Structure and Regulated Expression of the δ-Aminolevulinate Synthase Gene from Drosophila melanogaster*

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Inmaculada Ruiz de Mena‡§, Miguel A. Fernández-Moreno‡, Belén Bornstein‡, Laurie S. Kaguni§, and Rafael Garesse‡¶

From the ‡Department of Bioquímica, UAM, Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC-UAM Facultad de Medicina, Universidad Autónoma de Madrid c/Arzobispo Morcillo 4, 28029 Madrid, Spain and the §Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824-1319

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The structure of the single copy gene encoding the putative housekeeping isoform of Drosophila melanogaster δ-aminolevulinate synthase (ALAS) has been determined. Southern and immunoblot analyses suggest that only the housekeeping isoform of the enzyme exists in Drosophila. We have localized a critical region for promoter activity to a sequence of 121 base pairs that contains a motif that is potentially recognized by factors of the nuclear respiratory factor-1 (NRF-1)/P3A2 family, flanked by two AP4 sites. Heme inhibits the expression of the gene by blocking the interaction of putative regulatory proteins to its 5′ proximal region, a mechanism different from those proposed for other hemin-regulated promoters. Northern and in situ RNA hybridization experiments show that maternal alas mRNA is stored in the egg; its steady-state level decreases rapidly during the first hours of development and increases again after gastrulation in a period where the synthesis of several mRNAs encoding metabolic enzymes is activated. In the syncytial blastoderm, the alas mRNA is ubiquitously distributed and decreases in abundance substantially through cellular blastoderm. Late in embryonic development alas shows a specific pattern of expression, with an elevated mRNA level in oenocytes, suggesting an important role of these cells in the biosynthesis of hemoproteins in Drosophila.

Heme serves as the redox prosthetic group of respiratory cytochromes and other hemoproteins including oxygen carrier proteins. It also plays an important role in cellular homeostasis, participating in the regulation of many biological processes, such as transcription, translation, and protein translocation (1–4). In particular, heme may regulate the expression of a number of nuclear genes encoding mitochondrial proteins that participate in regulatory mechanisms involving the orchestration of changes in mitochondrial biogenesis in response to different metabolic conditions (5, 6).

δ-Aminolevulinate synthase (ALAS)†: succinyl-CoA:glycine C-succiny1transferase, EC 2.3.1.37) is the first enzyme in the heme biosynthetic pathway in animals (1, 7, 8). ALAS is a pyridoxal phosphate-dependent enzyme that exists as homodimer in the mitochondrial matrix, where it catalyzes the formation of δ-aminolevulinic acid by condensation of glycine and succinyl-CoA (9). In vertebrates, ALAS is encoded by two different genes (10) that have been isolated and characterized in several organisms, including humans (11). One gene (alas2 or alas-E) encodes two isoforms generated by alternative splicing that are expressed exclusively in erythroid cells, where they are required for the synthesis of hemoglobin (12, 13). The second gene (alas1 or alas-N) encodes the nonspecific or housekeeping isoform and is expressed in all cell types (including erythroid) with the highest level found in liver (7, 14), where it is required for the synthesis of cytochromes P450.

The expression of ALAS is regulated in vertebrates by a variety of transcriptional and post-transcriptional mechanisms that are different for each gene (1). Expression of alas1 is elevated in liver after treatment with porphyrogenic drugs (7, 15) and is repressed by heme (16–18). Heme also inhibits the transport of ALAS1 to mitochondria (19), probably through the heme response motif located in the amino-terminal region of the protein (4). Transcription of the alas2 gene does not respond to changes in heme concentration, but it is developmentally regulated (both isoforms in parallel) during erythroid differentiation, probably by erythroid-specific transcription factors such as GATA-1 or NFE-2 (20). At the post-transcriptional level, the translation of the alas2 mRNA is controlled by the iron content of the cell through the iron response element located in the 5′-untranslated region of the mRNA (21–23), a regulatory mechanism that is not present in the housekeeping gene. Similar to ALAS1, heme also regulates the transport to mitochondria of the ALAS2 isoforms via the heme response motif.

Because the majority of the studies on the alas genes have been carried out in liver and erythroid cells of vertebrates, very little is known about the mechanisms controlling the expression of the housekeeping gene in order to supply the necessary heme for the respiratory complexes (1) and coordinate the synthesis of hemocytocromes with the respiratory demand of the different tissues (24, 25). This coordination is central for understanding both the physiology and pathology of mitochondrial function (26, 27). Interestingly, the promoter of the alas1 gene (28) contains DNA binding sites recognized by NRF-1, a transcription factor involved in nucleo-mitochondrial interac-

NRF, nuclear respiratory factor; GST, glutathione S-transferase; Luc, luciferase; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; UTR, untranslated region; DPE, downstream promoter element.

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tion (29), reinforcing the important role of the enzyme in organelle biogenesis.

Heme biosynthesis has been studied in *Saccharomyces cerevisiae* by a combination of molecular and genetic strategies (5, 30, 31). In yeast, heme functions as a sensor of oxygen tension, and its level regulates the expression of genes involved in mitochondrial function, modulating the activity of the transcription factor HAP1, the only regulatory protein responding to heme that is well characterized in eukaryotic cells (32–34). Among animals, *Drosophila* also offers an excellent opportunity to study complex biological processes in *vivo* using molecular and genetic tools (35), including mitochondrial gene expression under differing physiological conditions such as embryogenesis or aging (36–38). As a first step to study the mechanisms controlling heme synthesis and its coordination with mitochondrial biogenesis, we have cloned a *Drosophila melanogaster* alas gene. In this paper, we describe the structure of this single copy gene, its spatio-temporal pattern of expression during development and the characterization of its proximal promoter region.

### EXPERIMENTAL PROCEDURES

#### Library Screenings—

*Library Screenings—* The probe used was a 450-bp alas DNA fragment amplified by PCR using rat genomic DNA as template and the primers 5'-GGTGAGGTGGGACAGCTAAGAT-3' (forward, from position 848 to 867 as numbered in Ref. 16) and 5'-GAGCCCTCTGCTAGTGGAC-CTC-3' (reverse, from position 1276 to 1256). Using this heterologous probe labeled with [α-32P]dCTP, a λ-EMBL3 *D. melanogaster* library was screened. 3 × 10^6 plaques were transferred to Zeta probe filters (Bio-Rad), hybridized at 68 °C in ZAP buffer (7% SDS, 0.25 M phosphate buffer, pH 7.2), washed in 0.5% SDS, 2× SSC at 55 °C (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate), and autoradiographed with intensifying screens at +70 °C. Positive clones were purified by two additional rounds of screening, and the phages were amplified using standard protocols. Four positive phages were shown to be identical by restriction enzyme and Southern hybridization analyses. In order to recover additional clones, a 1.8-kb *Sal*I fragment from one of the phages was subcloned in pBluescript (Stratagene) and used as probe to screen a λ-EMBL4 *D. melanogaster* library using the same conditions described above, except that the final wash was carried out at high stringency (0.5× SSC, 0.1× SSC, 68 °C). Several overlapping phage clones were recovered from the same genomic region that were further characterized by restriction enzyme and Southern hybridization analyses.

To isolate the *D. melanogaster* alas cDNA, an adult λ-gt11 library was screened under high stringency conditions using as probe the alas genomic 1.8-kb *Sal*I fragment labeled with [α-32P]dCTP. Several positive phages were then purified and amplified using standard protocols. Four positive phages were shown to be identical by restriction enzyme and Southern hybridization analyses.

#### DNA Sequencing—

The nucleotide sequences of the cDNA and genomic clones were determined using the dideoxy chain termination method with T7 DNA polymerase (Amersham Pharmacia Biotech) and electrophoresis in polyacrylamide gels (39) or using Taq polymerase and automatic sequencing (3T3 DNA sequencer, Applied Biosystems) following the manufacturer's instructions. Both strands of the DNA were sequenced in their entirety. Sequences were analyzed using the GCG programs of the University of Wisconsin (40) on a Digital Vax computer.

**Mapping of Transcriptional Initiation Sites—** For primer extension analysis, 5 μl of the oligonucleotide 5'-CCGCTAGAGTTGGACAAGCCTCCTC-3' (from position 1209 to 1185) was annealed to 50 μCi of [γ-32P]ATP and polynucleotide kinase. Aliquots of 30–100 μg of total RNA obtained from embryos or adults and 6 × 10^4 cpm of [32P]-labeled primer were used in each experiment. Hybridizations were carried out at 65 °C under conditions described previously (37), and the primer was extended with 20 units of avian myeloblastosis virus reverse transcriptase for 2 h at 42 °C. The extended products were analyzed in 8% polyacrylamide, 7 M urea gels. Sequencing reactions using the same oligonucleotide were run in parallel. Anchored PCR to amplify the 5'-ends of *alas* transcripts was carried out essentially as described (41), using the specific primer 5'-AACATGTTGCGCGGACG-3' (from position 1282 to 1266).

### RESULTS

#### Southern and Northern Analysis—

**Southern and Northern Analysis—** Total RNA from staged and overnight embryos and adults of *D. melanogaster* Oregon R were extracted as described (36). For Northern blot analysis, total RNA (20 μg) was electrophoresed in 1.2% agarose, 1.8 M formaldehyde gels, blotted to a Zeta probe membrane, and probed in ZAP buffer at 68 °C using the labeled [α-32P]dCTP alas cDNA clone 9a. Filters were washed in 0.5% SDS, 0.1× SSC at 68 °C and autoradiographed with intensifying screens at –70 °C.

**In Situ Hybridization—** In situ hybridization to *yu* embryonic embryos was carried out as described previously (42). An antisense alas riboprobe was prepared by in vitro transcription using as template the alas cDNA linearized by digestion with *Hind*III and DIG-labeled UTP in a volume of 25 μl. The transcription reaction was incubated for 2 h at 37 °C, and the RNA was hydrolyzed by the addition of an equal volume of carbonate buffer (120 mM Na_2CO_3 and 80 mM NaHCO_3, pH 10.2) and heating at 65 °C for 40 min. The reaction was terminated with the addition of 0.1× NaOAc, 0.5% acetic acid, pH 6.0. The RNA probe was precipitated by the addition of LiCl to 0.4 M, 100 μg of *Escherichia coli* RNA, and two volumes of ethanol, dissolved in 150 μl of hybridization buffer and stored at –20 °C. The riboprobe was heated at 80 °C, and used before hybridization. Hybridization was carried out overnight at 55 °C in a buffer containing 50% deionized formamide, 5× SSC, 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml heparin, and 0.1% Tween 80. The digoxigenin-labeled alas probe was detected using DIG-UTP antibody coupled to alkaline phosphatase, and the reaction was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. For double-labeling in situ hybridization, we followed a protocol described previously (42). Primary antibodies were incubated with embryos overnight at 4 °C and detected using a biotinylated secondary antibody and the Vectastain ABC elite kit according to instructions. The preparations were then washed and processed according to the standard whole mount *in situ* protocol described above. Anti-β-galactosidase was used at a 1:4000 dilution, and Elav-BFS9 (Developmental Studies Hybridoma Bank; University of Iowa) was used at a 1:1000 dilution.

#### Antibody Production and Immunoblot Analysis—

Antibody Production and Immunoblot Analysis—*In order to produce in *E. coli* a fusion protein with glutathione S-transferase (construct pALAS-GST), a cDNA fragment containing the complete *D. melanogaster* alas coding sequence was cloned in frame in the EcoRI site of the pGEX-4 vector (Amersham Pharmacia Biotech). The orientation of the fusion protein was induced in the presence of isopropylthio-β-galactoside following the manufacturer’s instructions. To obtain ALAS-specific antibodies, purified ALAS-GST fusion protein was cleaved with factor Xa, and the released ALAS protein was used to immunize rabbits (giant New Zealand strain). Polyclonal antibodies were purified in an affinity column prepared by coupling the pALAS-GST chimeric protein to Affi- graf (Affi- graf; Bio-Rad). The serum was passed over the column, and the antibodies were eluted with 0.2 M glycine-HCl, pH 2.5, dialyzed overnight against phosphate-buffered saline, concentrated by filtration, and stored at 4 °C with 0.02% sodium azide.

Immunoblot analyses were carried out using total protein extracts prepared from overnight embryos or adults. SDS-10% polyacrylamide gel electrophoresis was performed as described by Laemmli (43). Electrophoretic transfer onto Immobilon polyvinylidene difluoride membranes (Millipore Corp.) was performed essentially as described (44). After incubation of the filter with the anti-ALAS antibody, the reaction was visualized using ECL (Amersham Pharmacia Biotech) as described by the manufacturer.

**Promoter Constructs and Transfection Analysis in Schneider Cells—** A 1038-bp DNA fragment (from 1038; +1 corresponds to the first nucleotide of the ATG triplet specifying the initiator methionine) was amplified by PCR, using as primers two oligonucleotides mapping to the positions 95–112/1126–1.108 (accession number of the genomic sequence is Y14577) and containing XhoI restriction sites at the 5'-ends (forward, 5'-CAAGCCTGAGTTGGACGTTTCG-3'; reverse, 5'-ACAGCTCGGAGTTCGTTTGTA-3'). The resulting DNA fragment was purified by gel electrophoresis, cleaved with XhoI, and cloned into the XhoI site of pBluescript. This represents the parental fragment from which deletions were generated by digestion using the following restriction enzymes: HindIII, BamHI, DraI, PvuII, and BstI. After restriction with the selected enzyme, the ends of the linear DNA were filled in using Klenow DNA polymerase, digested with XhoI; the fragment was excised from agarose gels and inserted into the
**RESULTS**

Cloning and Structure of the Drosophila alas Gene—Two Drosophila genomic libraries were screened using a heterologous probe encompassing a well conserved region of the rat alas gene (see "Experimental Procedures"). Several independent positive clones were identified and plaque-purified. Two of them, covering a genomic region of roughly 20 kb, were isolated and from hemin-treated Schneider cells. Hemin treatment was made according to Soeller et al. (46) with some modifications. Streptomycin (100 μg/ml) and penicillin (100 IU/ml) were added to the medium, and cells were transfected with 10 μg of the vector pSV /gal (Promega) and 5 μg of the ppx constructs. After transfection, cells were incubated for 24 h at 25 °C, washed twice with phosphate-buffered saline, resuspended in 5 ml of fresh medium with or without 30 μM hemin (5 μl hemin stock was prepared according to Marziali et al. (47)), and incubated 60–80 h at 25 °C. To prepare extracts, cells were harvested by centrifugation, washed with phosphate-buffered saline and fresh TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl), resuspended in 100 μl of 0.1% Triton X-100, 5 mM hemin stock and subjected to five freeze-thaw cycles (−70 °C for 60 s followed by 37 °C for 60 s). Finally, cell debris was removed by centrifugation at 13,000×g for 5 min.

Promoter activities were calculated normalizing luciferase activities (pxp constructs) with β-galactosidase activity (pSV /gal). Luciferase activity was determined using the Luciferase Assay System (Promega) according to manufacturer’s recommendations, and β-galactosidase activity was measured according to Sambrook et al. (48).

**Electrophoretic Mobility Shift Assays—** Electrophoretic mobility shift assays were carried out using nuclear extracts prepared from untreated and from hemin-treated Schneider cells. Hemin treatment was made growing 5×10⁶ cells during 48–56 h in medium containing 30 μM hemin at 25 °C. To obtain nuclear extracts, 5–20×10⁶ cells were harvested by centrifugation (5 min at 400×g) and resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 200 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). After 10 min at 4 °C, cells were sedimented, resuspended in buffer A containing 0.5% Nonidet P-40, and incubated for 10 min at 4 °C. Nuclei were harvested by centrifugation under the same conditions and resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 200 mM EDTA, 200 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). After 30 min, nuclear membranes were removed by centrifugation at 9000×g for 20 min. For band shift analysis, 5 μg of total protein was incubated for 30 min at 4 °C with 5×10⁴ cpm of the selected probe in binding buffer (20% glycerol, 1 mM dithiothreitol, 20 mM HEPES, pH 7.9, 5 mM MgCl₂, 0.2 mM EDTA, and 200 mM KCl) and loaded in a 5% acrylamide gel containing 0.5×TBE. DNA probes were labeled using [γ-³²P]ATP and T4 polynucleotide kinase by standard procedures (48).
sequence of the D. melanogaster alas cDNA detects in embryonic or adult protein extracts a single band of approximately 55 kDa (Fig. 1D), corresponding to the predicted molecular mass for the mature ALAS protein. All of these results are compatible with the presence in Drosophila of a single gene encoding the presumptive ALAS housekeeping isozyme.

Developmental Pattern of Expression—To study the pattern of expression of the alas gene during Drosophila development, we have carried out Northern analyses using RNA extracted from staged embryos and adults. The results, shown in Fig. 2, indicate that the alas mRNA is present in Drosophila eggs. Its steady state level declines during the first hours of development and increases later on. This pattern of expression is similar to that found in Drosophila for other housekeeping genes encoding metabolic enzymes (50) and in particular for other mitochondrial genes (37, 38).

The spatio-temporal pattern of steady-state alas mRNA was studied by whole mount in situ hybridization using a digoxigenin-labeled antisense alas riboprobe. At the syncytial blastoderm stage, an overall staining pattern was observed, probably reflecting the storage of maternal mRNA (Fig. 3A). The maternal mRNA disappears very rapidly through cellular blastoderm (Fig. 3B) and early embryonic development (Fig. 3C), consistent with the very low signal detected at this time by Northern analysis. Interestingly, in latter stages of embryonic development, alas expression is concentrated in seven symmetrical groups of cells located in the lateral part of the a1–a7 abdominal segments, which apparently are the oenocytes. The alas mRNA is also concentrated in two groups of cells located in the anterior part of the embryo (Fig. 3D), which could be the Bolwig’s organ. At this time, alas expression is very low in other embryonic tissues, although a constitutive expression over the background signal is observed.

We have confirmed the location of alas expression by double staining using enhancer trap lines that express the enzyme β-galactosidase in oenocytes. As shown in Fig. 4, B and D, β-galactosidase and alas mRNA co-localize in these cells, indicating that oenocytes maintain an active expression of alas. On the other hand, to gain insight into the origin of the structures containing pHAlas, level of RNA loaded were evaluated by ethidium bromide staining (lower panel).

Initiation of Transcription and Structure of the Promoter Region—The transcriptional initiation site of the alas gene in both embryos and adults of D. melanogaster was determined using a combination of primer extension analysis and amplification of 5’-ends by anchored PCR. Using total RNA prepared from overnight embryos (0–20 h) and adults, primer extension revealed the existence of two major transcriptional initiation sites, located at 60 and 84 nucleotides 5’ upstream from the initiator methionine (Fig. 5); one is used mainly in embryos (−84) and the other in adults (−60). The presence of different transcriptional initiation sites was confirmed by anchored PCR, where amplified clones ending at positions −60, −71, −84, and −96 were obtained. These data suggest a heterogeneous initiation of transcription and excluded the presence of an intron in the region.

As has been described for many housekeeping genes, there are no TATA or CCAAT boxes in canonical positions. Both major transcriptional start sites (−60 and −84) contain initiator-related sequences. Surrounding the −60-position is the sequence TCACTT, which is completely conserved with the consensuse initiator sequence of vertebrates (PyPyAN(T/A)PyPy) and highly related to the Drosophila initiator (TCA(G/T)T(T/C)). At the −84 initiation site, there is also a sequence related to the Drosophila initiator, although in this case it is more divergent with conservation in four of six nucleotide positions (TCTTGC). In addition, the gene contains a downstream promoter element (DPE; Ref. 53) located at position −33 (see Fig. 6B). The sequence of the D. melanogaster alas DPE is highly conserved (GGTGGG) relative to the consensus recently described ((A/G)(G/A)(C)GTG) for this element.

We have searched for the presence of putative iron response element in this 5’-UTR. No secondary structure with the well conserved loop CAGT(A/N) (nucleotide A is not conserved to the consensus CAGTGN) present in the UTR of mammalian erythroid alas mRNA was found, a result consistent with the housekeeping function of the gene.

Functional Characterization of the Upstream Regulatory Region of the alas Gene—To identify functional elements in the proximal 5’ upstream region of the alas gene, a series of constructs containing specific DNA fragments were fused to the luciferase reporter gene using the vector pxp-1 (see “Experimental Procedures”). A construct containing approximately 1 kb of the 5’ upstream region of the gene (−1038pLuc construct) produced a significantly higher levels of luciferase activity as compared with the promoterless pxp1 vector (>1000-fold; Fig. 6A) that is orientation-dependent. Promoter activity is either maintained or increased with shorter constructs containing as few as the 238 proximal nucleotides (Fig. 6A), suggesting the possibility of negative regulatory elements in these regions. In particular the −691pLuc and the −238pLuc constructs direct a relative activity of 1.89 ± 0.39 and 2.04 ± 0.43 times higher, respectively, than the −1038pLuc construct. On the other hand, the construct containing the 151 proximal nucleotides retains 30% of the promoter activity detected in the −1038pLuc construct, while the activity is only of 1% in the −117pLuc construct. These results indicate that the region between positions −117 and −238 contains elements that are critical for full promoter activity in Schneider cells. This region contains a direct repeat (TGTTT) separated by 5 nucleotides; in addition, computer analysis reveals the presence of DNA sequence elements that are potential target sites for binding of regulatory proteins, including the Hunchback, Snail, GATA, CCAAT/enhancer-binding protein, and HNF factors (Fig. 6B). Most interestingly, there is a sequence located in the opposite orientation between −130 and −137 (TGTTGGCGT) with a 100% identity to the binding site recognized by the P3A2 factor (TGTTGGCGT(A)), a regulatory protein of the NRF1 family characterized in sea urchin (51, 52). This binding site corresponds approximately to a half-site of the NRF1 binding motif (TGCG-
CATGCGCA). Flanking both sites of the P3A2 motif, there are two DNA binding sites recognized by AP4 factors. It seems very likely that at least some of these DNA sequence elements are functional, because promoter activity is completely abolished in a construct (−117pLuc) that lacks this 5' proximal region.

**Effect of Heme on Drosophila alas Gene Expression**—In vertebrates, several reports suggest that *alas1* expression is regulated at the transcriptional level by heme, although these results are controversial (1). To determine if the expression of the *alas* gene is regulated by heme in *Drosophila*, we grew Schneider cells in the presence of 30 μM hemin (the oxidized form of heme) and quantitated the promoter activity of the

**FIG. 3. Whole mount in situ RNA hybridization of ALAS during D. melanogaster development.** Anterior is to the left, and dorsal is up. An overall staining pattern is observed in the syncytial blastoderm (A). Staining is absent in the cellular blastoderm (B) and early embryos through stage 13 (C). Staining in late embryos is concentrated in the distal part of abdominal segments 1–7 and in two symmetrical groups of cells located in the anterior part of the embryo (D).

**FIG. 4. Oenocyte localization of alas mRNA by double labeling in situ hybridization.** In situ hybridization to whole mount embryos. Enhancer-trap lines P706 (A and B) and P1385 (C and D) were obtained from the Bloomington stock center (P706, y w; Pw 'mc IacW' C3-2-2; P1385, Py'I7.2-ZP) dock 04723 cm1/eyO; ry506. β-Galactosidase (brown) was detected with an anti-β-galactosidase antibody (A and C) and *alas* mRNA (blue) with a digoxigenin-labeled antisense RNA *alas* probe. Double label (B and D) results in a darker staining in oenocytes (arrows). *E*, wild type embryo immunostained with the monoclonal antibody Elav-9F8A9, a marker of nervous system structures; the *arrowhead* shows the Bolwig's organ. *F*, double staining with Elav-9F8A9 antibody and digoxigenin-labeled antisense RNA *alas* probe. The *arrow* indicates the blue staining due to *alas* mRNA. A, dorso-lateral view; B, lateral view; C and D, ventral views; E and F, dorsal views of stage 14 embryos. Anterior is to the left, and dorsal is up.
series of constructs described in Fig. 6A. As control, we used the actin promoter fused to the luciferase reporter gene in the pxp1 vector. The hemin effect on the different promoter constructs is shown in Fig. 7. There is a 3-fold inhibition of the activity of the actin promoter, which may reflect some nonspecific effect of the hemin treatment at the cellular level, because the same range of response was detected using the human cytomelagovirus promoter (pCMV-Luc, data not shown). However, the inhibition detected in the alas promoter is dramatic (a 20-fold decrease) by comparison, and is maintained in all of the constructs tested, even in that containing the 117-bp proximal promoter/5'UTR (Fig. 7). To confirm the role of this region in mediating the heme response, we have cloned the 117-bp fragment in both orientations into the 3' region of the actin promoter and evaluated the effect of hemin on the activity of the chimeric constructs. A strong response to hemin is now detected in the actin promoter (Fig. 7), a result indicating that the 117-bp alas proximal region contains the necessary element to mediate the heme inhibitory effect.

To detect a possible effect of hemin on the binding of regulatory proteins to the alas 5' region, we carried out electrophoretic mobility shift assays using nuclear extracts prepared from Schneider cells grown in the absence and presence of hemin. In control untreated cells, a distinct pattern is detected using the 238-bp fragment (see Fig. 8A) as probe, with two major protein-DNA complexes that are specifically competed with an excess of free oligonucleotide (Fig. 8A). The addition of hemin directly to the assay does not influence the binding pattern (data not shown). However, using nuclear extract prepared from cells grown in the presence of hemin, the binding is largely abolished. The same result is obtained using the proximal 117-bp DNA fragment as probe (Fig. 8B). In this case, only one complex is clearly visible, which disappears completely using the nuclear extract prepared from cells treated with hemin. As a control, we carried out band shift experiments using a 121-bp DNA fragment containing the 5' half of the 238-bp fragment as probe. In this case, the pattern detected is nearly the same using nuclear extract prepared from cells grown in the absence or presence of hemin (Fig. 8C), documenting the specificity of the heme effect on the band shift of the 117-bp proximal fragment.

**DISCUSSION**

We have cloned and studied a Drosophila gene encoding the mitochondrial matrix enzyme 5-aminolevulinate synthase, which catalyzes the major regulatory step in the heme biosynthetic pathway. In animals, the enzyme has been characterized in vertebrates, mainly in mammals, which contain three ALAS isoforms encoded by two distinct genes. alas1 is expressed ubiquitously, and alas2 is expressed exclusively in erythroid cells. This is the first report of an invertebrate alas gene.

The D. melanogaster alas gene is located on the right arm of the second chromosome, spans a region of approximately 3 kb, and contains two exons interrupted by a single intron of 144 bp that is located at a position 375 bp from the 3'-end. In contrast to the situation in vertebrates, several experiments suggest that Drosophila ALAS is encoded by a single gene: repetitive screenings of several cDNA and genomic libraries recovered phages harboring DNAs corresponding to the same genomic region; Southern analyses under low stringency conditions detected only fragments from this DNA region; and Northern analyses revealed the presence of a single mRNA in embryos and adults. More importantly, immunoblot analysis using a polyclonal antibody raised against the complete ALAS protein detected a single band corresponding to the predicted mature protein. The logical interpretation of these results is that the Drosophila alas gene encodes the constitutive form of the enzyme. Accordingly, the 5'-UTR of the mRNA does not contain the iron response element, a characteristic feature of the eryth-
Hemin response of -fold inhibition of the promoter activity in the presence of hemin is components of energy metabolism (29, 57), including ALAS.

Drosophila D. melanogaster alas The gene has a TATA-less promoter that is developmentally regulated (51, 52) and contains a DNA-binding domain at position –60 and –84. During embryogenesis, the –84 initiation site is used preferentially, while adult mRNAs start predominantly at the –60-position. This raises the interesting possibility that the expression of the gene is regulated differentially in embryos and adults. In addition, the Drosophila alas promoter contains a DPE located in the 5'-UTR. DPEs substitute for the TATA-box to provide a binding site for TFIID and have been found recently to interact with TAF160 (54). They are conserved between Drosophila and humans and are critical for the function of a subset of TATA-less promoters (55, 56).

Using transient transfection analysis in Schneider cells, we have characterized the 5'-upstream region of the Drosophila alas gene. We have delimited the region responsible to direct maximal activity in Schneider cells to the proximal 238 nucleotides. In particular, the nucleotides encompassing the –238 to –117 region are necessary to recover full promoter activity. Within this region, we have identified several putative transcription factor binding sites and in particular one located at position –140 potentially recognized by P3A2. Notably, P3A2 is a regulatory protein identified in sea urchin that is itself developmentally regulated (51, 52) and contains a DNA-binding domain that shares high identity with the Drosophila ERECT WING and the mammalian NRF-1 factors (29). NRF1 is probably involved in a coordinated response of genes encoding key components of energy metabolism (29, 57), including ALAS.

Drosophila 6-Aminolevulinate Synthase Gene Expression

FIG. 7. Hemin effect on the activity of the D. melanogaster alas promoter. Hemin response of alas and actin promoter constructs. Schneider cells were transfected with 5 μg of the following constructs: PactinLuc, –1038pLuc, –117pLuc, or a single copy 117-bp alas heme response region cloned in both orientations (denoted by arrows) in the actin promoter. After transfection, the cells were incubated for 24 h at 25 °C, washed with phosphate-buffered saline, and incubated for 60 h in fresh medium in the presence (+) or absence (–) of 30 μM hemin. The -fold inhibition of the promoter activity in the presence of hemin is shown. The data represent the mean ± S.D. of at least five independent experiments.

FIG. 8. Protein binding to the 5' upstream region of the D. melanogaster alas promoter. Electrophoretic mobility shift analysis was performed with nuclear extracts and specific fragments from the 5' upstream alas region as probes. A, the 238-bp fragment from DraI to XhoI; B, the 117-bp fragment (heme response region) from BalI to XhoI; C, the 121-bp fragment from DraI to BalI. The positions of the restriction sites are shown in Fig. 7A. Radiolabeled double-stranded DNAs were incubated with nuclear extracts prepared from cells grown in the presence or absence of hemin. Lane 1, probe with no extract; lane 2, probe plus nuclear extract from untreated cells; lane 3, probe plus nuclear extract from hemin-treated cells; lane 4, probe plus nuclear extract from untreated cells and competed with a 50-fold excess of unlabeled DNA; lane 5, probe plus nuclear extract from hemin-treated cells competed with a 100-fold excess of unlabeled DNA. In all of the experiments, 5 μg of nuclear protein was used.

(28) Genetic and molecular analyses in Drosophila have demonstrated that erect wing plays an important role in the development of muscle and the nervous system (58, 59), two tissues with a very high energetic requirement that demand high mitochondrial activity and therefore require elevated synthesis of respiratory pigments.

ALAS activity is subject to a variety of heme-mediated negative control mechanisms including the inhibition of mRNA synthesis (11), which is one of the few examples of a feedback mechanism by end product at the level of transcription (2). In vertebrates, this mechanism has been detected only in the gene encoding the alas housekeeping isoform, although these data are controversial (1). We have detected a substantial decrease in the activity of the Drosophila alas promoter in Schneider cells treated with 30 μM hemin. Furthermore, we have delimited the sequence elements responsible for the heme-mediated inhibitory effect to a DNA fragment of 117 bp that includes 60 bp of the proximal core promoter and the 5'-UTR. Although we cannot rule out formally the possibility that the 5'-UTR mediates a decrease in mRNA stability of the luciferase transcript, the most likely interpretation is that the heme effect is exerted at the level of transcription and is mediated by the proximal 5’ upstream sequences and/or the UTR. Remarkably, the 117-bp fragment confers a heme response on heterologous promoters. Moreover, heme exerts a negative effect on the interaction of regulatory proteins and/or factors of the basal transcriptional machinery with the alas gene, blocking the binding of these proteins to the proximal 5’ upstream region of the gene.

This inhibitory mechanism is different from that described for other genes that are transcriptionally regulated by heme. For example, in yeast, heme also acts as an important regulator of gene expression (60). This effect is mediated, at least in some genes, by the zinc finger transcription factor HAP-1, which binds DNA in the presence of heme (61, 62). The presence of DNA regulatory proteins that bind the promoter of the mammalian ferritin gene in a heme-dependent manner has also been described recently, and in this case the effect is mediated by the ubiquitous transcription factor NF-Y (47, 63, 64). Another example of a gene regulated at the transcriptional level by heme is the tartrate-resistant acid phosphatase. This effect is mediated by the interaction of a heterogeneous complex...
comprised of Ku antigen, the redox factor protein Ref1 and a 133-kDa protein with a GAGGC tandem repeated motif (65, 66). Finally, the heat shock factor 1 mediates the transcription of the gene hasp70 mediated by heme; in this case, the mechanism could be indirect, involving the inhibition by heme of intracellular proteolysis (67).

Vertebrate alaS genes have been studied mainly in liver and cell culture. The characterization of the Drosophila alaS gene has allowed us to study the spatio-temporal pattern of expression of an alaS gene during development. alaS mRNA of maternal origin is homogenously distributed in the syncytial blastoderm at relatively high levels, and, consistent with Northern analyses, its concentration decreases rapidly and it is almost absent in cellular blastoderm. Interestingly, in later stages of embryogenesis after retraction of the germ band, the mRNA is expressed highly in oenocytes and in two symmetrical groups of cells located in the anterior part of the embryo. Oenocytes are a small group of cells of ectodermal origin, located in each of the abdominal segments that contain histoblasts (68). Their development is associated with the differentiation of fat cells, and some of the oenocytes invade the larval fat body and are found in its inner surface. Adult oenocytes are smaller than these larval ones and are clearly recognizable and distinct from the fat body cells (69). Some observations have suggested a potential role in secretion, although their function remains largely unknown. The specific pattern of expression of alaS in oenocytes suggests that these cells are highly active in the synthesis of hemoproteins, perhaps cytochrome P450, and may be involved in detoxification mechanisms in Drosophila.

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