Analysis of the blind *Drosophila* mutant *ninaB* identifies the gene encoding the key enzyme for vitamin A formation *in vivo*

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Visual pigments (rhodopsins) are composed of a chromophore (vitamin A derivative) bound to a protein moiety embedded in the retinal membranes. Animals cannot synthesize the visual chromophore *de novo* but rely on the uptake of carotenoids, from which vitamin A is formed enzymatically by oxidative cleavage. Despite its importance, the enzyme catalyzing the key step in vitamin A formation resisted molecular analyses until recently, when the successful cloning of a cDNA encoding an enzyme with β,β-carotene-15,15′-dioxygenase activity from *Drosophila* was reported. To prove its identity with the key enzyme for vitamin A formation *in vivo*, we analyzed the blind *Drosophila* mutant *ninaB*. In two independent *ninaB* alleles, we found mutations in the gene encoding the β,β-carotene-15,15′-dioxygenase. These mutations lead to a defect in vitamin A formation and are responsible for blindness of these flies.

The vertebrate and invertebrate phototransduction system is a rhodopsin-based G protein-coupled signaling cascade displaying exquisite sensitivity and broad dynamic range. The visual stimulus triggers a change of the ionic permeability of the photoreceptor cell membranes (1, 2). To analyze the molecular mechanisms of the visual process, *Drosophila* has served for decades as a model system for studies using electrophysiology, photochemistry, genetics, and molecular biology (1, 3, 4). Powerful screening strategies and subsequent genetic dissection led to the characterization of several mutants affected in different parts of the visual process, e.g., in the protein moiety of the visual pigments (*ninaE*; ref. 5), rhodopsin assembly (*ninaA*; ref. 6), and signal transduction (*norpA*, *rdgA*, *arr1*; refs. 7–9). The molecular identification of most of the components responsible for the phototransduction system has since been achieved. However, molecular characterization of mutants affecting the formation of the visual chromophores still is missing.

In both the vertebrate and invertebrate visual systems, vitamin A derivatives such as retinal or 3-hydroxyretinal serve as chromophores for the various visual pigments (rhodopsins; refs. 10 and 11). In vertebrates, besides a role in vision, vitamin A and its derivatives exert multiple functions in development, embryogenesis, and cell differentiation (12, 13), whereas in invertebrates vitamin A functions seem to be restricted to the visual system (14). Animals in general are not able to synthesize vitamin A *de novo*. For the formation of the visual chromophores, they rely on the uptake in the diet of carotenoids with provitamin A activity or on preformed retinoids from animal food sources. The key step in the formation of vitamin A was shown 40 years ago to be the oxidative cleavage of carotenoids by carotenoid dioxygenases (15). Molecular data on this important reaction have been missing since then, and there is a controversy regarding the reaction mechanism (16). A breakthrough in understanding the oxidative cleavage reaction of carotenoids came from earlier work on a carotenoid-cleavage enzyme in the pathway leading to the plant-growth regulator abscisic acid in maize and other plants. In plants, various carotenoid-cleavage products have been described, e.g., saffron in crocus, citraurin and other apocarotenals in citrus fruits, and abscisic acid. The analysis of the maize mutant, *vp14*, led to the identification of the gene and cDNA encoding a 9-cis-epoxycarotenoid dioxygenase, the key enzyme in the formation of abscisic acid (17, 18). By sequence similarity to this plant enzyme, a cDNA encoding a putative animal carotene dioxygenase could be isolated from *Drosophila melanogaster* in our laboratory (19). Heterologous expression in *Escherichia coli* and subsequent biochemical analyses revealed that the encoded protein, β,β-carotene-15,15′-dioxygenase (β-diox), was able to catalyze the symmetric oxidative cleavage of β-carotene to form two molecules of vitamin A aldehyde (retinal). Soon thereafter, a cDNA encoding its vertebrate homologue was identified (20). Furthermore, in the databases there are cDNAs encoding putative β-carotene dioxygenases from several species, including human. Sequence comparison showed a high overall sequence similarity among the animal enzymes and a significant similarity to some domains of the plant dioxygenase (Fig. 1). This similarity indicates that the animal proteins belong to a large and diverse class of dioxygenases heretofore described only in plants and microorganisms (21). The identification of cDNAs encoding β-diox may provide the key toward understanding vitamin A formation in animals. However, direct evidence that the encoded proteins catalyze vitamin A formation *in vivo*, and therefore the generation of the visual chromophores is still missing.

Therefore, we searched for *Drosophila* mutants affecting vision. Among the various mutants, two mutants, *ninaB* and *ninaD*, have been described (22) for which the corresponding genes have not yet been identified. Both mutants exhibit a strongly reduced visual sensitivity and display an abnormal electroretinogram (*nina*: *n*either *i*nactivation *n*or *a*fterpotential). The mutant phenotypes can be rescued by supplementing the diet with retinal, the direct precursor of the fly's visual chromophore 3-hydroxy-retinal. However, the *ninaD* phenotype can be rescued also by feeding high doses of β-carotene, and therefore it has been concluded that a function involved in carotenoid uptake, transport, or storage is affected by this mutation (23). In contrast, the *ninaB* phenotype can be rescued exclusively by feeding of retinal but not by high dosages of β-carotene (22). This finding led us to infer that a mutation in the recently characterized β-diox gene might cause this phenotype. This possibility was strengthened by the fact that the *ninaB* mutation has been mapped to the position 87E-F (22) in the *Drosophila* genome,coinciding with the physical location of the β-diox gene.

Abbreviations: β-diox, β,β-carotene-15,15′-dioxygenase; RT, reverse transcription.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ276682).

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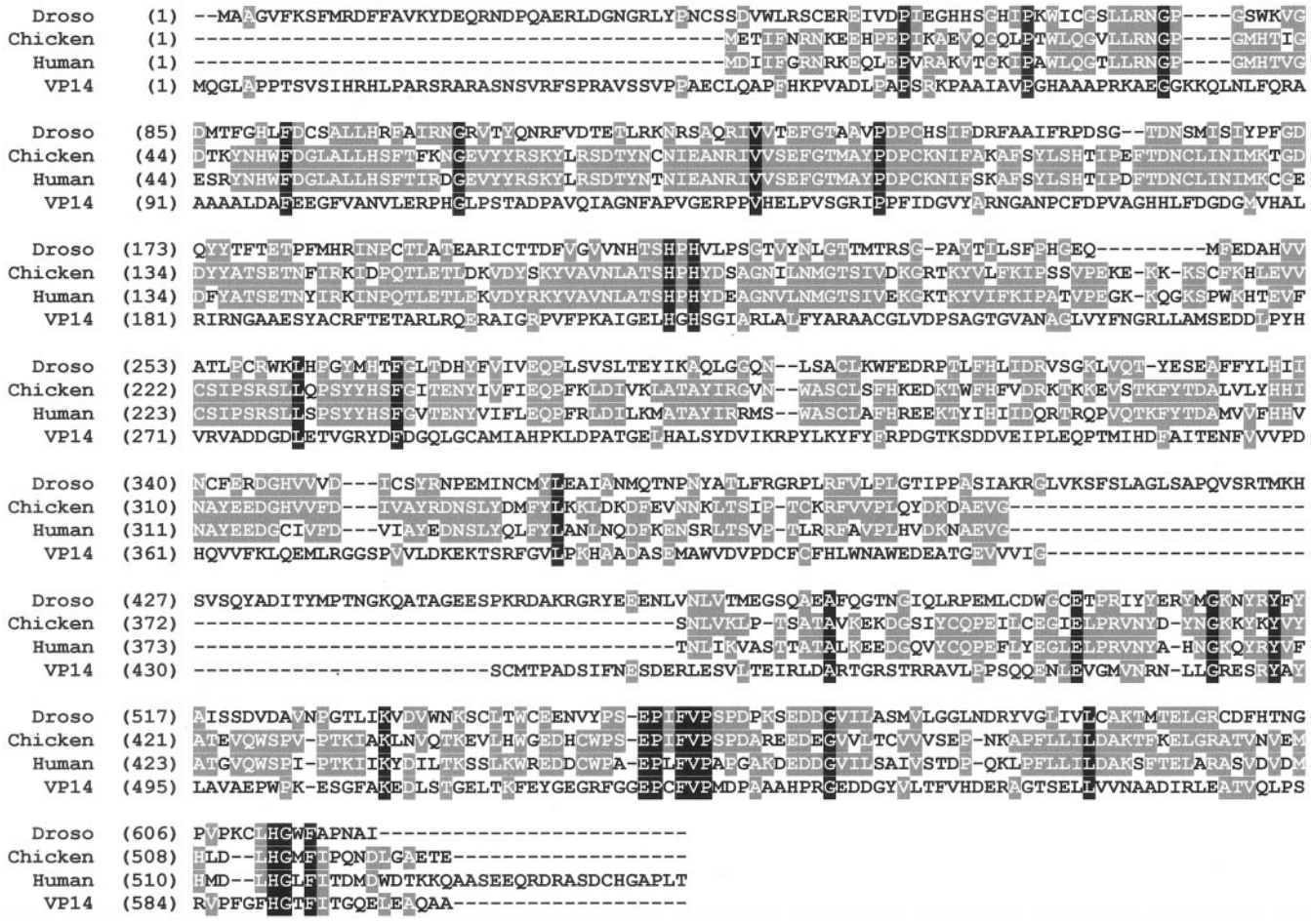

Fig. 1. Linear alignment of the deduced amino acid sequences of the β-diox from *Drosophila* compared with its vertebrate homologues from chicken (20) and human (GenBank accession no. AF294900) and to the plant epoxy-carotenoid cleaving enzyme (VP14) from maize (17). Identity of the amino acids in all four amino acid sequences is indicated in black, and identities in at least two amino acid sequences are indicated in dark gray.

Here we show by analyzing two independent *ninaB* alleles that β-diox indeed is encoded by this gene. Molecular and biochemical analyses of the β-diox function in both $ninaB^{P315}$ and $ninaB^{360d}$ *Drosophila* flies revealed defects leading to a loss of the ability to form the visual chromophore, thus identifying the *ninaB* gene to encode the key enzyme for vitamin A formation *in vivo*.

Materials and Methods

Flies Stocks and Growth Conditions. Homozygous $ninaB^{P315}$ and $ninaB^{360d}$ flies were reared on standard corn medium from a heterozygous w;;w $ninaB^{P315}/TM3,Sb,Ser$ stock and a heterozygous w;;w $ninaB^{360d}/TM3,Sb,Ser$ stock, respectively. For control experiments, heterozygous $ninaB^{P315}$ flies or *wtB* (Berlin) flies were used. For vitamin A deprivation, the flies were raised on carotenoid-depleted white corn (DWC) medium (24). β-Carotene or all-*trans* retinal were supplemented in ethanol to the appropriate medium.

Cloning of the β-diox from *ninaB* Flies. For cloning of β-diox from *wt*, $ninaB^{P315}$, and $ninaB^{360d}$ *Drosophila melanogaster*, we isolated total RNA from heads of adult flies obtained by hand dissection. Reverse transcription (RT) was performed by using the primer 5′-CTAAATGGCATTGGGTGCAAACC-3′ and superscript reverse transcriptase (Life Technologies, Gaithersburg, MD). Subsequently, PCR was performed with the primer 5′-GACGCCGGTGTCTTCAAGAG-3′ and the primer used for the RT assay and the Expand PCR system (Roche Molecular Biochemicals) and Pwo-Polymerase (PEQ-Lab, Erlangen, Germany), respectively. The PCR products were cloned directly in the vectors pTOPO-BAD and pCR2.1-Topo (Invitrogen), respectively. For cloning of the genomic DNA, a PCR was performed with the same set of primers by using genomic DNA as a template. The PCR products were cloned directly in the vector pCR2.1-TOPO (Invitrogen). To analyze the resultant plasmids and to confirm their structure, both strands were sequenced completely.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by PCR with a set of primers spanning the entire coding region: up, 5′- GACGCCGGTGTCTTCAAGAG-3′ and down, 5′-CTAAATGGCATTGGGTGCAAACC-3′. A set of reverse-oriented overlapping primers for the introduction of the desired base-pair alteration was used: for the Glu-to-Lys exchange at position 280 up, 5′-CTACTTTGTGATTGTGAAGCAGCC-3′ and down, 5′-ACGGACAACGGCTGCTTCACAATC-3′; for the Met-to-Leu exchange at position 471 up, 5′-GTCAATCTGGTTACCCTGGAGGGC-3′ and down, 5′-GCTTGACTGCCCTCCAGGGTAACC-3′; and for the Glu-to-Ala exchange at position 477 up, 5′-GGGCAGTCAAGCGGCGGCGTTTCA-3′ and down, 5′-CCCTGAAACGCCGCCGCTTGACTG-3′. For the Lys-to-Glu reversion at position 280 in the $ninaB^{P315}$ cDNA, the primers used were: up, 5′-CTACTTTGTGATTGTGGAGCAGCC-3′ and down, 5′-ACGGACAACG-

GCTGCTCCACAATC-3′. The PCR products were cloned in the expression vector pTOPO-BAD (Invitrogen), and the mutations in the plasmid constructs were verified by sequence analyses.

Estimation of the *ninaE* mRNA Levels by Semiquantitative RT-PCR Analyses. Total RNA was isolated from 10 flies reared on the appropriate media. Three different amounts of total RNA (100, 33, and 11 ng) were subjected to RT-PCR analyses as has been described (25). For *ninaE*, the following set of intron-spanning primers was used: up, 5′-TCTGTATTTCGAGACCTGG-3′ and down, 5′-TTCTCGGCATCCTCAGAGGAG-3′. Due to stochastic deviations between different sets of experiments, only samples from the same set of experiments were compared.

Extraction of β-Carotene and Retinoids from *E. coli* and HPLC Analysis. The *E. coli* strains were grown under a red safe light in 50-ml flasks in LB until the cultures had reached an OD_{600} of 1.0. Expression of β-diox was induced by the addition of L-arabinose (0.2% wt/vol) for 6 or 16 h. Then the bacteria were harvested by centrifugation. The pellets were extracted and HPLC analyses were carried out as has been described (19).

Visualization of the Deep Pseudopupil. For viewing the deep pseudopupil with orthodromic (direct-frontal) illumination at 573 nm in the compound eye of living white-eyed *Drosophila*, the eyes were adapted by saturating illumination at 450 nm, leading to a photo equilibrium with 20% rhodopsin (R480) and 80% metarhodopsin (M570). Monochromatic light was generated by a xenon lamp (Schölly, Freiburg, Germany) combined with an optical fiber with a filter wheel with interference line filters (Schott, Mainz, Germany). Photographs were taken under a binocular microscope (MZ12, Leica, Deerfield, IL) equipped with a VarioCam monochrome CCD video camera (PCO Computer Optics, Kelheim, Germany) with a variable time exposure, Peltier CCD cooling, and digital interface.

Results and Discussion

Cloning of the β-diox from *ninaB* Flies and Biochemical Characterization of the Encoded Protein. We established homozygous *ninaB*^{P315} and *ninaB*^{360d} flies from *TM3*,*Sb*,*Ser* balanced stocks and isolated genomic DNA and total RNA from homozygous *ninaB*^{P315} and *ninaB*^{360d} flies, heterozygotes, and *wt* flies (22). Because a putative mutation was expected to affect the β-diox function at several levels, we tested whether β-diox mRNA is still present in the *ninaB* flies first. For this purpose, we performed RT-PCR analyses by using a pair of primers spanning the entire coding region of β-diox. In both *ninaB* strains, the β-diox mRNA levels were similar to the wild-type control, and the amplified cDNAs had the same size. These results eliminated the possibility of a mutation affecting the transcriptional regulation of the β-diox gene. We next analyzed the ability of the encoded proteins to catalyze vitamin A formation. For this purpose, we cloned β-diox cDNAs from *ninaB*^{P315} and *ninaB*^{360d} homozygous flies and wild-type flies into the expression vector pTOPO-BAD. To test directly for enzymatic activity of the encoded proteins, we transformed the resultant plasmid constructs into the genetic background of an *E. coli* strain able to synthesize and accumulate β-carotene. With the aid of this test system, it is possible to assess β-diox enzymatic activity by a color change of the bacteria (19). This color change caused by the cleavage of β-carotene (yellow) to retinoids (colorless) became visible in the *E. coli* strain expressing the wild-type β-diox. In contrast, the *E. coli* strains expressing the β-diox encoded by the *ninaB* alleles remained yellow and therefore failed to catalyze retinoid formation (Fig. 2*A*). For more detailed analyses, overnight cultures were grown and HPLC analyses of lipids were performed as described (19). In the *E. coli* strain expressing β-diox isolated from *wt* flies, significant amounts of retinal as well as the corresponding retinols could be detected, whereas in the *E. coli* strain expressing β-diox isolated from the *ninaB* mutants, no traces of retinoids were detectable (Fig. 2*B*). Thus, the cDNAs isolated from *ninaB* homozygous flies did not encode functional β-diox proteins.

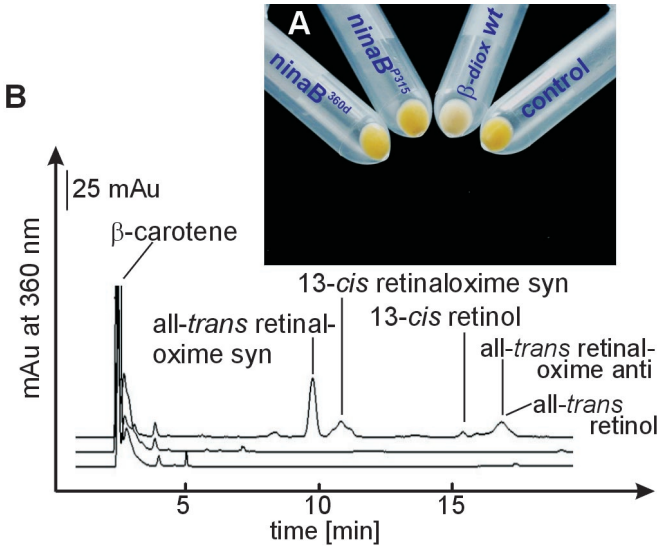

Fig. 2. Biochemical analyses of the β-diox encoded by the *ninaB*^{P315} and *ninaB*^{360d} alleles compared with the *wt* allele. (*A*) The picture shows the color shift of the β-carotene-producing and -accumulating *E. coli* strain from yellow to white caused by the formation of retinoids from β-carotene by the β-diox enzymatic activity. Although the *E. coli* strain expressing the β-diox encoded by the *wt* allele becomes white, the *E. coli* expressing β-diox encoded by the *ninaB*^{P315} and *ninaB*^{360d} alleles remain yellow. A control *E. coli* strain transformed with the expression vector (pBAD-TOPO) alone remains yellow. (*B*) HPLC analyses of the products formed in the different *E. coli* strains. In the *E. coli* strain expressing β-diox encoded by the *wt* allele, significant amounts of retinoids are found (upper trace), whereas in the *E. coli* strain expressing β-diox encoded by the *ninaB*^{P315} (middle trace) or *ninaB*^{360d} (lower trace) alleles, no retinoids become detectable.

A Single Lys-to-Glu Substitution in the *ninaB*^{P315} Allele and a Nonsense Mutation in the *ninaB*^{360d} Allele Abolish the β-diox Enzymatic Activity. To analyze the molecular basis for this defect, we performed sequence analyses of cDNAs isolated from *ninaB*^{P315} and *ninaB*^{360d} flies by three independent RT-PCRs and compared the sequence with cDNAs isolated from both *wt* and heterozygous flies.

The sequence analysis of the *ninaB*^{360d} allele revealed a nonsense mutation, TCG to TAG, at position 41 of the deduced amino acid sequence, whereas in the *ninaB*^{P315} allele, three base-pair exchanges compared with the *wt* allele were found (Fig. 3). In *ninaB*^{P315}, all three mutations lead to an alteration of the deduced amino acid sequence, but do not interfere with the ORF. To determine which of these three mutations is responsible for the loss of the β-diox enzymatic activity, we introduced each of these mutations separately into the *wt* allele by site-directed mutagenesis. The resultant expression plasmids harboring the three different mutant cDNAs were transformed into the β-carotene-producing *E. coli* strain and tested for β-diox enzymatic activity of the encoded mutant protein. The cDNA mutated at position 838 leading to a Glu-to-Lys exchange in the deduced amino acid sequence resulted in a loss of the β-diox enzymatic activity, whereas the mutations at the positions 1,411 (Met-to-Leu) and 1,430 (Glu-to-Ala) did not affect the β-diox enzymatic activity

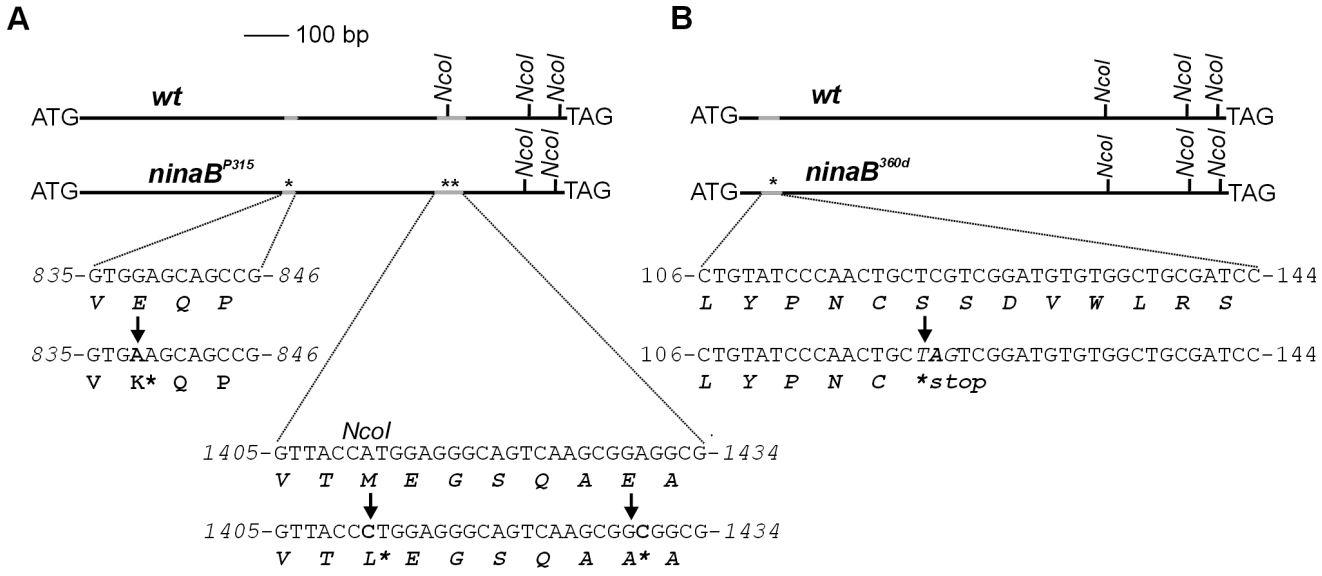

Fig. 3. Molecular analyses of the *ninaB* mutations. For sequence analyses β-diox cDNAs were cloned from homozygous *ninaB*^{P315}, homozygous *ninaB*^{360d}, heterozygous, and *wt* flies by RT-PCR with primers spanning the whole coding region. The alterations found in the *ninaB* alleles compared with the *wt* allele are indicated by arrows. (*A*) The sequence analyses of the cDNA isolated from *ninaB*^{P315} revealed three base-pair exchanges at positions 838, 1,411, and 1,430 with respect to the deduced start ATG compared with the cDNA isolated from *wt* flies (AJ276682). All three alterations led to an alteration of the deduced amino acid sequence. (*B*) In the *ninaB*^{360d}, a point mutation at position 122 (C to A) was found, resulting in a nonsense codon in the deduced amino acid sequence.

significantly as shown by the formation of retinoids in the *E. coli* test system (Fig. 4). This result was verified by converting the base-pair exchange at position 838 in the *ninaB*^{P315} cDNA to the *wt* sequence by site-directed mutagenesis, resulting in a restoration of the β-diox enzymatic activity in the *E. coli* test system (Fig. 4).

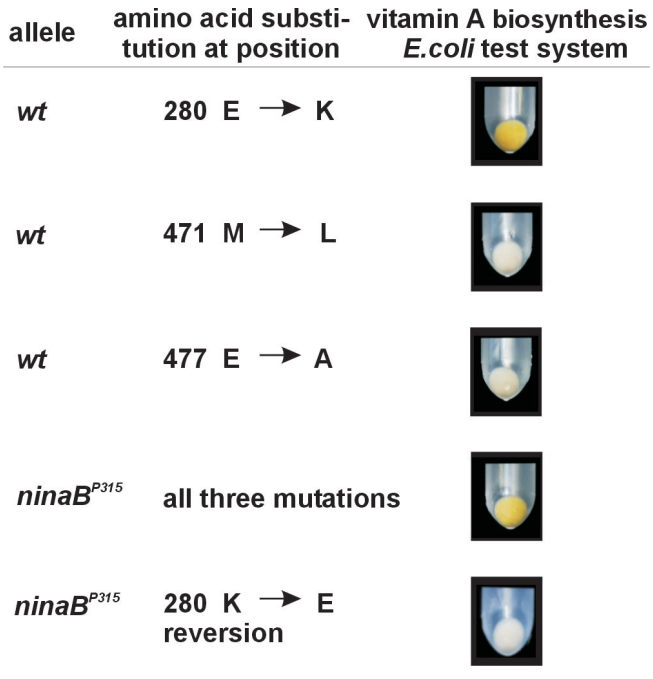

Fig. 4. A single Glu-to-Lys substitution leads to the loss of the β-diox enzymatic activity. The three different mutations found in the β-diox cDNA encoded by the *ninaB*^{P315} allele were introduced separately in the wild-type cDNA by site-directed mutagenesis, and the resultant plasmids were tested for enzymatic activity in the *E. coli* test system. Only the mutation at position 838 leading to a Glu-to-Lys exchange in the deduced amino acid sequence results in a loss of β-diox enzymatic activity, whereas the mutations at positions 1,411 and 1,430 do not affect the enzymatic activity of β-diox. By converting the mutation at position 838 in the *ninaB*^{P315} cDNA, the enzymatic activity of the encoded protein could be restored.

In summary, in both *ninaB* alleles, amino acid sequence alterations could be detected and shown to lead to a loss of function of the encoded β-diox protein. In the *ninaB*^{360d} allele, a nonsense mutation at position 41 interrupts the ORF, whereas in the *ninaB*^{P315} allele, the missense mutation at position 838 allele leading to a Glu-to-Lys exchange in the encoded β-diox protein is responsible for the loss of its enzymatic activity.

The Rhodopsin Content Is Drastically Reduced in *ninaB* Flies. We expected that the mutations found in the *ninaB* alleles abolished the ability to form the visual chromophore from carotenoids *in vivo*. To visualize this phenotype directly in the compound eyes of the living fly, we used the appearance of the deep pseudopupil as a measure for the visual pigment content (26–28). For this purpose, we used orthodromic illumination with 573-nm light, which is close to the metarhodopsin absorption maximum (M570) (29). For viewing the deep pseudopupil at 573 nm, the photo equilibrium between rhodopsin (P480) and metarhodopsin (M570) was shifted maximally to the M570 state with saturating illumination at 450 nm. As shown in Fig. 5, in heterozygous flies, a distinct pseudopupil became visible. In homozygous *ninaB*^{P315} flies, only a weak shadow was detectable, whereas in *ninaB*^{360d} flies, a pseudopupil could not be detected. To verify that this phenotype is caused by vitamin A deficiency, we raised *ninaB* flies on corn medium supplemented with all-*trans* retinal. This led to a recovery of the deep pseudopupil because of a restoration of the visual pigments. The weak remainder of visual pigments detectable in nonsupplemented *ninaB*^{P315} flies has been shown indirectly by electrophysiological measurements previously (22). It may be caused by a residual enzymatic activity of the *ninaB*^{P315} mutant protein *in vivo*. Sequence comparison uncovered no other gene with significant similarity to β-diox in the entire *Drosophila* genome. Thus, in *Drosophila*, the visual chromophores are formed exclusively by the symmetric cleavage of β-carotene catalyzed by β-diox.

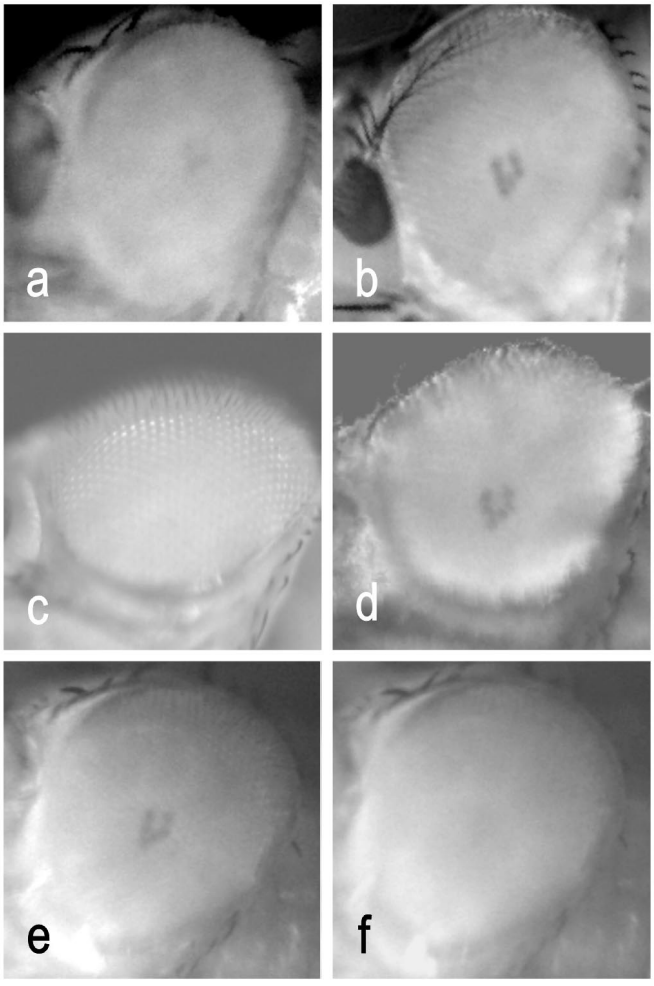

Fig. 5. Appearance of the deep pseudopupil at 573 nm in the compound eye of living flies after saturating illumination with light of 450 nm. (*a*) *ninaB*^{P315} homozygous flies raised on corn medium. (*b*) *ninaB*^{P315} homozygous flies raised on corn medium supplemented with all-*trans* retinal. (*c*) *ninaB*^{360d} homozygous flies raised on corn medium. (*d*) *ninaB*^{360d} homozygous flies raised on corn medium supplemented with all-*trans* retinal. (*e*) Visualization of the deep pseudopupil in heterozygous flies. (*f*) Disappearance of the deep pseudopupil in heterozygous flies after shifting the visual pigments from the metarhodopsin (570-nm) to the rhodopsin (480-nm) state with orange light.

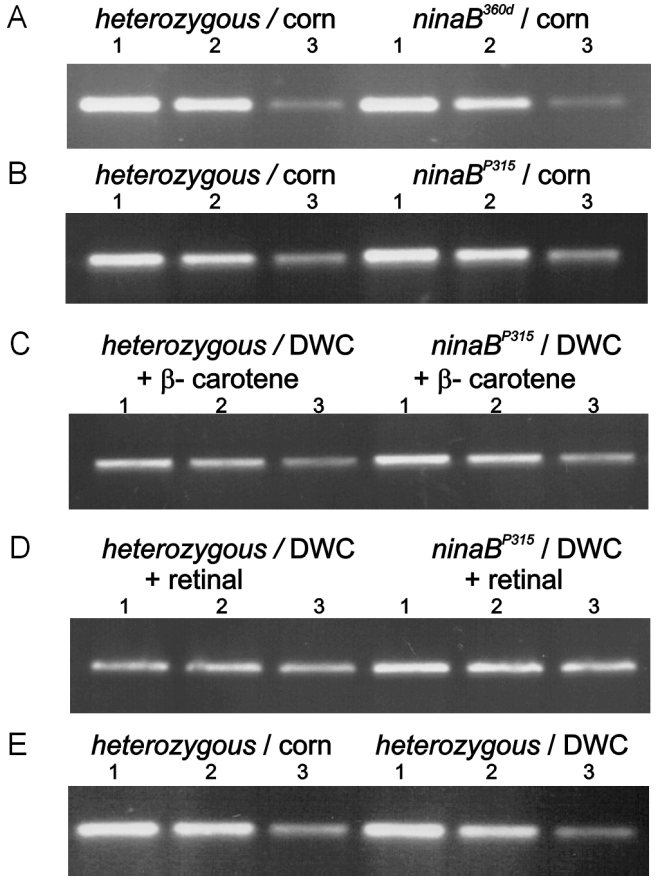

Fig. 6. Vitamin A deficiency does not influence the mRNA levels of *ninaE* (the major opsin) in wild-type flies and *ninaB* flies. Flies were reared on normal corn medium, DWC (carotenoid-depleted white corn) medium, or DWC medium supplemented with all-*trans* retinal and β-carotene, respectively. Total RNA was isolated, and RT-PCR was performed as described with three different amounts of total RNA. The RT-PCR products obtained from 100, 33, and 11 ng of total RNA (indicated by the numbers 1, 2, and 3, respectively, above the figures) were separated on an agarose (1.2%) gel containing 1× 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3 and 0.2 $\mu g \cdot ml^{-1}$ ethidium bromide. The photographs show the *ninaE* mRNA levels in heterozygous flies compared with *ninaB*^{360d} homozygous flies reared on standard corn medium (*A*), heterozygous flies compared with *ninaB*^{P315} homozygous flies reared on standard corn medium (*B*), heterozygous flies compared with *ninaB*^{P315} homozygous flies reared on DWC medium supplemented with β-carotene (*C*), heterozygous flies compared with *ninaB*^{P315} homozygous flies reared on DWC medium supplemented with all-*trans* retinal (*D*), and heterozygous flies reared on standard corn medium compared with heterozygous flies reared on DWC medium (*E*).

Vitamin A Deficiency Does Not Interfere with Opsin Gene Expression in *Drosophila*. Besides its role as the visual chromophore, vitamin A influences on opsin gene transcription, translation, and the maturation of the visual pigments have been reported (30–32) but are controversial. By using the *ninaB* mutants, which have a genetically caused vitamin A deficiency, we addressed this question and investigated the impact of this mutation on the regulation of the mRNA levels of the major opsin gene (*ninaE*) by semiquantitative RT-PCR. In flies raised on standard corn medium (with β-carotene as the source for vitamin A formation), no difference in the mRNA levels isolated from *ninaB* flies was detectable compared with heterozygous or *wt* flies (Fig. 6). Then we reared the flies on carotenoid-depleted medium and compared the mRNA levels of *ninaE* with those of flies reared on the same medium but supplemented with either β-carotene or all-*trans*-retinal. No differences were observed under these conditions. These results indicate that *ninaE* mRNA levels are not affected by vitamin A deficiency caused either by a genetic defect in the *ninaB* mutant or by deprivation of the provitamin in heterozygous and *wt* flies under the growth conditions applied. Furthermore, the inability to form retinal from β-carotene in the *ninaB* mutants did not seem to affect the β-diox mRNA levels, indicating that retinoids are not involved in its transcriptional regulation.

Conclusions

In summary, the molecular analyses of the *ninaB Drosophila* mutants revealed that their vitamin A deficient phenotype indeed is caused by a mutation in the β-diox gene and identified it as the gene encoding the key enzyme for vitamin A formation. In *Drosophila*, the *ninaB* mutation led to a vitamin A deficiency accompanied by a dramatically reduced content of visual pigments, but no other defects became phenotypically manifest. In the databases, several cDNAs encoding putative β-diox are found, including chicken (20),

mouse (AJ278064), and human homologues (AF284900). The results obtained by the analyses of *Drosophila ninaB* flies indicate that these vertebrate homologues likely exert the same *in vivo* function. However, it remains to be investigated whether the β-diox function in vertebrates is encoded by a single gene or by a small gene family. In vertebrates, a mutation comparable with *ninaB* probably would cause fatal effects considering the multiple vitamin A functions in development and cell differentiation as well as in vision. The molecular identification of the key enzyme for vitamin A formation will now open up further avenues of research. This research will include tissue specificity of vitamin A formation, the regulation of vitamin A homeostasis, and, especially in vertebrates, the impact of vitamin A formation on cell differentiation and developmental processes mediated by retinoic acid.

The significant similarity among plant and animal carotenoid-cleaving enzymes indicates that these enzymes belong to a diverse and widespread class of dioxygenases. In plants, a variety of carotenoid-cleavage products has been described, including all-*trans*-retinal in algal rhodopsins. The sequence homologies between algal and animal opsin genes (33) as well as genes involved in carotenoid cleavage in plants and animals support the hypothesis (34) that the various rhodopsin-based phototransduction systems most likely arose from a common ancestor.

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