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Rapid Communication

Transient Expression of the *Drosophila melanogaster cinnabar* Gene Rescues Eye Color in the White Eye (WE) Strain of *Aedes aegypti*

ANTHONY J. CORNEL,†‡§ MARK Q. BENEDICT,†‡ CRISTINA SALAZAR RAFFERTY,†
ANTONY J. HOWELLS,‖ FRANK. H. COLLINS*†‡

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The lack of eye pigment in the *Aedes aegypti* WE (white eye) colony was confirmed to be due to a mutation in the *kynurenine hydroxylase* gene, which catalyzes one of the steps in the metabolic synthesis of ommochrome eye pigments. Partial restoration of eye color (orange to red phenotype) in pupae and adults occurred in both sexes when first or second instar larvae were reared in water containing 3-hydroxykynurenine, the metabolic product of the enzyme kynurenine hydroxylase. No eye color restoration was observed when larvae were reared in water containing kynurenine sulfate, the precursor of 3-hydroxykynurenine in the ommochrome synthesis pathway. In addition, a plasmid clone containing the wild type *Drosophila melanogaster* gene encoding kynurenine hydroxylase, *cinnabar (cn)*, was also able to complement the *kynurenine hydroxylase* mutation when it was injected into embryos of the *A. aegypti* WE strain. The ability to complement this *A. aegypti* mutant with the transiently expressed *D. melanogaster cinnabar* gene supports the value of this gene as a transformation reporter for use with *A. aegypti* WE and possibly other Diptera with null mutations in the *kynurenine hydroxylase* gene. Published by Elsevier Science Ltd

Aedes aegypti Transient expression *cinnabar* gene

INTRODUCTION

Transgenesis will undoubtedly become an important component of molecular and genetic studies of agriculturally and medically important arthropods. In spite of the large number of different transposon-based transformation systems developed for use with *Drosophila melanogaster* since 1982, success in efficiently transforming other arthropods with a transposon-based gene delivery system has been limited to the Mediterranean fruitfly *Ceratitis capitata* (Loukeris *et al.*, 1995). One of the major limitations has been the availability of markers whose expression in the insect of interest can be verified without reliance on transformation itself.

In *D. melanogaster* and *C. capitata*, the favored marker has been the *white* gene, which encodes an ABC transporter protein responsible for the importation and assembly of pigments in the compound eyes of these and probably most insects (Ewart *et al.*, 1994). *White* genes have now been cloned from a number of insects in addition to *D. melanogaster* and *C. capitata* (O'Hare *et al.*, 1984; Zwiebel *et al.*, 1995), including the mosquitoes *Anopheles gambiae* (Besansky *et al.*, 1995), *An. albimanus* (Ke *et al.*, in press), *Aedes aegypti* (Coates *et al.*, 1997), and several Tephritids of the genus *Bactrocera* (S. Saul, personal communication). Mutations which result in white or abnormal eye color phenotypes are also

*To whom all correspondence should be addressed at: Department of Biological Sciences, Galvin Life Sciences, University of Notre Dame, Notre Dame, IN 46556, U.S.A. Tel: (219) 631 9245, Fax: (219) 631 7413; E-mail: frank.h.collins.75@nd.edu
†Entomology Branch, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway Chamblee, GA 30341, U.S.A.
‡Department of Biology, Emory University, Atlanta, GA 30322, U.S.A.
§Present address: University of California, Mosquito Control Research Laboratories, Kearney Agricultural Experimental Field Station, 9240h Riverbend Ave, Parlier, CA 93648, U.S.A.
‖Division of Biochemistry and Molecular Biology, Faculty of Science, The Australian National University Canberra, ACT, 2601 Australia

known from a large number of different insects, and at least in the cases of *An. gambiae* and *An. albimanus*, some of these lesions are due to mutations in the *white* gene (Benedict *et al.*, 1996; Ke *et al.*, in press). Unfortunately, the *white* gene product is cell autonomous, so validation of the utility of a particular *white* gene construct as a marker or testing of the ability of a white gene clone to complement known eye color mutations can be done only by transformation.

Another class of well characterized eye color mutations in insects may show more promise as transformation markers. These are in the genes encoding enzymes involved in the synthesis of ommochrome eye pigments from the amino acid tryptophan (Hiraga, 1964). Mutations in two of the *Drosophila* genes encoding enzymes in this pathway, *vermilion*, which encodes tryptophan oxygenase, and *cinnabar*, which encodes kynurenine hydroxylase, are known from several insects (Bhalla, 1968; Searles *et al.*, 1990; White *et al.*, 1996; Warren *et al.*, 1996). Furthermore, these enzymes are non-cell autonomous. Constructs based on cDNAs of the *D. melanogaster vermilion* (*v*) gene and its *An. gambiae* homologue *tryptophan oxygenase* (*to*) have already been evaluated in transient complementation experiments. The *D. melanogaster v* construct was shown to be capable of transiently rescuing eye color in a strain of *Musca domestica* with a mutation in the *tryptophan oxygenase* gene (White *et al.*, 1996), and an *An. gambiae to* construct successfully complemented a null *v* mutation in *D. melanogaster* (Besansky *et al.*, in press).

We have examined two strains of *A. aegypti* with mutations on different complementation groups that result in a white eye phenotype. One is a spontaneous mutation recently isolated from a wild-type colony by Stephen Higgs of Colorado State University (S. Higgs, unpublished data). The other, which was obtained from the *A. aegypti* stock center at the University of Notre Dame, is identified as the *A. aegypti* WE strain, which had been described by Bhalla in 1968 as having a mutation in the *kynurenine hydroxylase* gene.

Because the *D. melanogaster* homolog of this gene, *cinnabar* (*cn*), had recently been cloned (Warren *et al.*, 1996) and two potential *tryptophan oxygenase* reporter constructs were available (White *et al.*, 1996; Besansky *et al.*, in press), we determined whether these two *A. aegypti* strains had mutations in the ommochrome synthesis pathway and tested the available cloned *cn* gene and/or the *tryptophan oxygenase* constructs as potential reporters.

MATERIALS AND METHODS

Second instar larvae of the *A. aegypti* Higgs and WE mutant strains were reared at concentrations of 0.1 and 0.5 mg ml^{-1} of kynurenine sulfate and 0.05, 0.1, and 0.5 mg ml^{-1} of 3-hydroxykynurenine. Eye color change was monitored in fourth stage larvae, pupae and adults. In each of the pigment precursor solutions, the larvae were fed on rabbit chow pellets and kept at 27°C. Embryos of the *A. aegypti* WE strain were collected at 27°C for 30 min on damp filter paper disks in 100 × 15 mm polystyrene petri dishes. After aging the embryos for an additional 30 min until they had almost fully darkened, they were aligned side-by-side on damp filter paper in groups of approx. 30. A narrow strip of double stick tape (3M type 415) attached to a microscope slide was pressed onto the embryos, and the filter paper was removed. After drying the embryos until they had begun to show slight surface depression, they were covered with water-saturated mineral oil (Sigma Chemical Co., St Louis MO). They were immediately injected with approx. 0.5 pl of the Qiagen-purified plasmid pcnXK7.8 (Warren *et al.*, 1996) at a concentration of 0.5 $\mu g\ \mu l^{-1}$ in injection buffer (0.1 mmol $NaPO_4$, 5 mmol KCl, pH 7, Rubin and Spradling, 1982). This plasmid contains a 7.8 kb *Xho* I-*Kpn* I genomic fragment containing the entire *cn* gene, which possess two introns. Sequence and structure of this plasmid are described in Warren *et al.*, 1996. Needles were made from unbeveled thin-wall capillaries (World Precision Inst. no.TW120F-4). After injection, the oil was removed by blotting and the embryos were stored at 27°C for five days. Eggs were then hatched and examined at the larval, pupal and adult stages.

RESULTS AND DISCUSSION

The eyes of fourth instar larvae, pupae, and adults of the *A. aegypti* WE strain whose larvae were reared in solutions of 3 hydroxykynurenine (3HK) showed some rescue from white eye color to various shades of red (Fig. 1), visible in both the compound eye and in the stemma (ocellus-like light perceptive organs). At the lowest concentration of 3HK (0.05 mg ml^{-1}), approx. 10% of the larvae and pupae developed slight pigmentation in the stemma but not in the compound eye (Table 1). At the intermediate concentration of 3 HK (0.1 mg ml^{-1}), about 40% of the larvae and 60% of the pupae had some pigment deposition in the stemma of the immature stages and two females showed some slight red pigmentation in the compound eye in the pupal and adult stages. At the 0.5 mg ml^{-1} 3HK treatment concentration, more than 95% of the individuals showed distinct red eye color at all three stages. No pigmentation of the adult ocellus was observed at any of the 3HK concentrations.

No changes in the eye color were observed in the *A. aegypti* WE strain individuals reared in the 0.1 and 0.5 mg ml^{-1} solutions of kynurenine sulfate, although both of these treatment concentrations were sufficient to rescue eye color in a *D. melanogaster* strain with null mutations at both the *v* and *bw* loci (data not shown), indicating that the treatment concentrations should be sufficient for detecting mutations in the *tryptophan oxygenase* gene. [Note that while the only visible eye pigments in mosquitoes are the ommochromes (Beard *et al.*, 1995), *Drosophila* have both ommochrome and pterin

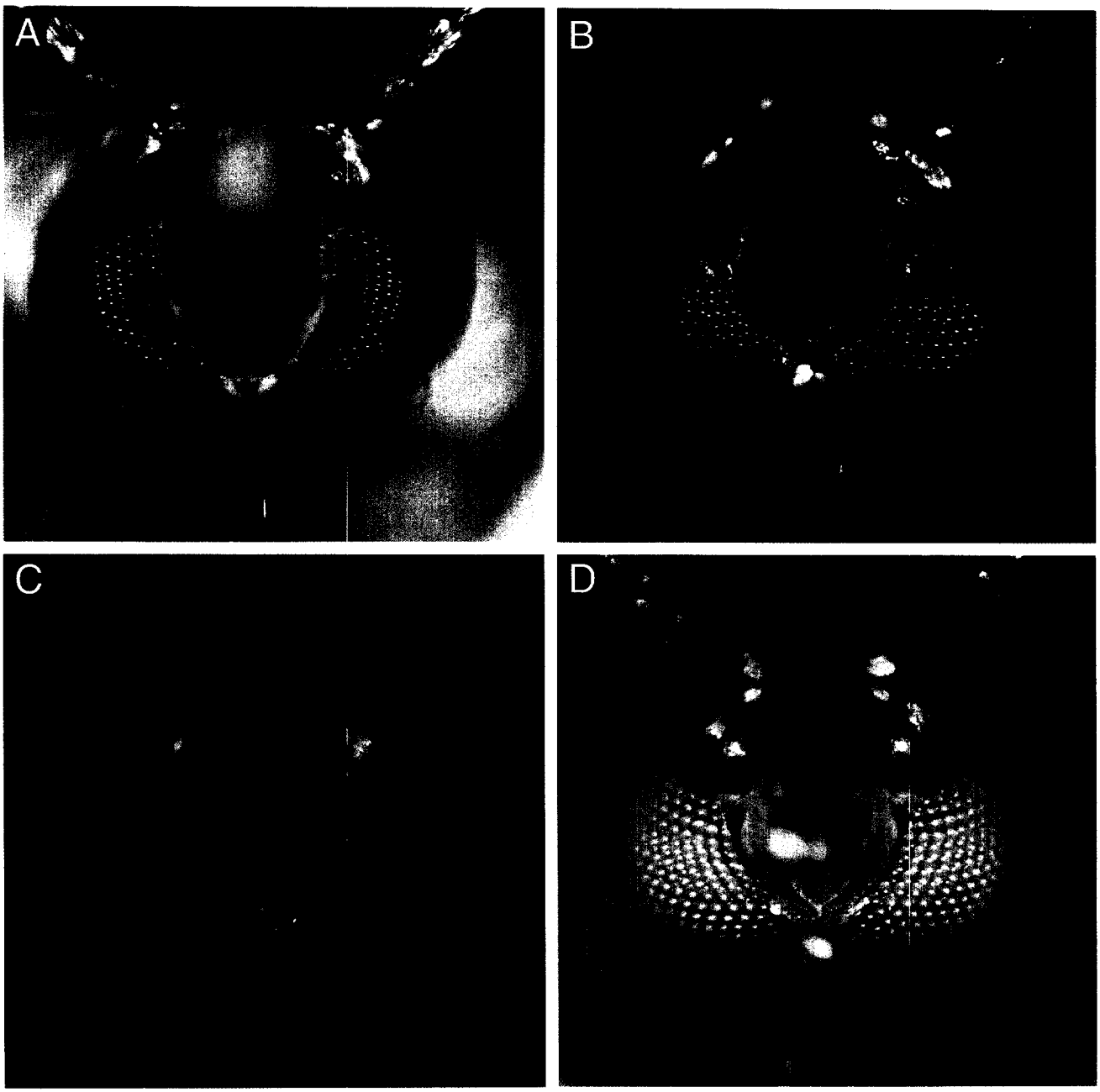

FIGURE 1. Compound eye color restoration of the *A. aegypti* WE strain. Adult compound eyes are shown in panels A–D, larval in E and F and pupal in G and H. (A) Wild-type *A. aegypti;* (D, E and G) *A. aegypti* WE strain; (B, F and H) shows eye color observed after rearing *A. aegypti* WE strain in a solution of 0.5mg ml^{-1} of 3 hydroxykynurenine; (C) shows eye color observed after injecting preblastula *A. aegypti* WE strain with a *D. melanogaster cn* genomic clone pcnXK7.8.

eye pigments. The *bw* locus is responsible for importation of pterin pigments, so the *bw, v* double mutant *D. melanogaster* have white eyes.] These results confirm that the mutation in the *A. aegypti* WE strain is in the kynurenine hydroxylase gene, as originally demonstrated by Bhalla (1968).

No change in eye pigmentation was observed in the *A. aegypti* Higgs strain reared in either kynurenine sulfate or 3HK. Genetic analysis suggests that the mutation in the *A. aegypti* Higgs strain may be a defect in pigment transport rather than pigment biosynthesis.

To test the ability of the *D. melanogaster cn* gene to transiently complement the *kynurenine hydroxylase* mutation in the *A. aegypti* WE mutant strain, the *D. melanogaster cn* gene with its native promoter, was injected into *A. aegypti* WE embryos. From 217 plasmid-injected embryos, 32 surviving G_0 adults were obtained. Three females and two males had partial restoration of eye color, which was first noted in the late pupal stage. In these individuals, the complementation was clearly evident and visible in varying intensities of red [Fig. 1(D)]. The remaining 27 individuals from the plasmid-injected group showed equivocal evidence of complementation, with some showing faint coloring suggesting low-level

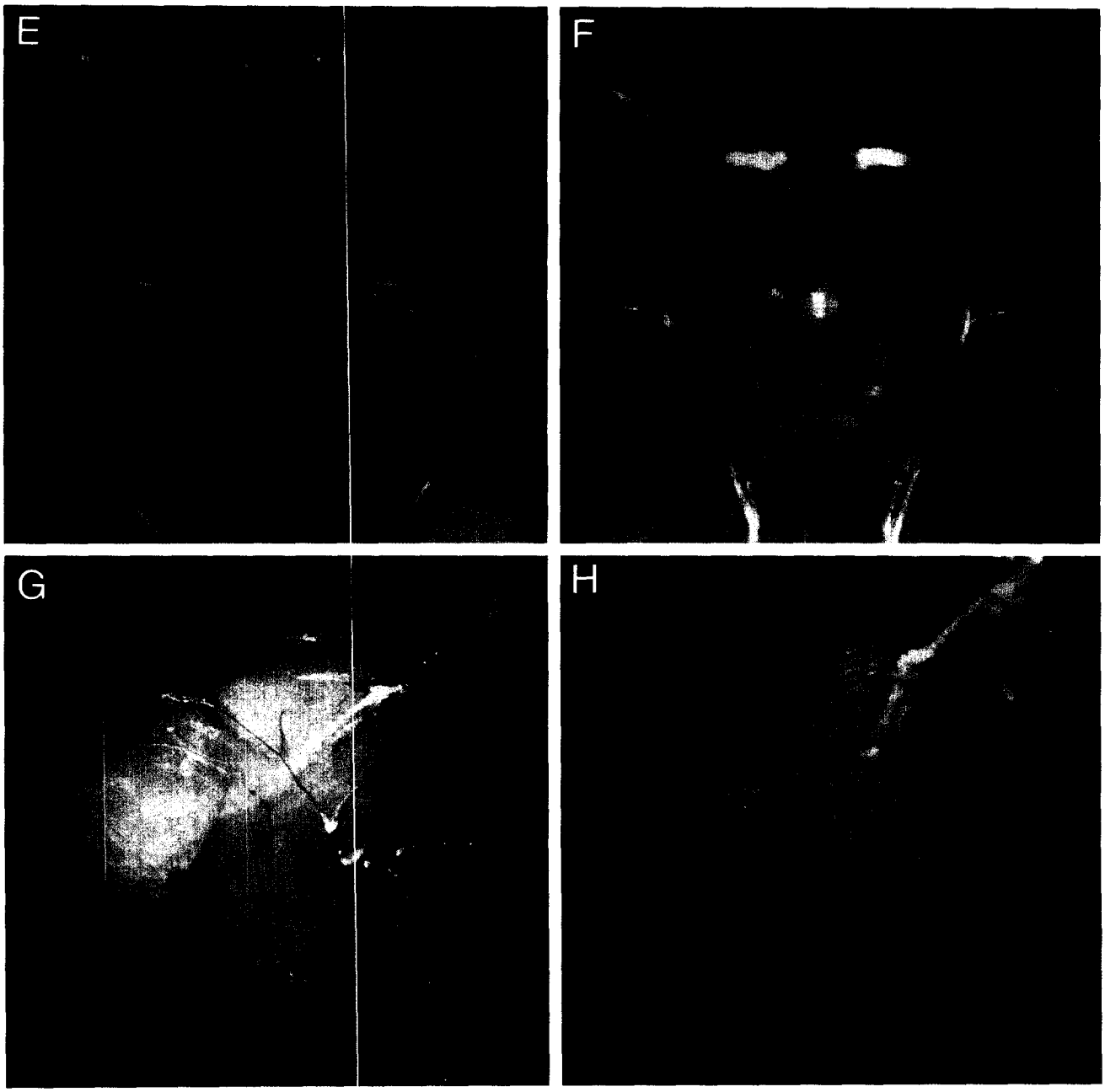

FIGURE 1.–*continued*

TABLE 1. Results of rearing *A. aegypti* WE strain larvae in solutions of 3 hydroxykynurenine

3HK conc.	Larvae		Pupae				Adults			
			Male		Female		Male		Female	
	p	np	p	np	p	np	p	np	p	np
0.05 mg ml^{-1} (39)	2	37	3	21	1	14	0	24	0	15
0.1 mg ml^{-1} (42)	17	25	18	2	8	14	0	20	2	20
0.5 mg ml^{-1} (61)	59	2	38	0	20	2	38	0	18	2

p = Pigment; np = no pigment; Numbers in parentheses refer to sample size.

complementation. No eye color rescue was observed in a control group of adults developing from buffer-injected embryos, and no reversion to partially wild type eye color phenotype has been seen among thousands of *A. aegypti* WE strain adults observed.

These results confirm that the white eye color of the *A. aegypti* WE strain is caused by a mutation in its *kynurenine hydroxylase* gene and show that the wild type *D. melanogaster cn* gene is capable of complementing this mutation. It is important to note that the *D. melanogaster cn* promoter is recognized and the two intron are apparently correctly sliced by *A. aegypti*. This argues strongly for the use of the native *D. melanogaster cn* gene as a transformation marker in *A. aegypti* WE strain mosquitoes.

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