Differential Regulation of the Two Glyceraldehyde-3-Phosphate Dehydrogenase Genes during *Drosophila* Development

XIAO-HONG SUN,[†] J. YUN TSO,[‡] JOHN LIS, AND RAY WU*

Section of Biochemistry, Molecular and Cell Biology, Wing Hall, Cornell University, Ithaca, New York 14853

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Drosophila melanogaster contains two genes encoding glyceraldehyde-3-phosphate dehydrogenase, *Gapdh-1* and *Gapdh-2*. The two genes are highly conserved in their coding sequences but not in their noncoding and flanking sequences. We report that both genes are expressed at higher levels in larval, late pupal, and adult stages than in embryonic, early, and midpupal stages. However, a major difference in the expression of the two genes is observed in the adult stage, during which the level of the *Gapdh-1* transcript decreases over fourfold, while that of the *Gapdh-2* transcript remains at a constant high level. In addition, the *Gapdh-1* transcript appears highly enriched in the thorax section compared with the head and abdomen sections, while the *Gapdh-2* transcript is evenly distributed. Analyses of the expression patterns of the two *Gapdh* hybrid genes, GAP1/2 and GAP2/1, revealed that the two genes have a distinct organization of their regulatory sequences. The principle regulatory sequences of *Gapdh-2* reside upstream of the translation start, while the principle sequences specifying the level and developmental pattern of *Gapdh-1* expression reside downstream of the translation start.

Gene duplication in eucaryotes is responsible for generating a family of genes that encode corresponding families of structurally and functionally related proteins. Members of a particular gene family can be either coordinately or differentially expressed. For example, the five *hsp70* genes of *Drosophila melanogaster* are expressed in a coordinate fashion (7, 8). In contrast, the *Drosophila* actin gene family consists of six unlinked genes that show several distinct forms of regulation (5, 6). Two actin genes appear to encode cytoplasmic actins in both differentiated and undifferentiated cells, while the remaining four genes show at least three distinct patterns of muscle-specific expression (5, 6).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in the glycolytic pathway. This pathway is responsible for converting glucose to pyruvate with the concomitant synthesis of ATP. The *Gapdh* gene family of *D. melanogaster* consists of two members which are located on different chromosomes (12). Although the two genes, *Gapdh-1* and *Gapdh-2* (located at cytological positions 43E-F and 13F, respectively), possess conserved coding sequences of 89% positional identity, no conservation is detected in their noncoding and flanking sequences. This observation motivated us to investigate whether the expression of the two genes is differentially regulated during development of *D. melanogaster*. In this paper, we report the developmental profile and tissue specificity of expression of the two genes and the dramatically different organization of their regulatory sequences. A detailed analysis of the upstream regulatory sequences of *Gapdh-2* has been published elsewhere (11).

MATERIALS AND METHODS

Construction of plasmids and *Drosophila* germ line transformation. Variant *Gapdh* genes were cloned into the Car-

negie 20 vector and introduced into *D. melanogaster* by P-element-mediated germ line transformation (10). The brief restriction maps of these plasmids are shown in Fig. 1. The plasmid construction procedures have been detailed elsewhere (X.-H. Sun, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1987).

Isolation of *Drosophila* RNA. The protocol for RNA isolation was derived from the guanidinium-hot phenol method of Feramisco et al. (4). *Drosophila* flies in various stages of development as well as Schneider II cells were homogenized with a Dounce homogenizer or a Polytron homogenizer, in 4 M guanidinium isothiocyanate mixture (4 M guanidinium isothiocyanate, 0.5 M Tris-hydrochloride [pH 7.6], 0.1 M EDTA, 2% homogenizer Sarkosyl) in a volume of 1 ml/100 flies. An equal volume of phenol was then added to the homogenate and vortexed vigorously, followed by the addition of 0.5 volume of 0.6 M sodium acetate and an equal volume of chloroform-isoamyl alcohol (24:1). The aqueous phase was obtained by centrifugation and extracted again with phenol-chloroform. RNA was then ethanol precipitated, and the pellet was suspended in water and extracted twice more with phenol. Finally, RNA was ethanol precipitated, and the concentration was determined by measuring the optical density.

RNA hybridization (Northern blot). Samples were prepared by mixing 1 volume of RNA with 2 volumes of loading dye (75% formamide, 3.3 M formaldehyde, 13 mM sodium phosphate [pH 6.5], bromophenol blue-xylene cyanol) and then heating the mixture at 50°C for 15 min and cooling it immediately on ice. The samples were loaded on a 1% agarose gel made in a formaldehyde buffer (2.2 M formaldehyde, 20 mM sodium phosphate, pH 6.5) and electrophoresed in a running buffer (1.87 M formaldehyde, 20 mM sodium phosphate, pH 6.5). The gel was blotted onto a nitrocellulose filter and baked to immobilize RNA. The filter was then hybridized to a nick-translated probe at 37°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate.

To ensure that equal amounts of RNA were loaded in each

* Corresponding author.

[†] Present address: Whitehead Institute, Cambridge, MA 02142.

[‡] Present address: Protein Design Laboratories, Inc., Palo Alto, CA 94304.

FIG. 1. Restriction maps of the *Gapdh* constructs. All constructs were cloned in the Carnegie 20 transformation vector (hatched boxes). *Gapdh-1* sequences as shown as solid lines, and *Gapdh-2* sequences are shown as broken lines. The arrows indicate the positions and lengths of the *Gapdh-1* and *Gapdh-2* transcripts. Symbols: Δ , 20-bp insertion sequence in *Gapdh-1*; $\blacktriangle$, 36-bp insertion sequence in *Gapdh-2*. Symbols for the restriction sites are: S, SalI; P, PstI; H, HindIII; E, EcoRI; X, XhoI; Hgi, HgiAI; Xmn, XmnI. The slash mark (/) shows the fusion of two restriction sites.

lane, 5 μ g (according to the concentration determined by optical density readings at 260 nm) of each RNA of interest was first electrophoresed on a 1% formaldehyde gel and stained with ethidium bromide to visualize the rRNAs. The gel was photographed with type 55 Polaroid film, and the negative was scanned by densitometry. The concentration of each RNA was normalized against the intensities of the rRNA bands.

RNAse mapping. RNAse mapping was performed essentially by the procedure of Melton et al. (9) with the following modifications. (i) Mini-prep plasmid DNA was used as the transcription template to obtain probes of acceptable quality, and (ii) the proteinase K treatment step for inactivating RNAse was omitted without affecting the results.

RESULTS

Developmental expression of the two *Gapdh* genes. To construct a developmental profile of the steady-state level of the two *Gapdh* transcripts, we collected animals at three different time points within each of the four major stages of *Drosophila* development—embryo, larva, pupa, and adult—and total RNA was isolated. Total RNA was also isolated from a *Drosophila* cell line, Schneider line II, and an equal amount of each RNA preparation was subjected to Northern (RNA) blot analysis (10). The nick-translated probes used for the hybridizations included a 138-base-pair (bp) *Pvu*II-*Aha*III fragment of *Gapdh-1* and a 311-bp *Taq*I-*Aha*III fragment of *Gapdh-2*, derived from the nonhomologous 3' noncoding sequences of the two genes (12) (Fig. 1), that can distinguish the transcripts from the two *Gapdh* genes. The developmental profile in Fig. 2 shows that both genes have two distinct modes of expression during development: a low- and a high-level mode. During the development of *D. melanogaster*, the difference between the highest level and the lowest level for *Gapdh-1* RNA was estimated to be about

100-fold, while that for *Gapdh-2* was about 20-fold. Both genes were expressed at low levels in 6- and 12-h-old embryos, early and mid pupae, and Schneider II cells. Both genes were expressed at high levels in larvae, late pupae, and adult flies. However, there were two major differences between the expression of the two genes. The first was that in 18-h-old embryos the level of *Gapdh-2* started to increase but that of *Gapdh-1* remained low.

The second difference occurred during the adult stage. The level of the *Gapdh-1* transcript decreased as adults aged but that of the *Gapdh-2* transcript remained constant. This conclusion was further substantiated by a more detailed examination of the expression of the two genes in the adult stage. Total RNAs were isolated from adult flies at the age of 8 h, 16 h, 1 day, 2 days, 3 days, 4 days, and 5 days. The level of the *Gapdh-1* transcript was detected by hybridization analysis and was found to decrease gradually over fourfold in 5 days as measured by densitometry of the autoradiograph (Fig. 3). In contrast, the level of *Gapdh-2* was relatively constant. The same filter was also hybridized to a probe specific to the *Drosophila* muscle-specific actin gene, *act79B* (6), that detects a 1.6-kilobase (kb) transcript. The accumulation of the *act79B* transcript during the adult stage produced a pattern similar to that of the *Gapdh-1* transcript. This result suggests that *Gapdh-1* shares some common features in its expression with the muscle-specific actin gene.

Tissue distribution of *Gapdh* transcripts in adults. Since GAPDH is involved in glycolysis, glycolytically active muscle is obviously one of the tissues in which to look for tissue-specific expression of the *Gapdh* genes. We dissected 2-day-old adult flies into three sections, head, thorax (including legs), and abdomen, and isolated total RNA from each section for RNA hybridization analysis. The thorax is composed mostly of flight muscle cells. Filters containing an equal amount of RNA on each lane were hybridized, respec-

FIG. 2. Developmental profile of the two *Gapdh* transcripts in *D. melanogaster*. RNA hybridization (Northern blot) analyses were performed on RNAs isolated from *Drosophila* animals of different developmental stages as indicated. Each lane contains 10 µg of total RNA. The concentration of the RNAs was standardized to ensure loading an equal amount of RNA on each lane. Filters were hybridized to *Gapdh-1* (A) and *Gapdh-2* (B) gene-specific probes at 37°C in 50% formamide–5× SSC–0.2% sodium dodecyl sulfate. The probes were labeled at similar specific activities. The filters were autoradiographed for 3 days at –70°C. The size of the major hybridizing band is approximately 1.5 kb.

tively, to specific probes from *Gapdh-1*, and *Gapdh-2*, and *Drosophila* adult-muscle-specific actin gene *act79B* (Fig. 4). The results showed that although the *Gapdh-2* transcript is almost evenly distributed in the three sections, the *Gapdh-1* transcript is highly enriched in the thorax section, a pattern resembling that of the *act79B* transcript. The difference in the levels of the *Gapdh-1* transcript between the thorax section and the head or abdomen sections as estimated by densitometry was about 10- to 20-fold.

Proper expression of modified *Gapdh-1* and *Gapdh-2* genes in vivo. To localize the regulatory sequences for the expression of *Gapdh-1*, we transformed variant constructs containing a modified *Gapdh-1* gene into the flies using a P-element vector. The modified *Gapdh-1* was created by inserting a 20-bp sequence into the coding region to distinguish it from the endogenous gene. Three derivatives, GAP1-8.5, GAP1-4.7, and GAP1-3.0, were constructed that possessed different amounts of 5'-flanking sequences (Fig. 1). The expression of these constructs in transformed flies was analyzed by an RNase protection assay, using a probe containing 230 bp of the coding sequence and the 20-bp insertion sequence. A 230-nucleotide-long protected band corresponding to the endogenous transcript was present in both wild-type Oregon R flies and transformed flies (Fig. 5). In addition, a 251-nucleotide protected band representing the modified *Gapdh-1* RNA was observed only in flies transformed with the three different constructs (Fig. 5, lanes 2, 3, and 4, respectively). Analyses of the RNAs isolated from the head, thorax, and abdomen sections of these transformants revealed that the modified *Gapdh-1* genes were also expressed in a pattern similar to that of the endogenous gene, i.e., higher levels in the thorax section compared with the head and abdomen

FIG. 3. Detailed profile of the two *Gapdh* transcripts in adult flies. RNA hybridization analyses were performed on RNAs isolated from adult flies of different stages, as well as from late pupae. Total RNA (10 µg) which had been standardized as described in the text was loaded on each lane. Filters were hybridized at 37°C in 50% formamide–5× SSC–0.2% sodium dodecyl sulfate with gene-specific probes of *Gapdh-1* (A), *Gapdh-2* (B), and *act79B*, a *Drosophila* muscle-specific actin gene (C). Filters were autoradiographed for 3 days at –70°C. The size of the major hybridizing band is approximately 1.5 kb in panels A and B and 1.6 kb in panel C. Under the hybridization conditions used in this experiment, it is possible that the *act79B* probe cross-hybridized with the *act87E* transcript (1.7 kb), which may have contributed to a 1.7-kb faint band in some of the lanes in panel C.

sections. An example of such an analysis is shown in Fig. 6c. These results indicated that the regulatory sequence responsible for proper expression of *Gapdh-1* was located within the 7.8-kb region, including 3.0 kb of 5'-flanking and 3.5 kb of 3'-flanking sequences and the 1.25-kb transcribed sequence.

FIG. 4. Tissue distribution of the two *Gapdh* transcripts in adult flies. RNA hybridization analysis of RNAs isolated from dissected body sections of 3-day-old adult flies. H, Head; T, thorax; and A, abdomen. Total RNA (10 µg) was loaded on each lane. Filters were hybridized with gene-specific probes of *Gapdh-1* (A), *Gapdh-2* (B), and *act79B* (C) at 37°C in 50% formamide–5× SSC–0.2% sodium dodecyl sulfate and autoradiographed for 3 days at –70°C.

FIG. 5. Expression of the modified *Gapdh-1* constructs in flies. Approximately 20 μ g of RNAs isolated from 2- to 3-day-old flies was analyzed by an RNase protection assay. The RNA probe, designated probe 1, contains the sequence derived from the coding sequence of *Gapdh-1* (solid line) plus a 20-bp inserted sequence (open triangle). The protected fragments of the modified and endogenous *Gapdh-1* transcripts are expected to be 251 and 230 nucleotides (nt) long, as demonstrated. The protected fragments were fractionated on a 6% polyacrylamide gel containing 7 M urea. RNAs were from Oregon R flies (lane 1), GAP1-8.5 transformants (lane 2), GAP1-4.7 transformants (lane 3), and GAP1-3.0 transformants (lane 4). The protected fragments of the modified and endogenous *Gapdh-1* are indicated by M and E, respectively.

We previously reported the detailed analysis of about 900-bp 5'-flanking sequences of *Gapdh-2* using the *Gapdh-2-lacZ* fusion constructs and concluded that the regulatory sequences of *Gapdh-2* located within the sequence 145 bp 5' to the transcription start were sufficient for a high level of expression (11). In this study, we constructed a modified *Gapdh-2* gene in which a 36-bp insertion was introduced into the coding sequence of *Gapdh-2*. This modified *Gapdh-2* gene contained about 300 bp of 3'-flanking sequence and about 900 bp of 5'-flanking sequence (Fig. 1). The modified *Gapdh-2* gene was transformed into flies, and its expression was assayed by RNase protection analysis. The probe used spans both 344 bp of the coding sequence and the 36-bp insertion sequence. The endogenous *Gapdh-2* transcript appeared to be 344 nucleotides long, and the modified *Gapdh-2* was 380 nucleotides long (Fig. 6b, lane 2). The expression levels of the modified and endogenous *Gapdh-2* were similar. Also, both genes were expressed in all three body segments (Fig. 6f).

Localization of major regulatory sequence responsible for tissue-specific expression of *Gapdh-1* to the region 3' to the translation start. The developmental pattern of expression of the two *Gapdh* genes is clearly different in adult stages. To locate sequences responsible for the differential regulation, we constructed two *Gapdh-1* and *Gapdh-2* hybrid genes, GAP2/1 and GAP1/2, using the parental plasmids GAP1-3.0 and GAP2-0.9. Both *Gapdh* genes had a unique *XmnI* site located at nucleotide 37 in the coding region that was used as the exchange point between the two genes (Fig. 1). Therefore, the 5' portion of each resulting gene refers to the sequence upstream from the *XmnI* site and the 3' portion refers to the sequence downstream from the site. Construct

GAP2/1 contained the 5' portion of *Gapdh-2* as in construct GAP2-0.9 and the 3' portion of *Gapdh-1* as in construct GAP1-3.0. Conversely, construct GAP1/2 had the 5' portion of *Gapdh-1* and the 3' portion of *Gapdh-2*.

The efficiencies as well as the tissue specificities of the expression of the two hybrid genes, GAP2/1 and GAP1/2, were examined by analyzing the RNAs isolated from adult flies transformed with each of the two hybrid genes by the RNase protection assay with the two probes described above. Comparing the expression patterns of the two hybrid genes with those of the two parental constructs, GAP1-3.0 and GAP2-0.9, gave clues to the location of the regulatory sequences. We found that construct GAP2/1 (Fig. 1) is expressed at a level higher than GAP1-3.0 (Fig. 6a). This was not surprising because we had previously shown that this 5' portion of *Gapdh-2* is sufficient for a high level of expression (11). However, as shown in Fig. 6d, the tissue-specific pattern of expression was similar to that of GAP1-3.0 (Fig. 6c), i.e., it was expressed at a higher level in the thorax section (presumably muscle cells) compared with the head and abdomen sections. On the other hand, construct GAP1/2 was expressed at a very low level in three independent transformants (Fig. 6b). The hybrid gene transcript was not enriched in the thorax even when the gel shown in Fig. 6e was exposed five times longer to visualize the low level of the GAP1/2 transcript (data not shown). Taken together, these results suggested that the sequence responsible for efficient as well as thorax-enriched expression of *Gapdh-1* is located 3' to the translation start of the gene. Although the 5' portion of *Gapdh-1* may be required for transcription, it is insufficient for tissue-specific expression.

DISCUSSION

Although the two unlinked GAPDH genes in *D. melanogaster* are structurally related in their coding regions, our analyses reported here showed that they are clearly regulated very differently. Since the *Gapdh* genes encode required glycolytic enzymes, at least one of the copies should function in every cell. The results of Northern blot analyses suggested that the *Gapdh-2* gene is likely to play a major role in supplying a constitutive level of the enzyme to all cells, since it is expressed at a higher level throughout development and its transcript is relatively evenly distributed in every segment of adult flies. In contrast, hybridization analysis of the RNAs from different sections of adult flies clearly showed that the *Gapdh-1* transcript is highly enriched in the thorax. This enrichment is likely due to its expression in the major tissue of the thorax, the flight muscle, although it could not be determined precisely whether this thorax-specific stimulation is restricted only to flight muscles. These questions can be addressed at higher resolution by the in situ hybridization technique (1). For the sake of simplicity of discussion, we refer to this *Gapdh-1* expression as muscle-specific expression.

Muscle cells are known to meet their extra energy requirement by increasing the rate of glycolysis. One way to do this is to increase the level of the mRNAs encoding glycolytic enzymes, including GAPDH. The level of *Gapdh* transcripts can be elevated by either transcribing a muscle-specific gene such as *Gapdh-1* or specifically increasing the transcriptional rate of a constitutive tissue-nonspecific gene such as *Gapdh-2*. Both mechanisms may be used at the stages at which muscles are well developed, e.g., larvae and adult flies.

The functional divergence of a multigene family is a common phenomenon. In *D. melanogaster*, one of the best

FIG. 6. Expression of the hybrid genes GAP1/2 and GAP2/1 in flies. RNAs were analyzed by an RNase protection assay, and the protected fragments were fractionated on a 6% polyacrylamide gel containing 7 M urea. Probe 1, used in the experiments shown in panels a, c, and d, is as described in the legend of Fig. 5. Probe 2, used in the experiments shown in panels b, e, and f, contains sequences from the coding sequence of *Gapdh-2* (broken line) and the 36-bp insertion sequence (solid triangle). The protected fragments of the modified (M) and endogenous (E) *Gapdh-2* are expected to be 380 and 344 nucleotides (nt) long, as illustrated. (a) Expression of GAP2/1 compared with that of GAP1-3.0. Lanes: 1, Oregon R; 2, GAP1-3.0; 3, 4, and 5, GAP2/1 (three independent transformants: 39-10, 39-24, and 39-32, respectively). (b) Expression of GAP1/2 compared with that of GAP2-0.9. Lanes: 1, Oregon R; 2, GAP2-0.9; 3, 4, and 5, GAP1/2 (three independent transformants: 41-10, 41-21, 41-55, respectively). The tissue specificities of the expression of GAP1-3.0 (c), GAP2/1 (d), GAP1/2 (e), and GAP2-0.9 (f) in the transformed flies were also examined by analyzing RNAs isolated from the head (H), thorax (T), and abdomen (A) sections. Owing to limited material obtained from the dissected flies, RNAs isolated were not well quantitated. The intensities of the protected fragments of the modified transcript were interpreted by comparison with the intensities of the endogenous transcript. The multiple bands observed for each of the modified and endogenous transcripts probably resulted from incomplete digestion by the RNase used.

examples is the actin gene family (6). *D. melanogaster* has six actin genes, two of which encode cytoplasmic actins, with the remaining four being muscle specific. Their transcripts were found to be present in a stage- and tissue-specific pattern. Two GAPDH isozymes, GAPDH-1 and GAPDH-2, are also present in *Caenorhabditis elegans* (3). They were shown to be encoded by at least two genes. One of the enzymes is localized in nonmuscle cells, but the other is present preferentially in the body wall muscle lineage where it is localized in the actin-containing zones. This is analogous to what we found in *D. melanogaster*, in which the two GAPDH genes have diverged into a tissue-nonspecific gene and a muscle-specific gene.

We have already shown that the efficient expression of *Gapdh-2* is driven by sequences up to 145 bp 5' to the transcription start (11). We now provide two additional results that support this conclusion. One is that a modified *Gapdh-2* gene containing a similar 5'-flanking sequence is expressed at a level comparable to that of the endogenous

Gapdh-2; the other is that the coding sequence and downstream sequences do not play a major role in the expression, as observed from the low level of *Gapdh* transcript in a transformant containing construct GAP1/2.

The expression of GAP1/2 also indicated that the 5' sequence of *Gapdh-1* is by itself insufficient for gene expression. In this regard, the mechanism of regulation for *Gapdh-1* is very different from that for *Gapdh-2*. Muscle-specific as well as efficient expression is conferred by sequences located in the 3' portion of the gene is demonstrated by the expression of construct GAP2/1. This 3' portion of *Gapdh-1* includes a large portion of coding sequences, the entire 3' untranslated sequence, and a 3.5-kb 3'-flanking sequence. Further analyses involving differently constructed hybrid genes, such as those performed with GAP1/2 and GAP2/1, are required to localize this regulatory sequence more precisely.

We propose two models to explain the efficient and muscle-specific expression of *Gapdh-1*. In the first, regula-

tion is mediated by a muscle-specific enhancer, to which a transcription factor specifically present in muscle cells binds. This factor can then interact with the transcription machinery assembled near the start site and activate transcription. Such examples in which the enhancers are located 3' to the transcription start sites have been found in the immunoglobulin genes, in which the B-cell-specific enhancer is located in the intron (2), and in several β -globin genes, in which the inducible enhancer is located 3' to the polyadenylation site (3). These enhancers greatly stimulate transcription over a long distance in appropriate conditions. According to this model, construct GAP2/1 contains both the regulatory elements of *Gapdh-2* and the muscle-specific enhancer of *Gapdh-1* and, as observed, is expressed at a higher level compared with that of *Gapdh-1*. In the second model, the efficiency and muscle specificity of *Gapdh-1* expression may be conferred by two separate events. The efficiency of transcription may be controlled by an enhancer element which may not be muscle specific, while the muscle specificity may be controlled by a different mechanism, such as the differential stability of the transcript in muscles compared with other tissues.

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