nt...tttagAATGTAAAACTTCCCGATGCGTACGAACGT 6723
TTGATTCTTGACGTGTTTTGCGGGTCGCAAATGCATTTTGTAAGGTCTGACGAACTTAGCGAAGCTTGGAGGATATTTAC 6803
GCCTTTGTTGCATGAAATCGAAGGCAACCGCGTCCAACCAATTCCTTACACATTTGGCAGCCGAGGGCCGAAAGAGGCAG 6883
ATGAGTTATTGAAAAAACATAACTTTAATTATACGGGTTCCTACAAGTGGTATAAATCGAACAA***GTA***GGAGAAAAGGTTA 6963
TAGGGCACCAATATGCAATATACACATTCCCTTTATAAAGCATTTTTATATCAGAAGTTGTATTGTTTATTCTGAAATTT 7043
GTTATATGGTTTTGGTTTTTTACTTCCGAACTTATACTTGTTAATTAAATGAAATAAATTATTTTGTTTAAAAAAAAAA 7123
AAAAAAAAA 7133

Figure 2. Exon (capital letters) and intron (lower case letters) sequences of the *G6PD* gene (the full sequence is deposited in GenBank under accession number AY845218). The first exon of the mRNA A isoform is underlined with an undulated line, and that of the B isoform with a straight line. The nontranslated regions are in italics and the initiation and stop codons in bold letters. The putative polyadenylation signals are underlined with dots.

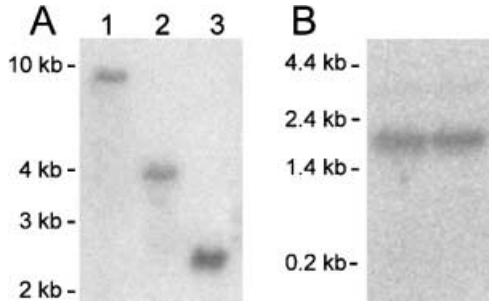

Figure 3. Southern and Northern analysis performed with a probe corresponding to exons IV and V. (A) Hybridization to the genomic DNA digested with *Eco*RI (lane 1), *Pst*I (lane 2), and *Xba*I (lane 3). (B) Northern blot of total RNA from the larvae (left lane) and adults (right lane).

obtained in all cases (Fig. 3A), which is consistent with the conclusion that *I. typographus* contains a single copy of the *G6PD* gene per haploid genome.

Both G6PD *transcripts occur in different developmental stages*

Because the A form cDNA includes, without the poly(A) tail, 1821 nt and the B form 1806 nt, the corresponding mRNAs could not be distinguished from one another on the Northern blots (Fig. 3B) that confirmed transcript presence in the larvae, adults and other developmental stages (data not shown). Developmental changes in the relative abundance of two *G6PD* mRNA isoforms were examined with semi-quantitative RT PCR. The primer sets FA/RC and FB/RC (Table 1) that amplified exons IA and IB, respectively, along with exon II and a part of exon III, were used. Measurements were carried out on individual insects with a random distribution of males and females (sex ratio is about 1 : 1 in *I. typographus*). The reliability of the procedure was checked with the set of primers F1/R1 (Table 1) generating most of the exon III which is present in both mRNA isoforms (data not shown). The results demonstrated the occurrence of both A and B isoforms in the larvae, pupae and adults of different ages (Fig. 4A). The expression of the shorter B isoform was consistently somewhat higher than that of the longer one; this difference was most obvious in the pupae. Additional analyses of reproducing males and females confirmed that both mRNA isoforms were expressed in both sexes (data not shown).

Deduced G6PD protein

The exons 1A and 1B each included an ATG initiator codon followed by a long ORF. Two cDNAs distinguished by these exons encoded two distinct enzyme isoforms A and B that shared 515 out of 540 (A) or 526 (B) residues and differ only in the N-terminal region (Fig. 5). Their predicted molecular weights are 61 970 and 60 495 Da, and the isoelectric points are 6.536 and 6.305, respectively. Consistent with

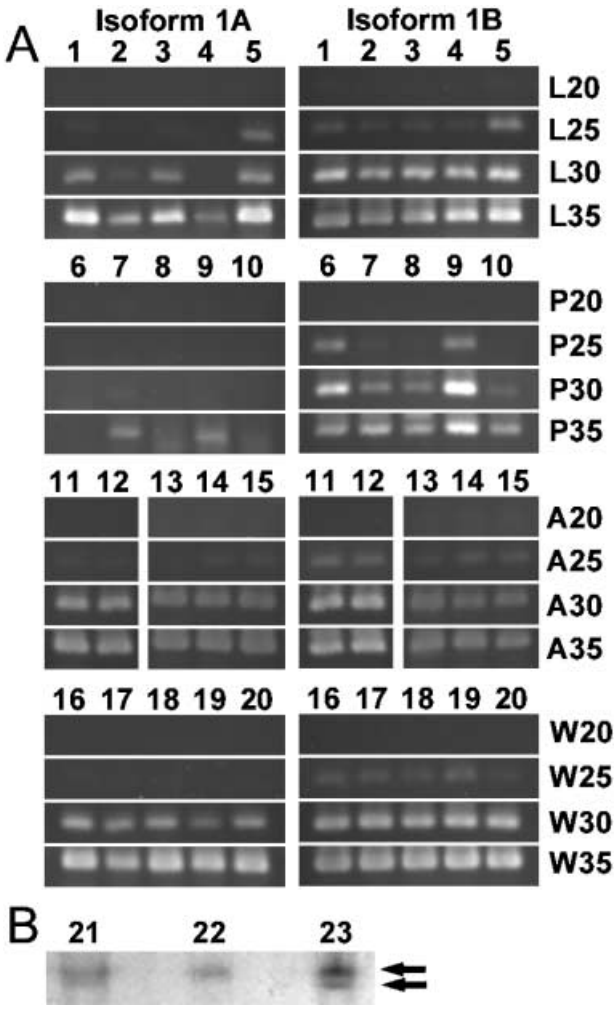

Figure 4. Semi-quantitative assessment of two *G6PD* mRNA isoforms and detection of two G6PD allozymes in individual insects (specified by lane numbers). (A) RT PCR amplification of sequences matching mRNA isoforms A and B, respectively, from the total RNA isolated from larvae (L), pupae (P), newly emerged adults (A, samples 11 and 12), reproducing one-week-old adults (A, samples 13–15), and old adults at the end of winter (W). The first strand cDNA was amplified with specific primers in twenty, twenty-five, thirty and thirty-five PCR cycles, respectively. (B) G6PD activity (arrows) in electrophoretically separated protein extracts from individuals labelled 21–23 (data taken from Kučerová, 2003).

the existence of two putative G6PD isoforms, two distinct enzyme forms were detected on the zymogram based on protein extracts from individual beetles taken from our standard stock culture (Kučerová, 2003).

Discussion

Structure and splicing of the G6PD *gene*

Our results demonstrate that the genome of the bark beetle *I. typographus* contains a single *G6PD* gene with eight exons (Fig. 6). Transcription begins either from exon IA or from exon IB that is situated in a long intron between the exons

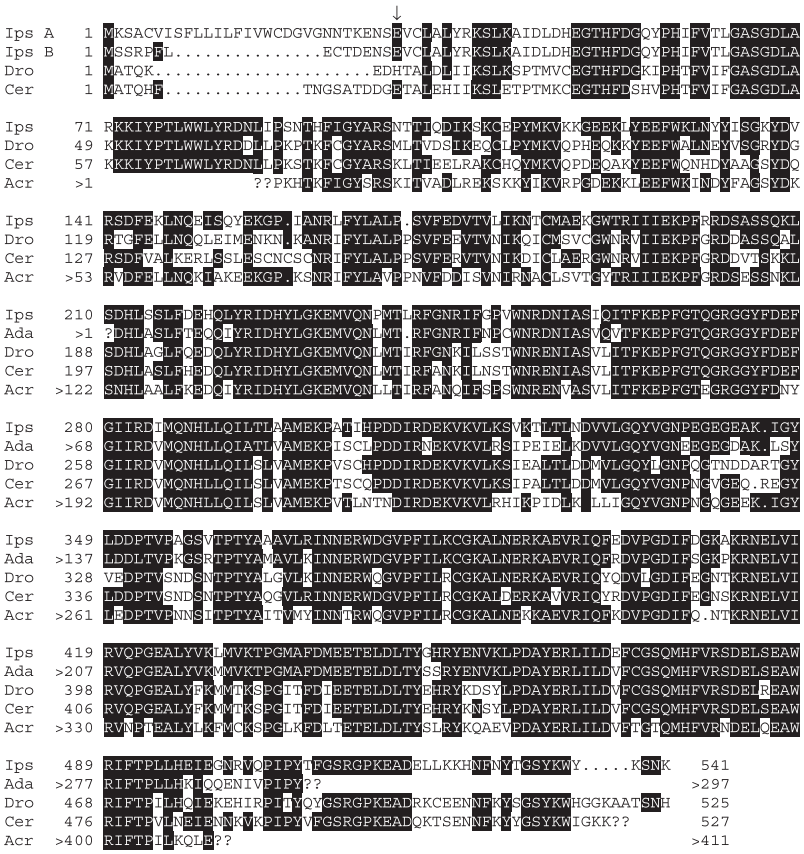

Figure 5. G6PD amino acid sequences deduced from available cDNAs of *Ips typographus* (Ips), ladybird beetle *Adalia decempunctata* strain DS8 (Ada; Jiggins, 2003b), *Drosophila melanogaster* (Dro; Fouts *et al*., 1988), Mediterranean fruit fly, *Ceratitis capitata* (Cer, Scott *et al*., 1993), and butterfly *Acraea encedon* (Acr; Jiggins, 2003a). The sequences Ips A and Ips B are identical from the position marked by the arrow (residue 27 in the A isoform). Residues present in the bark beetle sequence and conserved in at least one other species are printed in white on the black background.

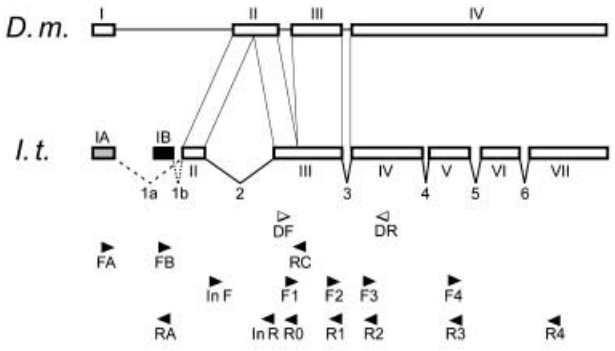

Figure 6. Comparison of *G6PD* gene splicing in *Drosophila melanogaster* (*D. m.*) and *Ips typographus* (*I. t.*) and the positions of the primers used to characterize the *I. t.* gene. The A and B mRNA isoforms of *I. typographus* are identical except for the first exons IA (grey) and IB (black), respectively. The exons IA, IB and II to VII include 115, 100, 119, 365, 379, 217, 206 and 258 nt, and the introns 1a, 1b and 2 to 6 include 3341, 67, 1792, 51, 53, 55–56 and 54–56 nt, respectively. Exact primer positions are given in Table 1.

IA and II. The use of two transcription starts leads to mRNA isoforms A and B with unique 5′ termini of 115 and 110 nt, respectively, and an identical remaining sequence of 1705 nt that is derived from exons II to VII. Among the insects, a full *G6PD* gene sequence is known in *D. melanogaster*. The gene of this fly harbours four exons (Fouts *et al*., 1988), the first of which is of similar length but apparently nonhomologous to exon IB of *I. typographus* (Fig. 6). Bark beetle sequence homologous to the second exon of *D. melanogaster* is broken by a long intron into the short exon II and a part of exon III. The rest of exon III is homologous to the third exon of the fly; the intron separating the second and third *Drosophila* exons is absent in the bark beetle. The coding sequence present in the last, fourth, exon of the *Drosophila* gene is split into exons IV–VII in *I. typographus.* Partial information available on the *G6PD* genes of other insects indicates a similar diversity in intron insertions. The sequence covered by the second exon in *D. melanogaster* is split into two exons in the Mediterranean fruit fly (He & Haymer, 1999) (organization of the remainder of the gene is not known). Three introns were found in the partial *G6PD* gene sequence of the ladybird beetle *Adalia decempunctata* (Jiggins, 2003b). The first lies within the region homologous to the bark beetle exon IV, indicating that *A. decempunctata* may have a similar number of exons as the bark beetle. However, the exon/intron boundaries identified so far in *A. decempunctata* are in different positions than in the bark beetle.

The presence of two transcription starts in the bark beetle *G6PD* gene is the major difference from its homologue in *D. melanogaster*. The existence of two transcription starts

may be linked to differential promoter usage and to separate transcription control indicated by differences in the abundance of the two transcripts, especially in the pupal stage. However, no obvious regulatory motifs were detected in the long intron 1a.

'Housekeeping genes' such as *G6PD* typically contain a high amount of GC pairs (Persico *et al*., 1986) but the *G6PD* gene of *I. typographus* contains only 31.6% GC (36.5% in the coding region).

Evolutionary diversification of G6PD

Alignment of the bark beetle G6PD amino acid sequence with that of other insects (Fig. 5) demonstrated variability of the N-terminal region encoded by the first exon (exons IA and IB in *I. typographus*) and the very beginning of the second exon. Most of the following sequence is highly conserved. The nine-residue sequence RIDHYLGKE in positions 236–244 is preserved in the G6PD of all organisms (Rowland *et al*., 1994). Certain amino acid variability occurs in all species in virtually identical positions, suggesting that it pertains to residues not essential for the function of G6PD. In many other cases, the variation consists of a replacement of residues with similar properties: for example, Val is replaced with Ale or Ile. The bark beetle enzyme contains a different residue in eight positions that are conserved in the G6PDs known from other insects. As expected, the bark beetle sequence is closer to the G6PD of the ladybird beetle *A. decempunctata* (Jiggins, 2003b) than to those of flies (Fouts *et al*., 1988; Scott *et al*., 1993) or the butterfly *Acraea encedon* (Jiggins, 2003a). Two diversified regions of seven to eight residues are present in the C-terminus of all insect sequences identified so far.

G6PD *as a marker in the population genetic studies of* Ips typographus

Cane *et al*. (1990) and Pavlíček *et al*. (1997) employed detection of G6PD activity in protein extracts separated by electrophoresis in the starch and polyacrylamide gels, respectively. The zymograms of Cane *et al*. (1990) disclosed one and those of Pavlíček *et al*. (1997) revealed up to three enzyme variants. Two variants were found in preliminary investigations performed by Kučerová (2003). It is possible that the bands shown in Fig. 4B correspond to the A and B peptide isoforms (Fig. 5) deduced from the DNA analyses. Differences in their isoelectric points (6.536 and 6.305, respectively) may render the isoforms distinguishable in native electrophoresis but the possibility cannot be excluded that the detected enzymes differ in post-translational modifications, which have been reported to occur in mammals (Biagiotti *et al*., 2000). Predictions performed by the NetPhos server (Technical University of Denmark, accessible through the ExPASy databank, http://www.expasy.ch) indicated a slight chance of glycosylation of Ser in position 212 (Fig. 5) and revealed a number of potential phosphorylation sites (data not shown).

DNA markers have only occasionally been applied to the population genetics of *I. typographus*. Stauffer *et al*. (1999) screened several bark beetle populations for variability in the *cytochromoxidase II* region of mitochondrial DNA and Salle *et al*. (2003) employed an analysis of microsatellites. Identification of the *G6PD* gene provides a new DNA tool for the studies in *I. typographus* and other beetles. We found eighteen polymorphic sites by sequencing four regions (each *c*. 500 nucleotides long) of the *G6PD* gene in two beetles from our standard stock, two from a local and two from a Swedish population of *I. typographus* (E. Doleželova, unpublished data).

Experimental procedures

Experimental insects

Most of the analysed individuals of the eight-toothed spruce bark beetle, *Ips typographus* (Scolytidae), came from a temporary laboratory culture established from a stock collected in the Šumava Mountains in southern Bohemia. The culture was maintained for three generations under long day conditions (18 L : 6 D) at 20 °C. A few analyses were carried out on beetles collected in the vicinity of České Budějovice and kept in culture for just two generations, and on insects from a long-term culture (118 generations) based on beetles caught in the Skyttorp region of Sweden.

Analysis and quantification of the G6PD *cDNAs*

Total RNA was extracted from the whole insects with a RNA-Clean kit (AGS, Heidelberg, Germany). The poly(A)+ RNA was subsequently isolated by batch-wise binding to poly(U)– Sepharose (Pharmacia, Fairfield, CT, USA) and used for the cDNA preparation with the aid of the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT) primer. A series of PCR reactions on the cDNA template was performed with primers specified in Table 1 and positioned as shown in Fig. 6.

The central, highly conserved gene region was amplified with the degenerate forward and reverse primers 5′-GCCAARAA-GAARATCTAYCC-3′ and 5′-TTYTGNACCATYTCYTTNCC-3′ (DF and DR, respectively) based on the *G6PD* sequences of humans (GenBank accession number HSG6PDGEN), hamsters (AF044676), kangaroos (MRU13899), a fish (FRG6PD), *Caenorhabditis elegans* (CEB0035), *Drosophila melanogaster* (DROG6PD02), and the Mediterranean fruit fly (S67872). The established central sequence of *I. typographus G6PD* cDNA allowed us to design the specific primers R0 and R1 for the 5′ RACE and primers F1 and F2 for the 3′ RACE. Standard protocols and anchored primers supplied with the Gibco BRL (Paisley, UK) RACE kits were used; the 'touch down' method was applied to amplify the 3′ end. RT PCR with the primer pairs FA/R0 and FB/R0, respectively, was used to verify the existence of two cDNAs differing in the 5′ region. Total RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA, USA) and the first strand cDNA was generated with Superscript II RNase H reverse transcriptase (Invitrogen). An identical procedure was used in the semiquantitative RT PCR based on RNA samples from individual insects. The aliquots of 0.5 μg total RNA were used for reverse transcription with the RT primer in 20 μl reaction mixtures and 1 μl aliquots of the first strand cDNA reaction mix were taken for the two PCR reactions run under identical conditions. The

cDNA isoforms amplified after twenty, twenty-five, thirty and thirty-five cycles, respectively, were visualized on gels.

Cloning and sequencing of the PCR products

PCR fragments were subcloned into the pGemTeasy vector (Promega, Madison, WI, USA) amplified in the DH5α strain of *Escherichia coli*. Nucleotide sequencing was performed with the Applied Biosystems (Foster City, CA, USA) automated DNA sequencer and BigDye Dideoxy Terminator cycle sequencing kits according to the manufacturer's instructions.

Northern blot analysis

Total RNA was isolated with the RNA Blue kit from Top-Bio (Praha, Czech Republic). RNA aliquots (5 μg) were separated by formaldehyde-agarose gel electrophoresis and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The membranes were stained with 1% methylene blue to verify the integrity of the rRNA bands. A hybridization probe covering the fourth and fifth exons was prepared by random priming of the gel-purified cDNA inserts with [α-^{32}P]dATP (Multiprime DNA Labelling System, Amersham Pharmacia Biotech). Hybridization at 65 °C and the washing conditions were as recommended by the membrane's manufacturer.

Genomic DNA analysis

Genomic DNA for both PCR and Southern analysis was isolated using the phenol-chloroform method (Sambrook *et al*., 1989). The PCR procedures and cloning of the products were similar to those used for the RT PCR. For the Southern analysis, 12 μg DNA was digested with the restriction enzymes *Eco*RI, *Pst*I and *Xba*I, respectively, blotted on a Hybond-N+ membrane (Amersham Pharmacia Biotech) and hybridized with a 0.5 kb cDNA probe covering the fourth and fifth exons (Fig. 2). The probe was prepared by random priming of gel-purified DNA inserts with [α-^{32}P]dATP (Multiprime DNA Labelling System, Amersham Pharmacia Biotech). Hybridization was carried out at 55 °C.

Protein analysis

Proteins were extracted from the abdomens of individual beetles and used for standard analysis of electrophoretic G6PD polymorphism according to Manchenko (1994).

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