Functional regulation of glutamine:fructose-6-phosphate aminotransferase 1 (GFAT1) of Drosophila melanogaster in a UDP-\(N\)-acetylglucosamine and cAMP-dependent manner

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INTRODUCTION

Glutamine:fructose-6-phosphate aminotransferase (GFAT; EC 2.6.1.16) catalyses the rate-limiting step of the UDP-\(N\)-acetylglucosamine (UDPNG) synthesis pathway, leading to various N-glycosylation reactions. In the past GFAT from different organisms has attracted attention because of its involvement in cellular growth and specific developmentally regulated processes. In yeasts like Saccharomyces cerevisiae and Candida albicans, GFAT activity is increased during budding due to extensive chitin synthesis [1,2]. Binding of the z-factor in a mating-type cell of S. cerevisiae induces GFAT expression prior to cell fusion [1]. The GFAT-mediated amino sugar pathway has been shown to be involved in the desensitization of adipocytes to insulin by glucose in non-insulin-dependent diabetes mellitus [3]. GFAT activity controls the expression of transforming growth factor-\(\beta\)1 in porcine glomerular mesangial cells [4].

In insects GFAT is involved in the synthesis of chitin, a homopolymer of \(N\)-acetylglucosamine. The synthesis of chitin to form a new cuticle precedes the different molts during development. Moultng itself is regulated by high-titre pulses of the moulting hormone 20-hydroxyecdysone [5]. Additionally, in Drosophila GFAT activity is stimulated for the synthesis of highly glycosylated salivary-gland glue proteins in late third-instar larvae [6]. The induction of glue-protein synthesis is a response to low-titre pulses of 20-hydroxyecdysone in salivary glands, while its termination is correlated with the high-titre pulse of 20-hydroxyecdysone at the end of the third larval instar stage [7]. In Drosophila virilis it has been demonstrated that the enzymic activity of GFAT in late third larval instar salivary glands is tightly coupled to the induction and termination of the synthesis of functional glue proteins [6] and we assume that the same holds true for Drosophila melanogaster. Thus, one can speculate that the expression of salivary gland glue (Sgs) genes and the GFAT enzymic activity may transcriptionally be regulated in salivary glands in a similar manner during late third larval instar development. To elucidate the mechanisms involved a knowledge of the molecular biology of the considered genes is necessary. The Sgs genes of D. melanogaster have been cloned and the regulation of their expression has been characterized extensively (for a review see [7]). Since molecular information on the Drosophila GFAT enzyme (Dmel/Gfat1) and the structure and expression of its gene was unavailable we started a project for the characterization and molecular analysis of GFAT expression in D. melanogaster. This was interesting because it demonstrated that in several organisms, including humans, GFAT activity is regulated by interconversion in connection with feedback inhibition by UDPNG, e.g. in Blastocepillidae emersonii it has been shown that GFAT activity during development is regulated by feedback inhibition by UDPNG [8]. Only the phosphorylated GFAT enzyme is susceptible to this regulation. Thus the activity of GFAT is regulated by protein kinase(s) and phosphatase(s). In B. emersonii protein phosphatases 2A and 2C are involved in this process [9]. The stimulation of rat GFAT by protein kinase A (PKA) has been demonstrated \(\textit{in vivo}\) and \(\textit{in vitro}\) [10]. However, the \(K_i\) value for the feedback inhibitor UDPNG was not altered by PKA treatment of GFAT, suggesting that PKA is not involved in the feedback regulation of GFAT mentioned above. In contrast, phosphorylation by arm of chromosome 3. By whole-mount \(\textit{in situ}\) hybridization specific expression of Dmel/Gfat1 was detected in embryonic chitin-synthesizing tissues and in the corpus cells of salivary glands from late third larval instar. Expressing Dmel/Gfat1 in yeast we showed that Dmel/Gfat1 activity is controlled by UDP-\(N\)-acetylglucosamine and PKA in the yeast total protein extract system. We propose a model for the independent regulation of the Dmel/Gfat1 enzyme by feedback inhibition and PKA.

Key words: amino sugar pathway, enzymic regulation, PKA.

Abbreviations used: 8-Br-cAMP, 8-bromo-cAMP; GFAT, glutamine:fructose-6-phosphate aminotransferase; hGFAT, human GFAT; Dmel/GFat, Drosophila melanogaster GFAT; PKA, protein kinase A; UDPNG, UDP-\(N\)-acetylglucosamine; GST, glutathione S-transferase; DIG, digoxigenin; AP, activator protein; TBP, TATA-box-binding protein.

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The sequences presented here are available from the EMBL Nucleotide Sequence Database under the accession numbers Y18627 and Y18628.
PKA was shown to inhibit the activity of a human GFAT1 (hGFAT1)–glutathione S-transferase (GST) fusion protein [11]. Here we show that during D. melanogaster development GFAT activity is not regulated in the same way as gene expression of structural genes for glycoproteins (e.g. the salivary gland glue proteins, Sgs). Instead, Dmel/GFAT is regulated on the post-translational level. UDPNG inhibits the activity of GFAT, but it can be stimulated by PKA by supplying 8-bromo-cAMP (8-Br-cAMP) to a yeast expression system in order to activate endogenous PKA. The inhibition of GFAT activity by UDPNG can overcome the stimulation by PKA.

**EXPERIMENTAL**

**Strains and libraries**

*D. melanogaster* wild-type strain Oregon S was used for *in situ* hybridization experiments and RNA isolation. Functional expression of *Dmel/GFAT1* was achieved in *S. cerevisiae* strain ENY-MR.17 (his3 ΔI/ his3 ΔI, ura3-52/urA3-52, leu2-3, 112/leu2-3,112, MAL2-8*/ MAL2-8*, MAL3/MAL3, SUC3/ SUC3 [12]). *Escherichia coli* strains DH5α [12], XL-1 Blue (Stratagene), BL23 (Novagen, Madison, WI, U.S.A.) and P2392 (Stratagene) were used in general molecular biology techniques. For DNA isolation a *D. melanogaster* genomic DNA library in the λ Fix II vector (Stratagene), a *D. melanogaster* late third-instar larvae salivary gland cDNA library in the λZAPII vector (Stratagene) and a pNB40 vector-borne cDNA library of 12–24 h *Drosophila* embryos (constructed by Nicolas Brown [13]) were applied.

**DNA cloning**

Standard screening procedures were applied for the isolation of *D. melanogaster* genomic DNA. Lambda phages were plated with *E. coli* strain P2392, and nitrocellulose membranes for plaque hybridization were BA85 from Schleicher & Schuell. Probes were labelled with [γ-32P]dATP, or [γ-32P]dCTP, or commercial sequencing (Replicon Corp., Berlin, Germany, and MWG Biotech. AG, Ebersberg, Germany) using T3 and T7 universal primers, were applied for determination of genomic and cDNA sequences.

**DNA sequencing**

Radioactive cycle sequencing (Amersham Life Science kit no. 71036) with [α-35S]dATP, or [α-35S]dCTP, or commercial sequencing (Replicon Corp., Berlin, Germany, and MWG Biotech. AG, Ebersberg, Germany) using T3 and T7 universal primers, were applied for determination of genomic and cDNA sequences.

**PCR**

For whole-mount *in situ* hybridization probes were labelled with digoxigenin (DIG) by PCR. Primers GFA Tm5 (5'-GCGGAC- CACTATGGCGTACCC-3') and GFA Tm6 (5'-CATGGACA- ATCTCGTGAACTGCC-3') were used to amplify a 400 bp fragment of *Gfat* cDNA using 2.5 units of *Taq* polymerase (Life Technologies) and 5 μl of DIG DNA labelling mix (Roche Biochemicals, catalogue no. 1 277 065) per 50 μl reaction. It should be noted that the PCR DIG labelling mix of the same manufacturer (catalogue no. 1 585 550) was not used due to weak labelling. Standard PCR was performed in a Techne PHC-3 thermocycler. The protocol for 30 cycles was 94°C for 1 min, 62°C for 1 min and 72°C for 1 min. Samples were purified using the GFX™ PCR DNA and gel band purification kit (Amersham Pharmacia Biotech) after checking the results of the PCRs by agarose-gel electrophoresis.

**Site-directed mutagenesis**

Site-directed mutagenesis was performed by PCR using primers GFA Tm3 (5'-GTTGTTAAGCCTCAGTATGTTGGAATAT- TTGATCATC-3') and GFA Tm4 (5'-CGTCCTGACCATT- CACG-3'), sequence added to the genomic DNA of *Gfat* is underlined. PCR reactions were as detailed above except that the annealing temperature was 52°C. Site-directed mutagenesis of the *Dmel/Gfat* cDNA cloned into the pET21a vector (Novagen) was performed using the MORPH™ system kit (Peplab GmbH, Erlangen, Germany) and primer GFA T12-T6 (5'-CGACGTC- CTTTTTATTATCGCACATCGTGAAG-3'). Mutated clones were identified by PRM PCR method [14].

An artificial linker for mutation of the multiple cloning site of the SK + vector was obtained by annealing of the oligonucleotides HNSX-1 (5'-AGCTTACATATGCGTGCACT-3') and XSNH-2 (5'-TGGATGATCGACATATGGTA-3'). Oligonucleotides were 5'-phosphorylated by T4 polynucleotide kinase prior to annealing.

**Functional expression**

For expression in yeast the p425GAL1 vector [15] was used. ENY-MR.17 cells were transformed as described in [16]. Transformed cells were grown to D₅₀₀ = 0.6–0.8 in SSC-Leu medium in 0.1 M NaH₂PO₄/0.05 M Na₂HPO₄/1%, SDS at 65°C. After prehybridization and hybridization using standard protocols individual positive colonies were identified and checked by plasmid mini-preparations and restriction analysis.

Five-Truncated *Dmel/Gfat* cDNAs were isolated from a library of *D. melanogaster* late third-instar larvae salivary gland cDNAs in the λZAPII vector. Phages were plated with XL-1 Blue *E. coli* cells to a density up to 50000 plaques/145 mm-diameter plate. Positive plaques were identified and isolated using standard approaches. cDNA cloned in the SK + bluescript vector was rescued from the lambda DNA by co-transformation of positive λZAPII clones with EXASSIST helper phage according to the manufacturer’s instructions (Stratagene).
(synthetic complete medium with 0.67% yeast nitrogen base and amino acids except leucine) containing 5% glycerol and 0.1% glucose, and were harvested by centrifugation. Cells were resuspended in fresh medium for repression (SSC-Leu containing 2% glucose and 2% fructose) or induction (SSC-Leu containing 4% galactose) and grown for an additional 4 h. For enzymic assays cells were harvested by centrifugation (2000 g for 5 min), resuspended in water and centrifuged again. Pellets were ground by vortexing for 2 min with glass beads (0.5 mm diameter; Braun Corp., Melsungen, Germany) and 500 µl of ice-cold lysis buffer 180 (50 mM NaH_2PO_4, pH 6.8/10 mM EDTA/12 mM glucose 6-phosphate/5 mM GSH/1 mM PMSF). Another 500 µl of lysis buffer 180 were added, and the mixture was cleared of cell debris by centrifugation for 10 min at 10500 g. The supernatant (200 µl) was mixed with 440 µl of test buffer 181 (50 mM Tris/HCl, pH 6.8/4 mM EDTA/5 mM GSH), 80 µl of 100 mM fructose 6-phosphate and 80 µl of 100 mM glutamine. Other chemicals (NaF, ATP, MgCl_2 and 8-Br-cAMP) were added at this point to the appropriate concentrations. Blank controls were boiled for 3 min in a water bath, and all samples were incubated for 20 min at 37 °C. The enzymatic reaction was stopped by incubation in a boiling-water bath for 3 min. Aliquots of 200 µl were added to 1 ml of glacial acetic acid. The mixture was incubated for 10 min at 20000 g to obtain the supernatant.

Glucosamine 6-phosphate was assayed in a modified Morgan-Elson procedure: 400 µl of the supernatant were incubated with 66.4 µl of fresh 0.15% acetic acid anhydride in water-free acetone and 333 µl of 0.7 M K_2B_4O_7, pH 9.1, for 3 min at room temperature. The remaining acetic acid anhydride was destroyed by incubation in a boiling-water bath for 3 min followed by chilling on ice for 3 min. Aliquots of 200 µl were added to 1 ml of p-dimethylaminobenzaldehyde solution (1 g in 1.25 ml of 10 M HCl/100 ml of glacial acetic acid). The mixture was incubated after vortexing at 37 °C for exactly 20 min and then chilled on ice for 2 min. Absorption at 585 nm was determined immediately on ice for 2 min. Absorption at 585 nm was determined immediately after trichloroacetic acid precipitation [17] determined by the method described in [18].

**In situ hybridization**

For in situ hybridization on polytene chromosomes DNA probes (0.5–1 µg) were labelled with biotin (BioNick™ Labelling System; Life Technologies) and checked for functionality on a Southern blot. Hybridization and staining on chromosomes and whole-mount hybridization on *Drosophila* third larval instar salivary glands were performed according to [19]. In situ hybridization on *Drosophila* embryos was performed using a modified version of the Tautz and Pfeifle protocol [20]. Fixation of embryos after dechorionization was performed in 1 ml of PEMS 98 (100 mM Pipes/2 mM MgSO_4/1 mM EGTA, pH 6.9), 0.25 ml of 37% formaldehyde and 5 ml of n-heptane by vigorous shaking for 40 min. The water phase was then removed completely, and the heptane was reduced to 1 ml. Methanol (5 %) was added and the embryos were devitellinized by vortexing for 1 min. Embryos were washed twice with 5 ml of methanol and three times with 100% ethanol. Embryos were equilibrated to PBT (0.1% Triton X-100/0.13 M NaCl/7 mM Na_2HPO_4/3 mM NaH_2PO_4) [20] by a 30 min incubation in ethanol/PBT (1:1). The following washes were performed as described in [20]. An aliquot of embryos was separated at this step for pre-absorption with anti-DIG-Fab fragments (Roche Biochemicals) to suppress unspecific binding on embryos. Prehybridization was performed overnight at 45 °C, and embryos were hybridized overnight in 100 µl of fresh hybridization solution and heat-denatured probe [20]. Washing, antibody incubation and staining were performed as described in [20]. Embryos were dehydrated in an ethanol series and then rehydrated in water/glycerol. Prior to mounting embryos were placed in 87% glycerol. Photographs were taken through a Zeiss Axiophot microscope.

**Northern blotting**

Preparation of total RNA from *D. melanogaster* was performed using Trizol (Life Technologies). Between 10 and 200 insects, depending on age, were rinsed in fresh, ice-cold *Drosophila* Ringer’s solution, the solution was minimized, and the flies were ground by 30 strokes of a Teflon pestle in an Eppendorf reaction vial (1.5 ml). Trizol reagent (1 ml) was added, vortexed and incubated at room temperature (≈ 23 °C) for 5 min. After adding 200 µl of chloroform, the solution was vortexed for 15 s, incubated for 3 min at room temperature and then centrifuged at 4 °C and 12000 g. RNA from the supernatant was precipitated with 500 µl of propan-2-ol for 10 min at room temperature, and RNA was pelleted by centrifugation at 12000 g and 4 °C. Total RNA was washed twice with 80% ethanol, dried briefly and resuspended in 100 µl of 0.1% diethyl pyrocarbonate in double-distilled water. The quality of the RNA was checked by TAE (40 mM Tris/acetate/2 mM EDTA, pH 7.9) agarose-gel electrophoresis, and the total yield was determined spectrophotometrically. Equal amounts of total RNA from different stages were applied to standard RNA gel electrophoresis and Northern blotting. A complete *Dmel/Gfat1* cDNA was radioactively labelled with ^32P by random priming, and was used for standard hybridization and detection.

**Computing**

DNA and protein sequences were analysed using programs of the GCG Wisconsin sequence-analysis package, SEQED, MAP, BESTFIT, DISTANCES, GROWTREE and PILEUP [21]. Promoter analysis was performed using the analysis programs of [22].

**RESULTS**

Cloning and analysis of *Dmel/Gfat1* genomic DNA and cDNA

Genomic DNA of *Dmel/Gfat1* was cloned by screening a lambda phage library of *Drosophila* genomic DNA using mouse *Gfat1* cDNA as a radioactive screening probe. A BamHI/BamHI fragment of 2.0 kb of mouse *Gfat1* cDNA was isolated and radioactively labelled. Positive phage inserts were subcloned and fragments were sequenced on one or both strands. Sequences similar to the mouse *Gfat1* coding sequence were identified by comparison of deduced amino acid sequences from the genomic DNA. Fragments containing these sequences were used to screen a late third larval instar salivary gland cDNA library of *D. melanogaster*. Several positive clones were isolated and sequenced. However, all of them were lacking the 5’ end of the *Dmel/Gfat1* cDNA. Thus a library of 12–24 h *D. melanogaster* embryonic cDNA was screened using the previously isolated C-terminal cDNA fragments as screening probes. Full-length cDNA clones V.1, V.2, V.3 and V.4 were isolated from this library. Clone V.1 was sequenced completely on both strands. By comparison with the genomic DNA the exon/intron structure of the gene was determined (Figure 1). The *Dmel/Gfat1* gene spans approx. 8 kb including eight introns varying between 47 bp and 2.5 kb in size. The first intron was not sequenced completely, thus approx. 1.5 kb are missing in the presented sequence. From the cloned cDNAs a single transcription start site was deduced (genomic DNA position 1471). The nucleotides surrounding this
Figure 1  Schematic presentation of the Dmel/Gfat1 gene

Genomic DNA is depicted as a horizontal line. Exon sequences are given as boxes. Open boxes show untranslated sequences, filled boxes represent translated ones. Exons are consecutively termed A, B, C etc., introns are labelled with italic numbers. Lines with arrows below the sequence show extension of the two lambda phages used for the determination of genomic DNA sequences. Arrowheads 5 and 6 show localization of PCR primers used for DIG-labelled cDNA probe synthesis (hatched boxes).

point (5'-ctcAGTG-3'); the transcription start site is underlined) correspond to consensus sequences of Drosophila transcription initiation [23]. A TATAAA box was identified 40 bp in the 5' direction from the putative transcription start site. This result is in good agreement with the distances observed between TATA boxes and transcription start sites in Drosophila [23].

Polyadenylation signals [24] TATAAA and AATAAA were found in positions 7154–7159 and 7201–7206 of the genomic DNA, respectively. They are respectively located approx. 80 and 40 nucleotides 5' of the two polyadenylation sites deduced from the cloned cDNAs. The processed Dmel/Gfat1 mRNA has a total length of 2406 or 2413 nucleotides, excluding the poly-A tract, depending on the polyadenylation site. A continuous open reading frame of 2082 nucleotides was determined. The nucleotides in the vicinity of the translation start codon, which is located seven nucleotides from the 3' end of exon B (Figure 1), are consistent with the consensus sequence for Drosophila translation-initiation sites (MAAMATG) [25]. For the localization of the Dmel/Gfat1 gene on Drosophila chromosomes, phage A2.7.1 DNA (Figure 1) was labelled with biotin, and the probe was hybridized in situ on Drosophila larval salivary gland polytene chromosomes. A single signal was detected at position 81F of the right arm of chromosome 3 (results not shown). This is in agreement with the data obtained from the Drosophila genome project [26].

Determination of the Dmel/GFAT1 amino acid sequence and sequence analysis

The deduced Dmel/GFAT1 protein comprises of 694 amino acid residues and has a calculated molecular mass of 78124 Da. The Dmel/GFAT1 protein consists of two different domains. The N-terminus has been proposed to represent the glutamine-binding aminotransferase domain, and the C-terminus was postulated to form the fructose 6-phosphate-binding isomerase domain [27]. Comparison with GFAT sequences from other sources showed some intriguing features. First, a similar sequence encoded by the CG1345 gene was identified by computer search of the D. melanogaster genome at position 93C. The deduced gene product has been annotated as a glutamin:fructose-6-phosphate amido-transferase by the Drosophila genome project [26]. In contrast to the Dmel/Gfat1 gene (Figure 2), the CG1345 gene contains just a single intron. Alignment of the two Drosophila GFATs with the hGFAT1s and the yeast GFA1p showed the D. melanogaster GFATs to be much more similar to the hGFATs than to GFA1p (Figure 2). Whereas hGFAT1 and hGFAT2 can be distinguished by the presence of two (hGFAT1) and one (hGFAT2) postulated PKA phosphorylation sites (Figure 2 [10]), this difference is not shared by Dmel/GFAT1 and Dmel/GFAT2. However, in Dmel/GFAT1 the putative second PCA site has been conserved except for the reactive serine residue (KKLC). In Dmel/GFAT2 no such conservation takes place; its amino acid sequence is totally different compared with hGFAT1 and Dmel/GFAT1 at this particular position. In the phylogram shown in Figure 3 the computer program used assigned the discovered Dmel/GFAT to the group 1 enzymes. Taking this and the conservation of the PKA sites, we termed the newly discovered gene Dmel/Gfat1, and so accordingly the CG1345 gene encodes the putative Dmel/GFAT2.

GFAT enzymes from bacteria to humans share some short sequences that are absolutely conserved at the amino acid level (Figure 4). These conserved peptide stretches, which may be involved in the function of the enzyme, are scattered along the entire sequence. Amino acids that have been shown experimentally to be important for function [28–30] are located within these conserved peptide stretches. Both the glutamine-binding aminotransferase domain and the fructose 6-phosphate-binding isomerase domain [27] stressed above are hinged by a non-conserved peptide stretch which shows differences in length and sequence between species (Figures 2 and 4). Yeast GFAT is elongated by 38 amino acid residues in this region compared with hGFAT1 and Dmel/GFAT1 and sequence between species (Figures 2 and 4). The two interconnecting exons between introns 4 and 6 are extremely short. Exons E and F are 27 and 24 bp in length, respectively (Figure 1).

Computer analysis of the Dmel/GFAT1 promoter

Several hundred bp of DNA sequences upstream (5') of the putative transcription start site of the Gfat gene were analysed in
<table>
<thead>
<tr>
<th>Source</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>hum1</td>
<td>MQEFAYLNYHVTREILS</td>
</tr>
<tr>
<td>hum2</td>
<td>---M---R-----FE---</td>
</tr>
<tr>
<td>me11</td>
<td>---G------T-L-K--</td>
</tr>
<tr>
<td>me12</td>
<td>LT-KS-Q-V-GLQ-</td>
</tr>
<tr>
<td>Yeast</td>
<td>---G-------E-S-G---</td>
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**Figure 2** Alignment of GFAT amino acid sequences from different sources

Amino acids are given in the single letter code. Gaps are introduced where necessary, and are presented by dots. Identical amino acid residues with reference to the hGFAT1 (hum) sequence are symbolized by dashes. Numbers give extension of the respective sequences. Amino acids of the Dmel/GFAT sequences whose positions are hit by the exon/intron splicing of the corresponding pre-mRNA are bold and underlined. Conserved putative PKA phosphorylation sites are indicated by bold italic letters. hum1, hGFAT1 [38]; hum2, hGFAT2 [46]; me11, Dmel/GFAT1 (this work); me12, CG1345 gene product (accession no. AAF56785), putative Dmel/GFAT2; yeast, S. cerevisiae GFAT [1].
silico for consensus transcription-factor binding sequences (Figure 5). In contrast to several of the Sgs genes, no ecdysone-response elements nor a forkhead-binding site were identified. The latter has been shown to be functional in the promoters of Sgs genes [31–33]. In close proximity to the TATAAA element, which also serves as a putative recognition site for binding of the B-factor, the Engrailed protein, TATA-box-binding protein (TBP), transcription factors TFIIB, TFIIFα and TFIIFβ, and three putative GC boxes were identified (Figure 5). In mammals, the Sp1 transcription factor binds to such sites. Two Drosophila homologues of the Sp1 factor have been identified, buttonhead [34] and D-Sp1 [35]. However, their DNA-binding properties have not been characterized so far. Recently, three functional binding sites for the Sp1 protein have been identified within 120 bp of the promoter of the mouse Gfat gene [36]. Also, the consensus binding site for Sp1, GGGCGG, normally occurs only very rarely in Drosophila promoters, as pointed out in [23]. The other factor for which putative binding sites were identified is activator protein (AP)-1 [37]. In approx. 500 bp 5’ to the transcription start site a second consensus sequence for the binding of the B-factor and the TBP was identified (Figure 5). However, it should also be noted that the Dmel/Gfat1 gene is located close to the heterochromatin on chromosome 3; thus effects of heterochromatic gene suppression cannot be excluded at the moment.

Expression of the Dmel/Gfat1 gene

The in vivo expression of the Dmel/Gfat1 gene at the mRNA level was studied by Northern-blot analysis using total Drosophila RNA from whole animals (Figure 6). Two bands were detected:
Regulation of glutamine:fructose-6-phosphate aminotransferase 1

Figure 6 Expression of the Dmel/Gfat1 gene
Northern blot of total RNA from first (L1) and third (L3) larval instars. Arrows point to detection of Dmel/Gfat1 mRNA.

Figure 7 Expression of Dmel/Gfat1 detected by whole-mount in situ hybridization using a DIG-labelled cDNA probe
Expression is visualized by an Nitro Blue Tetrazolium/X-phosphate staining reaction by alkaline phosphatase covalently coupled to anti-DIG-Fab fragments. (A) Expression in embryos of stages (a) 13, (b) 16 and (c) 17. Arrowheads point to areas of early or strong expression. All images were taken from a single staining experiment causing a dark stain in strongly expressing tissues. (B) Expression in the salivary gland of late third larval instar.

A prominent band of approx. 2.5 kb, the size of which corresponds to the size of the isolated cDNAs of 2400 bp plus the attached poly-A tail. The second band was approx. 7–8 kb in size. Putatively, this band represents the unspliced primary transcript of the Dmel/Gfat1 gene. A pre-mRNA of similar size can be deduced from the positions of the transcriptional start and termination sequences on the chromosomal DNA of the Dmel/Gfat1 gene. It should be noted that the ratios of the amounts of the putative unspliced and spliced Dmel/Gfat1 mRNAs vary between different stages of Drosophila development (Figure 6).

For detection of tissue-specific expression of the Gfat gene whole-mount in situ hybridizations were performed on embryos of different stages and on late third-instar larval salivary glands. A probe of 350 bp was labelled with DIG by PCR (Figure 1). The probe was located in a region where the Dmel/Gfat1 and Dmel/Gfat2 sequences show less-conserved nucleotide sequences and can be distinguished by performing high-stringency hybridization experiments. In embryos Dmel/Gfat1 exhibited specific expression during the late phases of development (Figure 7A). Expression begins at stage 16 dorsally at the developing tracheae, and extends to stage 17 to the whole cuticule-forming tissue. Additionally, the Dmel/Gfat1 gene is expressed along the oesophagus where synthesis of the chitinous masticatory organs takes place. In late third larval instar salivary glands Dmel/Gfat1 is expressed exclusively in the corpus cells (Figure 7B). This corresponds to the synthesis of the highly glycosylated Sgs proteins in the corpus cells, which are the prominent targets of N-glycosylation reactions at this stage of development. We conclude from these results that Dmel/GFAT1 is involved in chitin synthesis and glycosylation reactions as well. It can be speculated that a mechanism of regulation similar to mammalian GFATs for the glycosylation can be expected, but a different type of regulation is proposed for the GFAT activity involved in the synthesis of chitin.

Regulation of the enzymic activity of the Dmel/GFAT1 protein
Since the different organization of the Dmel/Gfat1 and Sgs gene promoters excludes common transcriptional control, we investigated the role of post-translational and allosteric modifications of the Dmel/GFAT1 protein in the regulation of the enzymic activity. In order to determine the enzymic function of the cloned Dmel/GFAT1, the cDNA was cloned into the p425GAL1 expression vector. This construct (p425GFAT) was used to transform ENY.MR-17 yeast cells. The expression vector p425GAL1 without a GFAT insert was used to transform ENY.MR-17 cells as a control. Positive transformants were checked by re-isolation of the transformed plasmid, transformation of DH5α E. coli cells, preparation of plasmids and restriction analysis.
Enzymic activities were determined in cell-free extracts of the transformed yeast cells in repressed (glucose) and induced (galactose) conditions. In contrast to the hGFAT1 enzyme that was reported to be most active at pH 7.5 when expressed in E. coli [38], we selected pH 6.8 for enzymic assays of the Dmel/GFAT1 in accordance with [39]. Glutathione was applied as a reducing agent in extraction buffer 180 as well as in reaction buffer 181, as determined for D. viridis GFAT [39]. Dmel/GFAT1-transformed cells displayed up to 5-fold increased activities as compared with cells transformed with the p425GAL1 vector alone (Figure 8a). However, the relatively high internal GFAT activities of the transformed yeast cells in repressing medium (4% glucose; Figure 8a, compare repressed 1 and induced 1) depended in part on a faster growth rate compared with the induced cells growing on 4% galactose (results not shown). Thus faster chitin synthesis in glucose medium was achieved by a higher synthesis rate of amino sugars involving an elevation of internal yeast GFA1p activity. In general, the transformed control cells (p425GAL1) showed only minor activities compared with the Dmel/GFAT1-transformed cells (Figure 8, compare repressed 1 and induced 1 in all panels).

As expected, transformed Dmel/GFAT1 activities were induced 2.5-fold upon induction by galactose (Figure 8a). The measured Dmel/GFAT1 activities were susceptible to feedback inhibition by the addition of UDPNG to the reaction (Figure 8b). Stimulation of the yeast PKA by the addition of 8-Br-cAMP, ATP, NaF and MgCl₂ activated Dmel/GFAT1 1.7-fold compared with the induced and non-stimulated enzyme (Figures 8a and 8d). This stimulation is even greater if the portion of the internal yeast control is subtracted (Figures 8a and 8d, induced 2 minus repressed 2). Incubation with MgCl₂, ATP and NaF without 8-Br-cAMP was performed as a control for unstimulated but optimized internal yeast PKA activity (Figures 8c and 8e). NaF was added as an unspecific phosphatase inhibitor. We concluded that yeast PKA is active in cell extracts at a lower level (Figure 8a), and can be optimized by appropriate supplements (Figure 8c) and induced additionally by external 8-Br-cAMP (Figure 8d). The activity of Dmel/GFAT1 was down-regulated by UDPNG independently of the stimulation by PKA via cAMP (Figure 8d). The Attach map of Dmel/GFAT1 was down-regulated by UDPNG independently of the stimulation by PKA via cAMP (Figure 8d). The activity of Dmel/GFAT1 by 8-Br-cAMP (Figure 8f). The suppression is not affected by supplements of NaF, MgCl₂, and ATP, since in the presence of these kinase activators and unspecific phosphatase inhibitors the suppression of Dmel/GFAT1 activity by UDPNG is similar with or without addition of 8-Br-cAMP (Figures 8a, 8e and 8f). We conclude that suppression by UDPNG can overcome the Dmel/GFAT1 activation by PKA.
DISCUSSION

The GFAT enzyme catalyses the first and rate-limiting step of the amino sugar synthesis pathway. In insects the control of its expression is the key step for the synthesis of UDPNG, which is needed for cuticle synthesis in the cuticle prior to the various molts during development. Additionally, in D. melanogaster GFAT is involved in the synthesis of highly glycosylated proteins, like the salivary gland glue proteins (Sgs), at the end of larval life. Thus, in late third-instar salivary glands when the enzymatic activity of GFAT is tightly coupled to the synthesis of glycosylated glue proteins we expected GFAT activity to be regulated in a similar manner to that of the Sgs genes. However, our analysis of the cloned Dmel/Gfat1 gene revealed other facets of a complex expression-regulation pattern.

Expression of the Dmel/Gfat1 gene

In contrast to the Sgs genes which are transcribed from puffed DNA loci of late third-instar polytene chromosomes [32] the Dmel/Gfat1 gene is located in chromosome subdivision 8F. The region is localized close to the centromeric heterochromatin of chromosome 3 and does not show a puff at all during late third larval instar development. In late third larval instar salivary glands the Dmel/Gfat1 gene is expressed exclusively in the corpus cells of the salivary glands that synthesize the functional glue proteins at this stage of development, but it is not detected in the colurn cells. This result points to a tight regulation of Dmel/GFAT1 enzymic activity linked to glycoprotein synthesis, which is distinct from other regulation pathways leading to cutin synthesis in insects. Several of the promoter elements of the Sgs genes mediating tissue and developmental specific expression have been characterized (for a review see [7]). None of the elements responsible for proper Sgs gene expression have been found in the Dmel/Gfat1 promoter by computer analysis. Instead, putative GC boxes have been identified. In mammals, the ubiquitous transcription factor Sp1, which is involved in the expression of many genes [40], binds to such boxes. The DNA-binding properties of the Drosophila homologues buttonhead and D-Sp1 have not been characterized yet, and a consensus binding sequence has not been determined. However, the detection of multiple putative binding sites within the Dmel/Gfat1 promoter correlates with the functional characterization of Sp1-binding sites in the promoter of the mouse Gfat gene [36]. Additionally, in the mouse promoter putative binding sites for transcription factors of the AP family have been identified although they were not shown to be functional by DNA-shift experiments [36]. Similarly, in the Dmel/Gfat1 promoter putative binding sites for the transcription factor AP-1 of Drosophila were identified (Figure 5). It should be noted that the functionality of a specific binding site for a transcription factor may depend on the proper bending and presentation of the respective DNA sequence by the nucleosome [41]. Therefore, the lack of binding of a transcription factor to its consensus binding sequence shown by DNA-shift experiments does not consequently exclude the functionality of the respective site in vitro. In contrast to the mouse GFAT promoter, a full-match TATAAA box was identified in the Dmel/Gfat1 promoter (Figure 5). In mouse the lack of a consensus TATAA box led to the characterization of Gfat as a ‘housekeeping gene’ [36]. However, the activity of GFAT in different rat tissues varies up to 200-fold depending on the more or less high expression rates of glycoproteins [42]. The tissue and developmental specific expression of Dmel/GFat1 mRNA and the lack of expression during most stages of embryonic development does not characterize Gfat as a typical housekeeping gene. This specific expression of Dmel/Gfat1 was shown in the last stages of embryonic development to occur in tissues involved in the synthesis of cutinin for cuticle formation and for the chitinous parts of the mouth armature of the developing first instar larva (Figure 7A). The expression detected later in development by Northern blotting (Figure 6) correlates with the synthesis of chitin for the new cuticle prior to molting. However, it should be noted that the Northern-blot detection of Dmel/GFAT1 mRNA from a defined larval stage does not discriminate between early or late onset of GFAT expression during larval development. Thus a more detailed ontogenetic study has to be performed to elucidate whether the Gfat gene is regulated constitutively, or whether the expression is under developmental control.

Regulation of GFAT activity by PKA phosphorylation

Until recently, the activation of GFAT by PKA phosphorylation seemed to be a doubtless feature of eukaryotic GFAT enzymes. However, the study by Chang et al. [11] renders this general assumption questionable. Etchebehere and Da Costa Maia [8] showed the activation of B. emersonii GFAT by the addition of exogenous PKA in an extract. However, the effect was not normalized to their standard conditions except the depletion or addition of the PKA. The stimulation of GFAT from rat aortic smooth-muscle cells via in vivo induction of endogenous PKA has also been shown by Zhou et al. [10]. This activation was approx. 2-fold upon stimulation of PKA by the addition of 8-Br-cAMP, forskolin or isobutylmethylxanthine to the cell-culture medium. Two putative phosphorylation sites for PKA located in the less-conserved hinge region were postulated for hGFAT1 by Zhou et al. [10]. They reported a repression of recombinant hGFAT1 activity by PKA phosphorylation of Ser395, which corresponds to the single putative PKA site conserved in Dmel/GFAT1 (Figure 2). Our results are in contrast to these data; Dmel/GFat1 expressed in yeast was stimulated 1.7-fold by adding 10 μM 8-Br-cAMP under optimal conditions, including supplements of MgCl2 and ATP (Figures 8a and 8d). A comparative discussion is difficult,
because there are major differences in the three systems used for monitoring GFAT activity stimulated by PKA phosphorylation.

(i) All three systems analysed GFAT enzymes from different sources. Zhou et al. [10] assayed rat GFAT. These cell extracts presumably contained a mixture of unknown proportions of GFAT1 and GFAT2. Since hGFAT1 and hGFAT2 differ in the number of PKA phosphorylation sites, similar features of rat GFATs can be anticipated regarding the close to 100% conservation of the human and mouse GFAT enzyme sequences. As long as no separate measurements of the enzymic properties of the GFAT2 with respect to PKA treatment have been reported, the PKA effects on a mixture of both enzymes cannot be estimated unequivocally. On the other hand, Chang et al. [11] used recombinant hGFAT1, although their PKA phosphorylation was performed in vitro in an artificial environment (glutatione affinity beads). The data in the present paper were accumulated from recombinant Dmel/GFAT1, of which the major difference from both the rat and human systems is the lack of a second PKA site (Figure 2). Although GFAT in insects is involved in chitin synthesis, which has no equivalent in the vertebrate metabolism, Dmel/GFAT1 is involved also in the synthesis of highly glycosylated proteins. Its functional regulation implies similar mechanisms to those reported for rat and human glycoprotein synthesis.

(ii) The determination of GFAT enzymic activities performed by the different authors involved different methods. However, the methodological diversities seem not to have caused the contradictory results.

(iii) The main differences in the systems used are related to the mode of PKA action on the GFAT molecules. Zhou et al. [10] applied the most ‘biological’ way, the in vitro method. Both enzymes, PKA as well as GFAT (or mixture of GFATs), originated from the same species. The PKA reaction was performed in intact cells. However, the true phosphorylation status of the GFAT used for further enzymic testing was not shown. Our system is ‘semi-biological’. Although Dmel/GFAT1 synthesis is performed in a living cell where phosphorylation by PKA can occur naturally, the induction of PKA activity is performed in crude cell extracts. Additionally, the PKA and Dmel/GFAT1 used did not originate from the same source. Modified action of yeast PKA on Dmel/GFAT1 may be possible. Again, this may be not important since the single Dmel/GFAT1 PKA phosphorylation site matches the consensus sequence for yeast PKA sites too. On a lower level this semi-biological system also provides activation of internal yeast GFA1p by PKA activation (Figure 8d).

Compared with the former two applications the system of Chang et al. [11] is artificial. First of all, it should be noted that the GST fusion protein was attached directly to the C-terminus of the hGFAT1 protein. By comparison of GFAT proteins from different sources (Figure 4), the C-termini of GFATs from E. coli to humans are composed of 14 absolutely conserved amino acid residues. Thus, we propose a functional relevance for this conserved domain which may be altered completely if the C-terminus is fused to another protein, e.g. GST. This alteration may also change molecular properties located somewhere else in the protein, including its phosphorylation sites. More importantly, hGFAT1–GST fusion proteins are bound to the glutathione beads preferentially as monomers. In that condition they are good substrates for PKA when applied as shown by Chang et al. [11]. However, GFAT seems to be active as a homodimer, which has no equivalent in the vertebrate metabolism, where GFAT is involved in chitin synthesis, which has no equivalent in the vertebrate metabolism. Dmel/GFAT1 is stimulated by PKA via 8-Br-cAMP or not, enzymic activity is suppressed completely by UDPNG (Figures 8e and 8f). The possibility cannot be excluded that the phosphorylation of GFAT by PKA is not involved in feedback inhibition by UDPNG. Whether Dmel/GFAT1 is stimulated by PKA via 8-Br-cAMP or not, enzymic activity is suppressed completely by UDPNG because the degree of PKA phosphorylation of expressed Dmel/GFAT1 prior to yeast PKA induction is not known. Alternatively, the stimulating effects on Dmel/GFAT1 activity by PKA phosphorylation are dominantly suppressed by UDPNG independently of the phosphorylation status by PKA. This is the issue central to our proposed model of eukaryotic GFAT regulation (Figure 9). A second, independent kinase/phosphatase system for the regulation of GFAT1 enzymic activity besides the stimulation upon phosphorylation by PKA seems to be highly probable. Many putative phosphorylation sites for

UDPNG contra cAMP

Whether the deduced PKA site of D. melanogaster GFAT1 is functional remains to be proved at the molecular level. In B. emersonii, two-dimensional PAGE of partially purified GFAT phosphorylated by 32P showed an elongated protein spot, suggesting multiple sites of phosphorylation responsible for the susceptibility to UDPNG inhibition [8]. This would contradict the identification of only a single PKA site in Dmel/GFAT1, or it would lead to speculation that the phosphorylation of GFAT by PKA is not involved in feedback inhibition by UDPNG. Whether Dmel/GFAT1 is stimulated by PKA via 8-Br-cAMP or not, enzymic activity is suppressed completely by UDPNG (Figures 8e and 8f). The possibility cannot be excluded that the phosphorylation of GFAT by PKA is responsible for the reversible susceptibility of GFAT to allosteric feedback inhibition by UDPNG because the degree of PKA phosphorylation of expressed Dmel/GFAT1 prior to yeast PKA induction is not known. Alternatively, the stimulating effects on Dmel/GFAT1 activity by PKA phosphorylation are dominantly suppressed by UDPNG independently of the phosphorylation status by PKA. This is the issue central to our proposed model of eukaryotic GFAT regulation (Figure 9). A second, independent kinase/phosphatase system for the regulation of GFAT1 enzymic activity besides the stimulation upon phosphorylation by PKA seems to be highly probable. Many putative phosphorylation sites for
other protein kinases can be deduced from the GFAT amino acid sequence.

Therefore, we propose the following model of Dmel/GFAT1 activity regulation (Figure 9): Dmel/GFAT1 can be phosphorylated by PKA and, independently, by a second, to date unknown, kinase. Phosphorylation by PKA activates Dmel/GFAT1. Phosphorylation by the second kinase, using a phosphorylation site different from that of PKA, renders Dmel/GFAT1 susceptible to feedback inhibition by the allosteric binding of UDPPNG, abolishing Dmel/GFAT1 activity. The feedback inhibition abolishes Dmel/GFAT1 activity regardless of whether the enzyme is activated by PKA or not. De-phosphorylation by unknown phosphatases makes the system reversible.

Conclusions

Dmel/GFAT1 activity is regulated independently by feedback inhibition by UDPPNG, and by phosphorylation by PKA. The Dmel/GFAT1 gene is transcribed in a tissue- and development-specific manner in embryonic chitin-synthesizing tissues, and in the corpus cells of late third larval instar salivary glands. However, a similar mechanism of expression to the Sgs genes for the synthesis of functional glue proteins in late third larval instar salivary glands is not probable. Thus the co-ordinated synthesis of structural protein moieties and low-molecular-mass substrates for the production of functional glycoproteins in the salivary glands does not depend on similar modes of gene expression in Drosophila.

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