Isolation and Characterization of the Xanthine Dehydrogenase Gene of the Mediterranean Fruit Fly, Ceratitis capitata

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ABSTRACT

Xanthine dehydrogenase (XDH) is a member of the molybdenum hydroxylase family of enzymes catalyzing the oxidation of hypoxanthine and xanthine to uric acid. The enzyme is also required for the production of one of the major Drosophila eye pigments, drosopterin. The XDH gene has been isolated in many species representing a broad cross section of the major groups of living organisms, including the cDNA encoding XDH from the Mediterranean fruit fly Ceratitis capitata (CxDH) described here. CxDH is closely related to other insect XDHs and is able to rescue the phenotype of the Drosophila melanogaster XDH mutant, rosy, in germline transformation experiments. A previously identified medfly mutant, termed rosy, whose phenotype is suggestive of a disruption in XDH function, has been examined for possible mutations in the XDH gene. However, we find no direct evidence that a mutation in the CxDH gene or that a reduction in the CxDH enzyme activity is present in rosy medflies. Conclusive studies of the nature of the medfly rosy mutant will require rescue by germline transformation of mutant medflies.

The development of improved control strategies for insects that act as biological pests and disease vectors is vitally important for the prevention of the spread of human disease and for the alleviation of damage to economically important domestic animals and plant species. One of the most notorious agricultural pests is the Mediterranean fruit fly (medfly), Ceratitis capitata. The medfly has migrated from its origins in Africa throughout the Mediterranean region and into the Americas within the last 100 years (Haymer et al. 1997; Malacrida et al. 1998; Davies et al. 1999) and is responsible for the annual loss of billions of dollars in fruit crop production. As a consequence, medflies are the subject of intense control efforts in many parts of the world, including Central America, Europe, and the United States (Kahn et al. 1990; Barinaga 1991; Carey 1991). The medfly is particularly destructive because it has a very wide host plant range, being able to infect some 200 fruit varieties, and because females puncture the fruit when laying eggs, allowing for larval opportunistic microbial invasion (Saul 1986; Robinson 1989). It is hoped that a detailed understanding of the biology of the medfly will eventually lead to the development of the tools needed to effectively manage its populations. Germline transformation is one of the tools likely to contribute to the design of novel and effective control strategies.

To facilitate germline transformation, selectable or visible phenotypic markers are required to separate transformants from nontransformants. Many such visible markers have been used to great effect in the widely studied insect model system, Drosophila melanogaster. Currently, the two genetic markers most routinely used are the eye color genes white and rosy (Ashburner 1989). In Drosophila, white encodes an ABC family transporter that is responsible for import of eye pigments into photoreceptor cells (reviewed in Higgins 1992) while rosy encodes xanthine dehydrogenase (XDH), a member of the molybdenum-containing dehydrogenase family of enzymes (Keith et al. 1987). The Drosophila rosy/XDH gene enzyme system has been studied in great detail, and it has long been established that XDH is required for the production of drosopterin eye pigment as well as for the conversion of purines into uric acid (reviewed in Chovnick et al. 1990; Wootton et al. 1991; Hille and Nishino 1995). The use of these genes in Drosophila transformation experiments has led to the suggestion of their similar application as phenotypic markers for germline transformation in the medfly and other insects (Zwiebel et al. 1995). Several medfly eye color mutants have been described, including white, rosy, light eye, andPurple eye (reviewed in Saul 1985, 1986) and, to date, white is the only one whose gene has been fully isolated from medfly (Zwiebel et al. 1995; Gomulski et al. 2001). As in Drosophila, the medfly white mutants completely lack eye pigmentation (Rossler and Koltin 1976; Rossler and Rosenthal 1992). Significantly, the medfly white gene is homologous to Drosophila white, and two naturally occurring mutations have been defined at the DNA level (Zwiebel et al. 1995; Gomulski et al. 2001).
Indeed, partial phenotypic rescue of white mutants has been successfully carried out in medfly using the medfly white cDNA as a dominant marker for two transposon-based transformation vectors, Minos and piggyBac (Loukeris et al. 1995; Handler et al. 1998).

As is the case in Drosophila, the characterization of multiple molecularly defined phenotypic markers for transformation in the medfly will facilitate increased flexibility among different applications. As part of the effort to expand the range of germline transformation systems in this important agricultural pest insect, we have undertaken the isolation and characterization of the medfly XDH gene. By analogy to the white gene, Ceratitis XDH (CcXDH) has the potential to be used as an additional phenotypic marker, but only if a corresponding medfly XDH mutant with an easily distinguishable phenotype can be identified. A previous study has isolated and characterized a C. capitata mutant, termed rosy, which phenotypically displays burgundy eyes as well as a sensitivity to purine-supplemented media (Saul 1982), two phenotypes that are characteristic of many of the rosy alleles of D. melanogaster (reviewed in Chovnick et al. 1990). This would suggest that the molecular basis for the rosy mutant may be a defect in the CcXDH gene. Alternatively, these phenotypes could be the result of a mutation in a gene affecting CcXDH function because the enzyme requires the actions of several cofactors and gene products for its normal function in Drosophila (Hughes et al. 1992; Hughes 1992). For example, the product of the ma-l gene is required for XDH activity (Forrest et al. 1956; Glassman and Mitchell 1959) where it is apparently responsible for the ability of XDH and other enzymes to incorporate sulfur (Wahl et al. 1982). Mutations in ma-l and other loci in Drosophila, including low xanthine dehydrogenase (lxd) and cinnamon (cin), also display a rosy eye phenotype and have a corresponding low level of XDH activity (reviewed in Kamdar et al. 1997). It will be critical to elucidate whether a lesion in XDH causes the medfly rosy mutant because it has the potential to be used in germline transformation.

To directly examine this issue we have cloned the full-length medfly CcXDH cDNA and have tested the hypothesis that the medfly rosy phenotype is caused by a defect in CcXDH. Our studies demonstrate that the CcXDH gene is capable of functionally rescuing the Drosophila XDH rosy mutant and may therefore be useful as a marker for medfly germline transformation in a CcXDH mutant background. However, we find no evidence for a defect in the XDH gene in the medfly rosy mutant at the levels of DNA, RNA, or enzyme activity in vitro. Final resolution of this question requires the use of CcXDH cDNA in an attempt to rescue the medfly rosy mutant by germline transformation. We are prevented from conducting such studies due to the absolute quarantine against live medflies that is currently in place in the continental United States.

MATERIALS AND METHODS

Medfly strains: Two independently derived medfly laboratory strains were used in this study: Benakeion, which is associated with the XDH allele and has wild-type eye color, and the rosy eye mutant strain, in the Wiedemann genetic background. The wild-type eye color strain Benakeion was originally established in the laboratory by P. A. Mourikis (Benakeion Institute of Phytopathology, Athens, Greece) with flies from the Southern Peloponnesse (Greece) and Palermo (Italy; Rina and Savaki 1991). The rosy strain was established in the laboratory of Stephen Saul (Saul 1982).

C. capitata XDH cloning: CcXDH clones were isolated using degenerate PCR primers designed using the medfly XDH cDNA as a sensitivity to purine-supplemented media (Nakamura et al. 2000) on conserved domains of rat XDH and two available insect sequences, D. melanogaster and C. vicina. The primers were LjZVI 5′-ACG GCG GGY GGY TGY GGI CCW CAR GGI ATG-3′ (corresponding to the amino acid sequence TAFRGFGPGQM) and LjZVIII 5′-YTG ICC RAT RTC ICC DGG RTT RAA IGA IGA ICC-3′ (corresponding to the amino acids GQSSLNAIDIQG). PCR generated an 860-bp product that was TOPO TA cloned (Invitrogen, San Diego) and sequenced to verify that it was XDH from medfly. This sequence corresponds to amino acids starting from position 1155 of the full CcXDH peptide VGDD...etc. The 860-bp fragment was radiolabeled with [32P]dATP and used to isolate both genomic and cDNA clones from λ-phage libraries (Sambrook et al. 1989). Genomic clone 623 remains uncharacterized while λzap (Stratagene, La Jolla, CA) cDNA clone 114-1 was subcloned into the KpnI and SalI restriction sites of vector pSP73 (Promega, Madison, WI).

Poly(A)+ RNA was isolated from adult medflies (C. capitata) and used to synthesize double-stranded cDNA followed by adapter ligation using the Marathon cDNA amplification kit protocol (Clontech, Palo Alto, CA). Adapter oligonucleotide primer AP2 5′-ACT CAC TAT AGG GCT CGA GCG GC-3′ was used in combination with oligonucleotide primer XDH-3′ 5′-AGC RAT RTC ICC DGG RTT RAA IGA IGA ICC-3′ to PCR amplify the 5′ end of the XDH cDNA from the medfly rapid amplification of cDNA ends (RACE) library under the following conditions. A premix of 17.5 μl 10× Clontech RACE PCR buffer, 14 μl [10 mM] dNTP, 3.5 μl of Advantage Taq polymerase, and 119 μl H2O was mixed and kept on ice. Each reaction contained 21.5 μl of premix, 2.5 μl of a 1:200 dilution of adapter ligated RACE library, and 0.5 μl of each [10 μM] primer. A positive control reaction containing 2.5 μl of control cDNA and 0.5 μl of oligos AP1 5′-CCA TCC TAA TAC GAC and TFR3 5′-ATT TCG CAG ATG CTG AGA AAA CAG ACA GA-3′ produced the expected 2.9-kb product. Negative control reactions containing single primers produced no products. Reactions were carried out in a PerkinElmer (Norwalk, CT) 9700 thermal cycler as follows: 94°C for 2 min; 5 cycles of 94°C for 5 sec and 72°C for 4 min; 5 cycles of 94°C for 5 sec and 70°C for 4 min; and 25 cycles of 94°C for 5 sec and 68°C for 4 min.

Digesting the 5′ RACE subclone with XhoI/BstEII restriction endonucleases generated the full-length medfly cDNA clone (2.8 kb). This fragment was ligated into a SalI/BstDII-digested vector containing the 3′ end of XDH. The full-length cDNA, pSP73:CcXDH, was sequenced in an ABI377 automated sequencer as described (Perkin-Elmer). CcXDH was conceptually translated and alignments with similar peptides were performed using CLUSTAL W software (Thompson et al. 1994). The PAUP software package (Swofford 1991) was used to determine the phylogenetic placement of our medfly XDH sequence in relation to those of the full-length XDH genes previously sequenced and analyzed from other insect taxa (Komoto et al. 1999). These included sequences from two species, D. melanogaster and C. vicina.
different XDHi loci from *Bombyx mori* as well as single copies from the drosophilid species *D. melanogaster*, *D. pseudoobscura*, and *D. subobscura*, and from the calliphorid *Calliphora vicina*. *B. mori* is the only insect reported to possess more than one XDHi gene, each apparently serving a different current function (Yasukochi et al. 1998; Komoto et al. 1999). Our initial analyses used mouse XDHi as an outgroup, while later analyses deleted mouse and used *Bombyx* sequences instead. Equally weighted XDHi data sets were analyzed as both nucleotide and amino acid sequences using maximum parsimony, neighbor joining, and maximum likelihood methods. The bootstrap (Felsenstein 1985) was used to assess statistical support for relationships via branch and bound analysis of 100 pseudoreplicated data sets.

**D. melanogaster transformation:** The Drosophila Pelement vector, pPCaSpeRhs/act (GenBank accession no. U60735), for vector map see http://www.hhmi.genetics.utah.edu/thum mel/pelement.html, mini prep DNA (1 μg) was digested with *Not* and BamHI and pSP73:GXDH mini prep DNA (1 μg) with *Not* and *BgII*. Digests were run on 0.7% agarose (TAE) gels. The 9.2-kb vector and 4.9-kb XDH insert sequences were excised from the gel with sterile razor blades and DNA was isolated from the gel slices using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Ligations were set up as follows and were allowed to proceed overnight at 16°C: 1 μl pCaSpeRhs/act fragment; 1 μl or 2 μl of pSP73:GDH *Not*/*BgII* fragment; 1 μl T4 ligase buffer; 0.3 μl T4 ligase [400 units/μl]; dH2O to 10 μl. Each ligation (2 μl) was transformed into XL1-Blue competent cells (40 μl) by electroporation (2.5 kV). Transformants were plated onto Luria broth (LB) + ampicillin (100 μg/mL) agar and incubated overnight at 37°C. Fresh amp’ colonies were picked with a sterile toothpick and used to inoculate an overnight LB culture (3 ml) (pH 7.5). Each homogenate was centrifuged for 30 min at 13,000 rpm and 4°C. Supernatant was transferred to a new tube and centrifuged as before. Protein concentrations of each extract were measured using the bicinchoninic acid method and according to the manufacturer’s protocol (Pierce Chemical). Five microliers of each extract were loaded onto cellulose acetate gels that had been pretreated in a running buffer of 64 μm Tris, 4 mm EDTA, and 13.6 mm citric acid (pH 7.5). Gels were run at 100 V for 20–30 min. Gels were stained in Tris buffer with 1.4 mm hypoxanthine, 2.4 mm NAD, 0.4 mm phosphlane methosulfate (PMS), and 1.2 mm nitro blue tetrazolium (NBT) (or xanthine:PMScytC or xanthine:NAD+).

**RNA Analysis:** *C. capitata* total RNA was isolated from 1-day-old pupae of the *rosy* (Wiedemann) mutant and from embryos, pupae, and adults of wild type (Benakeion), using the RNeasy RNA isolation kit (Qiagen). RT-PCR was performed using the Titan One-Tube RT-PCR kit (Roche Molecular Biochemicals). The manufacturer’s protocol was followed except that reactions were scaled down from 50 to 25 μl by using half the amount of each reagent. About 0.5 μg of each RNA sample and a 0.2-μm final concentration of XDHi left and XDHi primers were used for each reaction. First-strand synthesis was performed at 50°C for 10 min. This step was followed immediately by 10 cycles of 94°C for 30 sec, 53°C for 30 sec, and 68°C for 45 sec, and then 30 cycles of 94°C for 30 sec, 53°C for 30 sec, and 68°C for 45 sec + 5 sec per cycle. Reactions were concluded at 68°C for 7 min. Five microliers of each reaction was analyzed on a 1.5% agarose gel.

**XDHi enzyme assay:** Crude extracts were prepared by homogenizing single medfly pupae or five Drosophila adult flies in 1.5 ml Eppendorf tubes in 80 μl cold buffer: 100 mM Tris, 1 mM EDTA, 0.5 mM NAD, and 0.05% 2-mercaptoethanol (pH 7.5). Each homogenate was centrifuged for 5 min at 13,000 rpm and 4°C. Supernatant was transferred to a new tube and centrifuged as before. Protein concentrations of each extract were measured using the bicinchoninic acid method and according to the manufacturer’s protocol (Pierce Chemical). Five microliers of each extract were loaded onto cellulose acetate gels that had been pretreated in a running buffer of 64 μm Tris, 4 mm EDTA, and 13.6 mm citric acid (pH 7.5). Gels were run at 100 V for 20–30 min. Gels were stained in Tris buffer with 1.4 mm hypoxanthine, 2.4 mm NAD, 0.4 mm phosphlane methosulfate (PMS), and 1.2 mm nitro blue tetrazolium (NBT) (or xanthine:PMScytC or xanthine:NAD+).

**RESULTS**

**C. capitata cDNA:** The full-length *C. capitata* cDNA sequence is 4397 bp in length, including the 5’ and 3’ untranslated sequences (UTR; GenBank accession no. AY014961). The coding region spans 4041 bp, with the ATG translational start codon located at position 223 and the TGA stop codon located at position 4264. The ATG at position 223 is presumed to be the correct translational start because it is the first methionine following several cryptic stop codons in the 5’ UTR, including one that is just eight codons upstream in the same reading frame. Furthermore, this start site facilitates the longest possible open reading frame that is consistent with the sizes of closely related XDHi sequences while the next potential start point lies 65 codons downstream. Within the 5’ UTR, a potential polyadenylation signal sequence, AATAAA, precedes the observed polyadenylation site of the cDNA by 20 bp. The cDNA encodes a peptide of 1347 amino acids when conceptually translated (Figure 1A). Highly con-
**Figure 1.**—Medfly XDH gene. (A) Structure of the CcXDH gene. Boxes represent exons 1 through 6 of indicated size and thin lines denote introns A through G. Conserved functional areas of enzyme are 2Fe/2S, iron-sulfur domains (stippled box); NAD$^+$, nucleotide binding site (shaded box); and MoCo, molybdenum cofactor (solid boxes). (B) Partial amino acid sequence alignments of highly conserved domains. Identical residues are boxed while conserved residues are shaded. Asterisks denote the eight cysteines and the single tyrosine implicated in XDH function.
served structural genes for XDH have been cloned from a wide range of species including bacteria, fungi, plants, insects, birds, and mammals (Keith et al. 1987; Houde et al. 1989; Riley 1989; Amaya et al. 1990; Terao et al. 1992; Ichida et al. 1993; Glatigny and Scazzocchio 1995; Sato et al. 1995; Berglund et al. 1996; Comeron and Aguade 1996). Of these, CxXDH is most similar in size and sequence to other insect XDH peptides. Overall, it is 74–75% identical to XDH peptides from C. vicina, D. melanogaster, D. pseudoobscura, and D. subobscura, all of which are dipteran flies. The medfly peptide is somewhat less similar to the two XDH peptides identified in the silkworm moth, B. mori (order Lepidoptera), displaying 58 and 52% identity to the B. mori 1 and B. mori 2 peptides, respectively. A partial alignment (Figure 1B) of the seven peptides from the six available insect species shows that XDH is highly conserved within the regions thought to bind iron-sulfur (2Fe-2S), flavin adenine dinucleotide, and molybdenum (MoCo) cofactors (Hughes et al. 1992; Sato et al. 1995; Doyle et al. 1996).

Notably, there are eight completely conserved cysteine residues, four in each iron-sulfur domain, that are likely to participate in the binding of iron-sulfur cofactors and a conserved tyrosine residue at position 407 that is likely to bind NAD\(^+\) (asterisks, Figure 1B). The cysteine residues correspond positionally with cysteines of the aldehyde oxido-reductase (Mop) from Desulfovibrio gigas that, on the basis of crystal structure analysis, have been directly implicated in iron-sulfur binding (Romao et al. 1995; Romao and Huber 1997). The tyrosine corresponds to the same residue of the chicken xanthine dehydrogenase that is reported to participate in NAD binding (Nishino and Nishino 1989).

Genomic DNA Southern blot analysis indicates that CxXDH is a single copy gene in the medfly (Figure 4A), as is the case in Drosophila (Keith et al. 1987). Furthermore, all phylogenetic analyses provide statistical corroboration of the findings of Komoto et al. (1999) where Drosophila XDH sequences formed a monophyletic grouping within a more inclusive dipteran clade, with the two Bombyx moth sequences being united in a separate clade. While our medfly sequence was firmly placed inside the dipteran clade, the relationships among medfly, C. vicina, and Drosophila XDH sequences varied among analyses. Some estimates of dipteran phylogeny suggest that the Tephritidae (including the medfly) are more closely related to the Drosophilidae than either is to the Calliphoridae (McAlpine 1989). However, more recent estimates made by comparing the sequences of two dipteran genes, glucose-6-phosphate dehydrogenase, G6pdh (Soto-Adames et al. 1994), and white (Gomulski et al. 2001), suggest that the Tephritidae are more closely related to the Calliphoridae than to the Drosophilidae. In this study, XDH from the calliphorid C. vicina was observed to group with either the Drosophila or with C. capitata in certain analyses. Nonetheless, the relationships among these three lineages were not strongly supported by bootstrap in any single analysis and are presented here as unresolved (Figure 2).

PCR analysis indicates that at least four introns are found within the CxXDH gene, and evidence is described for a fifth. In these studies, genomic DNA was used as a PCR template for a series of primers covering the XDH coding region, and products that were larger than cDNA control fragments were subcloned and sequenced. In this manner, four small introns (introns 2 through 5) were identified, ranging in length from 59 to 85 bp (Figure 1A and Table 1). The positions of three of these introns, D, F, and G (Komoto et al. 1999), are absolutely conserved when compared with the positions of introns of other insect species (Table 1), all of which are bounded by gtg splice site consensus sequences (Breathnach and Chambon 1981). We confirm the results of Tarrio et al. (1998), who discovered that one of these four introns (intron U in Figure 1A) is absent in all other reported insect XDH genomic sequences (Tarrio et al. 1998). This unique medfly intron does not correspond to the position of intron E (Komoto et al. 1999) and it is believed to be a duplication of intron D because their sequences are very closely related in the medfly (Tarrio et al. 1998).

There are also considerable lines of evidence suggesting the existence of a large first intron in medfly. Many of the introns within insect XDH genes are positionally conserved and all of the known insect XDH genes contain a commonly located intron A as their first, and largest, intron (Komoto et al. 1999). The size range of the first intron varies from 815 bp in D. melanogaster to 15 kb in C. vicina (Keith et al. 1987; Houde et al. 1989). Furthermore,

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**Figure 2.** XDH phylogram. Nucleotide sequences of insect XDH genes were aligned and phylogenetic relationships were assigned as described in MATERIALS AND METHODS. Numbers indicate bootstrap values. The XDH gene from Mus musculus was used as an outgroup.
TABLE 1

<table>
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<tr>
<th>Conserved intron positions</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<td>—</td>
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<td>85</td>
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<td>—</td>
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<td>—</td>
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<td>—</td>
<td>62</td>
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<td>455</td>
<td>76</td>
<td>—</td>
<td>—</td>
<td>165</td>
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</table>

Conserved intron positions A through G are lettered according to Komoto et al. (1999). The size of each intron is listed in base pairs except where indicated in kilobase pairs. Hyphens denote the lack of a particular intron within a species. Intron U is unique to C. capitata (Tarrio et al. 1998).

Southern blots of medfly genomic DNA, probed with PCR products derived from cDNA spanning the putative splice site, hybridize with large molecular weight bands, indicating an intron size of >5 kb (data not shown), and several attempts to PCR amplify the first intron region of the XDH gene from wild-type genomic DNA failed. Nonetheless, genomic PCR reactions designed to amplify the exons immediately surrounding the putative intron site gave products of the expected length for cDNA and therefore indicate that these regions themselves are uninterrupted by introns (data not shown).

**Rescue of Drosophila ros[309] mutant:** To demonstrate that CxXDH encodes a functional xanthine dehydrogenase enzyme in vivo, phenotypic rescue experiments were carried out using the well-established P-element transformation protocols available in D. melanogaster (Rubin and Spradling 1982; Ashburner 1989). Prior to transformation, and as an indication that the CxXDH cDNA could be fully translated, a peptide of ~150 kD corresponding to the expected size for the full-length XDH monomer (Edwards et al. 1977; Keith et al. 1987) was observed in an in vitro rabbit reticulocyte translation system (data not shown). The full-length CxXDH cDNA was subsequently cloned into the P-element transformation vector, pP[CaSpeR-hs/act], which utilizes a white mini gene for transformation selection, a 3′ heat-shock promoter to drive expression of the insert, and 3′ Act 5C sequences to promote transcript stability. This construct was introduced into the germline of white, forked recipient D. melanogaster flies that were then crossed into an appropriate ry− background. Ultimately, two transformed lines, B and I, each having X chromosome insertions, were chosen for detailed rescue analyses. The results from one such experiment with line B are presented in Figure 3 and are representative of repeated analyses with both lines B and I. In these studies, all G4 females inherit the CxXDH transgene and those with stubble bristles are homozygous for the ry mutation (see Materials and Methods). Under heat-shock conditions, all these flies have wild-type eye pigmentation, indicating that the CxXDH transgene was both functional and apparently able to fully complement the ry mutant phenotype (Figure 3D). In contrast, the G4 stubble males, which do not carry the X-linked XDH transgene and are also ry homozygotes, retained the ros[309] eye mutant phenotype under the same heat-shock conditions and served as an internal negative control (Figure 3C). This experiment establishes the CxXDH as the functional ortholog of the Drosophila XDH.

**Medfly ros[309] mutant:** To initially address the possibility that the medfly ros[309] mutant carries a defect in the XDH gene, we carried out comparative Southern blot and PCR analyses between genomic DNA prepared from wild-type and ros[309] medflies. Inasmuch as identical bands were observed in the genomic PCR reactions designed to amplify the exons immediately surrounding the putative intron site gave products of the expected length for cDNA and therefore indicate that these regions themselves are uninterrupted by introns (data not shown). That the medfly ros[309] mutant carries a defect in the XDH gene, we carried out comparative Southern blot and PCR analyses between genomic DNA prepared from wild-type and ros[309] medflies. Inasmuch as identical bands were observed in the genomic PCR reactions designed to amplify the exons immediately surrounding the putative intron site gave products of the expected length for cDNA and therefore indicate that these regions themselves are uninterrupted by introns (data not shown).
observed in a series of side-by-side reactions covering the entire coding region of the gene, these comparisons show the medfly rosy XDH gene to be indistinguishable from the wild-type gene (Figure 4A; PCR data not shown). Furthermore, RT-PCR comparisons demonstrated the presence of indistinguishable XDH transcripts in RNA isolated from three developmental stages of wild type and one stage of the rosy mutant all show XDH transcript expression. Oligonucleotide primers spanning intron F result in products of 743 and 658 bp in genomic and cDNA controls, respectively.

Figure 4.—XDH gene analysis in medfly. (A) Southern blot displaying single copy nature of medfly XDH gene. + and ry indicate Benakeion and rosy genomic DNAs, respectively, while numbers indicate sizes in kilobase pairs. Bands of 0.9 kb and 1.3 kb were expected for AccI digests while a band of 2.5 kb was expected for AvaII digests. (B) RT-PCR analysis of RNA isolated from three developmental stages of wild type and one stage of the rosy mutant all show XDH transcript expression. Oligonucleotide primers spanning intron F result in products of 743 and 658 bp in genomic and cDNA controls, respectively.

DISCUSSION

XDH cloning: The XDH gene of C. capitata shares considerable nucleotide and amino acid sequence identity with XDH genes of other insect species (Figure 1B). Comparison of the known, complete XDH nucleotide sequences produces the phylogenetic tree in Figure 2. Not surprisingly, the C. capitata XDH protein sequence is most closely related to other sequences from dipteran species and less similar to the sequences of the two XDH genes of the lepidopteran, B. mori. Given that the dipteran XDHs are all single copy genes, their relationships might reasonably be considered orthologous, or all derived from a common ancestor gene by speciation in the insect lineages. Further evidence for XDH orthology was presented by Komoto et al. (1999), who noted that upstream of BmXDH2 is a region homologous to 1(3)12s, a gene that is similarly located upstream of the XDH genes of D. melanogaster, D. pseudoobscura, and D. subobscura (Riley 1989; Dutton and Chovnick 1991; Comeron and Aguade 1996). The apparent XDH duplication event within B. mori thus occurred after the divergence of the dipteran and lepidopteran orders (Komoto et al. 1999).

As indicated for the white genes of several insect species (Gomulski et al. 2001), the evolutionary relationships of the reported species are also reflected by the conservation of intron positions within the XDH gene (Komoto et al. 1999). For example, the intron positions A, B, F, and G of the C. capitata XDH gene are conserved when compared with the intron positions for several other insect species (Komoto et al. 1999). It is interesting to note that the medfly gene lacks introns B, C, and E, introns that may have been lost during the evolution of the XDH gene. This is reminiscent of the loss of introns that has been suggested for introns B, C, E, and F of the XDH gene of D. melanogaster (Komoto et al. 1999). Alternatively, those introns may have been gained later.
Figure 5.—XDH enzyme activity. (A) Extracts from wild-type and rosy mutant medflies display XDH enzyme activity when hypoxanthine or xanthine are used as substrates. Similarly prepared Drosophila extracts serve as controls. (B) The chemical reactions catalyzed by XDH include the conversion of hypoxanthine to xanthine and xanthine to uric acid.

in XDH evolution and therefore persist in the genes of B. mori and mammals (Komoto et al. 1999). Recent evidence supports the introns-gained hypothesis through the comparison of several partial XDH sequences (Tarrio et al. 1998).

We find 35 polymorphic nucleotides between our sequence and the partial sequence of 2085 bp reported by Tarrio et al. (1998), which would lead to eight amino acid substitutions. These differences probably reflect XDH allelic differences within the same laboratory strain, since both studies used Benakeion DNA, a fact that is not surprising given that several XDH allozymes have already been described in the medfly (Malacrída et al. 1992). Furthermore, multiple XDH alleles have been identified in a laboratory population of C. vicina (Rocher-Chambonnet et al. 1987; Houde et al. 1989) and a high level of heterozygosity has been observed at the XDH locus in D. pseudoobscura (Singh et al. 1976).

The XDH gene of C. capitata was shown to encode an active XDH enzyme by its ability to rescue the eye color phenotype of the D. melanogaster mutant, ry506, which lacks any detectable XDH activity (for review see Chovnick et al. 1990). Homozygous ry506 Drosophilas that carried a heat-shock-driven CxXDH cDNA had normal, bright red eyes, while those lacking the CxXDH had the deep red eyes characteristic of the rosy mutant (Figure 3, C and D) even under heat-shock conditions. This result demonstrates that the CxXDH cDNA encodes a functional enzyme that is also biologically active in heterologous species separated by over 100 million years of evolution (Beverly and Wilson 1984). Heterologous rescue of the Drosophila rosy mutant has been previously shown using a chimerical XDH containing the C-terminal portion of the D. melanogaster XDH including the 5’ UTR (Tiveron et al. 1991). These results indicate that CxXDH has the necessary functionality for use as a marker for germline transformation within the medfly.

rosy-like mutant: The most likely C. capitata XDH mutant isolated to date, rosy, displays a deep red eye color and a sensitivity to purine supplemented growth media, both characteristic of Drosophila rosy (i.e., XDH) mutants (Saul 1982). Although red drosopterin pigments have not yet been identified in wild-type medflies, XDH allelic differences within the same laboratory strain, since both studies used Benakeion DNA, a fact (Ziegler and Feron 1965), it remains unclear as to whether or not they are present in medfly eyes. Indeed, the existence of the medfly rosy mutant provides strong, albeit circumstantial, evidence for their presence. Further study of eye pigments in the medfly may also lend insight into the nature of the medfly rosy mutant. For example, because the Drosophila rosy mutant specifically lacks isoxanthopterin and accumulates 2-amino-4-hydroxypterin, the pteridine product and substrate of the XDH enzyme, respectively (Reaume et al. 1991), a similar pattern might be expected to occur in rosy medflies. It is also formally possible that the medfly lacks drosopterin pigments and the rosy phenotype is the result of a defect in a gene unrelated to XDH.

Interestingly, the medfly XDH gene has been genetically mapped by allozyme analysis to the same position as the medfly rosy mutant on linkage group D, the genetic element that has subsequently been renamed chromosome 2 (Saul and Rossler 1984; Saul 1986; Malacrída et al. 1990, 1992). In fact, cytological mapping by in situ hybridization to polytene chromosomes of the CxXDH cDNA reported here has further refined its location to section 4C of the long arm of chromosome 2 (A. R. Malacrída and C. Torti, personal communica-
tion). As more genetic markers become available in medfly, finer mapping studies of rosy should be possible, either confirming or rejecting its potential correlation to XDH. Given the similar phenotypes of medfly rosy and Drosophila rosy, as well as the correlation of CxXDH with the rosy mutant on the medfly genetic map, we examined the medfly rosy mutant for potential abnormalities at the XDH locus.

While we were unable to detect gross DNA differences such as large deletions, insertions, or chromosomal rearrangements between the wild type and rosy mutant medflies in our genomic analyses (Figure 4A), we could not eliminate the possibility that point mutations, small deletions, or small insertions may lie within the XDH coding region of the rosy mutant. This is especially relevant since the rosy mutant was generated by formaldehyde treatment of medfly eggs, a process thought to cause small DNA mutations (Saul 1982). To further compare the XDH gene products, we carried out a reverse transcription/PCR analysis of wild-type and rosy RNAs. In these studies, indistinguishably sized XDH transcripts could be amplified from several development stages of wild-type medflies as well as rosy pupae (Figure 4B). These data show a similar developmental pattern of XDH transcript expression in wild-type medflies as in wild-type Drosophila (Covington et al. 1984). Furthermore, the XDH expression that was detected in rosy pupae occurs at a time that is relevant for eye color development in Drosophila and is therefore likely to be equally important for the medfly (Barrett and Davidson 1975). Lastly, the primers used for RT-PCR expression studies were located near the 3' end of the gene and as such indicate that the transcript is likely to be full length and stable. Taken together, these data suggest that the rosy mutant might not be the result of the alteration or loss of XDH transcripts. Again, as with the DNA analysis, these data neither rule out the possibility that small, mutagenic changes exist within the XDH coding region and are responsible for the mutant phenotype, nor eliminate the possibility that the transcript is otherwise improperly translated but the protein remains functional in vitro.

In the absence of a functional antiserum against medfly XDH that might be used to directly assay protein levels, we attempted to address the possibility that the medfly rosy mutant lacks or has aberrant levels of XDH enzymatic activity. In these studies, we examined the ability of crude extracts from wild type as well as rosy medflies to reduce xanthine or hypoxanthine in vitro and to produce a colorimetric reaction product on cellulose acetate gels. Importantly, we were able to take advantage of the availability of well-characterized Drosophila wild-type and y^50k strains for the preparation of extracts to serve as positive and negative controls for XDH activity, respectively. These data (Figure 5A) clearly demonstrate that the medfly rosy mutant retains considerable, if not wild-type, levels of XDH enzymatic activity in vitro. Whether or not the mutant has XDH activity in vivo has not been directly examined. In Drosophila, XDH is synthesized in the fat bodies surrounding the eye and must be transported into the eye for normal pigmentation to develop (Barrett and Davidson 1975; Reaume et al. 1989, 1991). It is possible that the medfly rosy mutant may produce an enzyme in vivo that is either inactive or not expressed in the proper tissues or at the proper time during development. It is also formally possible that our assay system using crude extracts cannot detect specific mutations, particularly point mutations, within the XDH enzyme that would alter, but not eliminate, XDH activity. Several detailed Drosophila studies have been carried out that define functional domains of the XDH peptide where known single amino acid changes in rosy mutants affect XDH activity (Hughes et al. 1992; Hughes 1992; Doyle et al. 1996). In those experiments, extracts from some rosy mutants, such as G800E and G1011E, show relatively high levels of in vitro activity in some assays, especially following mild oxidation (Hughes et al. 1992; Hughes 1992; Doyle et al. 1996). Therefore we cannot rule out the possibility that the rosy mutant of medfly may be the result of a point mutation in XDH that is not discernible in our enzyme activity assays or that mild oxidation of XDH occurs during the extraction process.

On the basis of our examinations of the CxXDH locus at the levels of DNA, RNA, and enzyme activity, we cannot support, nor conclusively rule out, the possibility that the medfly rosy phenotype is caused by a mutation at the CxXDH locus. Definitive evidence would best be obtained by performing medfly transformation rescue experiments using rosy medflies and an appropriate expression construct with the functional, wild-type cDNA reported here along with either the Minos or piggyBac vectors that have been previously used in the generation of transgenic medflies (Loukeris et al. 1995; Handler et al. 1998). However, because of the absolute quarantine against live medflies in the continental United States, we are prevented from carrying out this final test. Such an experiment would help define the potential of the rosy mutant to be used in combination with the wild-type cDNA as a marker for germline transformation experiments in medfly. Aside from its proposed function as a visible marker, the wild-type CxXDH may be useful as a selectable marker if it can restore purine resistance alone in the medfly rosy mutant and may thereby contribute to population control strategies.

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