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Krüppel homolog (*Kr h*) is a dosage-dependent modifier of gene expression in *Drosophila*

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Summary

A lethal mutation in the *Krüppel homolog* (*Kr h*) was isolated in screens of *P*-element insertion mutations for modifiers of *white* gene expression. The mutation occurs in the 5′ untranslated region of the *Kr h* gene and causes a lightening of the eye colour for several alleles of *white* due to a decrease in *white* steady-state mRNA levels at pupal stages. Two related genes, *scarlet* and *brown*, were significantly affected as well in early pupae. Genetic analysis of different *white* alleles suggests that enhancer sequences are necessary for interaction with KR H. Thus, the *Kr h* gene is a member of the dosage-dependent hierarchy effective upon *white*.

1. Introduction

Modification of gene expression involving *trans*-acting dosage effects has been hypothesized to be responsible for various biological phenomena associated with chromosomal copy number, for example aneuploid syndromes and dosage compensation (Birchler & Newton, 1981; Birchler *et al*., 1990; Birchler, 1996; Bhadra *et al*., 1999). Earlier studies in maize and *Drosophila* have revealed that for almost every gene, several chromosomal segments can be found for which variation of dosage would affect the expression of the target gene in a dosage-dependent manner (Sabl & Birchler, 1993; Guo & Birchler, 1994). Our data from *Drosophila* show that the effects produced by varying the dosage of chromosomal segments are due to distinct loci. The effects of mutations in these loci on target genes fulfil the prediction for being responsible for the whole chromosomal segment effects (Bhadra *et al*., 1998). Recently, we conducted a screen of *P*-element insertion lethal mutations (Spradling *et al*., 1995) for isolation of a set of dosage-dependent modifiers of gene expression. The screen for prospective modifiers was carried out on the *apricot* allele (w^a) of the *white* eye colour gene as a target. Modifiers of the *white* gene define a set of regulators that also usually effect expression of two unlinked homologous genes, *scarlet* and *brown*, in a coordinate manner (see, for example, Rabinow *et al*., 1991; Benevolenskaya *et al*., 1998; Bhadra *et al*., 1998; Frolov *et al*., 1998). The premise of the screen is that in the sensitized w^a background, a reduction in the dose of a *white* gene modifier (i.e. by mutating one copy of two present in the diploid genome as a result of *P*-element insertion) will alter the expression of *white* and thereby visibly modify the *apricot* eye colour phenotype. A dominant enhancement of the w^a phenotype was found with *P*-element insertions in the *Kr h* locus. The phenotype shows a positive correlation between dosage of normal alleles and *white* steady-state mRNA level.

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2. Materials and methods

(i) Drosophila *stocks*

Two mutations in the *l(2)10642* locus, $l(2)10642^{05208}$ (*l(2)05208* herein) and $l(2)10642^{10642}$ (*l(2)10642* herein), are caused by *P*[*lacZ*, $rosy^+$] insertions (Mlodzik & Hiromi, 1992; FlyBase, http://flybase.bio.indiana.edu:82). The stocks were obtained from the Indiana University Drosophila Stock Center. To generate a revertant, *l(2)05208/CyO*; ry^{506}/ry^{506} females were crossed to *Sp/CyO*; *delta 2–3*, *Sb/TM6*,

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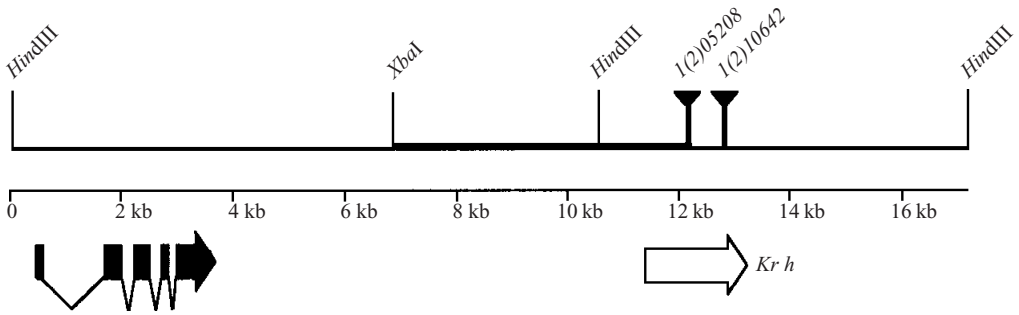

Fig. 1. Molecular map of the *Kr h* region with indication of *P*-element insertion sites in the *l(2)05208* and *l(2)10642* alleles. The rescued genomic fragment originating from the *l(2)05208* allele is depicted by a bold line. Two *Hin*dIII genomic subclones from P1 clone DS02136 were used for screening of cDNA libraries. Both *P*-element insertions (*PZ* elements shown by triangles) occur in the 5′ UTR of the *Kr h* gene. The exon–intron structure of another transcription unit identified in the region is shown on the left.

Ubx males (Robertson *et al.* 1988). The F_1 *l(2)05208/CyO; delta 2–3, Sb /ry*^{506} males were crossed individually to three-five *Sp/CyO*; *ry*^{506}*/TM6*, *Ubx* females. The *Cy* non *Sb* progeny (*l(2)05208/CyO*; *ry*^{506}*/ry*^{506}) were screened for *rosy*$^{-}$ flies, which were mated to individuals carrying the *In(2LR)Gla/SM6a*, *Cy* balancers to establish stocks. For the developmental Northern analysis, genetic crosses were performed essentially as described in Birchler *et al.* (1994).

(ii) *Cloning and analysis of gene expression*

RNA isolations and synthesis of ^{32}P-labelled antisense RNA probes were performed as described (Benevolenskaya *et al.*, 1998). Kr h RNA was detected using a subcloned *Eco*RI fragment derived from the smaller *Hin*dIII genomic fragment (depicted in Fig. 1), which was linearized with *Sal*I. When used as a DNA template for *in vitro* transcriptionn with T7 RNA polymerase (Promega), this probe will detect exon sequences downstream of the insertion site in the *l(2)05208* allele. An *rp49* RNA probe was used as a loading control. The signals were quantified with a Fuji Phosphorimager. Reporter gene expression in embryos and larvae was investigated by beta-galactosidase histochemical staining. DNA clones were isolated from a cDNA library generated from 2-week-old Canton S flies (IZAP II vector, Stratagene). About 1 200 000 phage from an adult cDNA library have been screened and the isolated cDNA clones were sequenced using the Sequenase 2.0 kit (Amersham) according to the manufacturer's recommendations. GenBank and Swiss Protein databases were searched using BLAST (Altschul *et al.*, 1990). The primary structures were aligned utilizing DNA STAR software (DNASTAR, Madison, WI). Domain structure was determined applying Profilescan software at ISREC-Bioinformatics (http://www.isrec.isb-sib.ch).

3. Results and discussion

(i) *Genetic characterization of a novel modifier of white expression*

Two *PZ* element insertions in the *l(2)10624* locus, *l(2)05208* and *l(2)10642* (Spradling *et al.*, 1995), dominantly enhance the *apricot* phenotype. To verify that the *P*-element insertion is responsible for the mutant phenotype, the original *P*-element in the *l(2)05208* allele was removed by supplying a source of transposase. Several strains with precise *P*-element excisions were generated as revealed by Southern analysis. The established revertant line, *l(2)05208*^{rev}, did not produce any effect on *w*^{a} and was homozygous viable. Thus, a mutation in a gene caused by the *P*-element insertion is the basis of the enhancement of the eye colour.

The effect of *l(2)05208* was investigated on 15 *white* alleles containing point mutations or inserted sequences which result in interrupted regulatory regions, premature termination of transcription or improper splicing (Lindsley & Zimm, 1992; FlyBase). *l(2)05208/SM6a Cy* males were crossed to females of each stock and the progeny males segregating for *l(2)05208* were compared for the intensity in their eye colour. Since *w*^{a} is caused by the insertion of a *copia* retrotransposon into an intron of the *white* gene, we addressed the question of whether the interaction is specific to transposable elements. *l(2)05208* enhances the phenotypes of point mutations, *w*^{a3} and *w*^{Bwx}. The *w*^{h}, *w*^{e} and *w*^{IR2} alleles, which are revertants of *w*^{I} due to secondary insertions into the *Doc* element responsible for the latter, and the *w*^{IR1} allele generated by hybrid dysgenesis, demonstrate suppression (*w*^{h} and *w*^{e}) or enhancement (*w*^{IR1} and *w*^{IR2}) by *l(2)05208*, while three other retrotransposon insertions, *w*^{bf}, *w*^{co} and *w*^{bf2}, are all unaffected. These unusual interactions may reflect an effect on transposable element expression as well as *white* given that the effect on the point mutations matches that on the level of normal *w* RNA (see below). The series revealing promoter-

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Table 1. *Quantitation of the effect of the l(2)05208 mutation on the mRNA levels of white, scarlet and brown*

Developmental stage	*white*	*scarlet*	*brown*
Early pupae			
Male	0·41 ± 0·05*	1·41 ± 0·06*	1·54 ± 0·04*
Female	0·61 ± 0·03*	1·53 ± 0·11*	1·38 ± 0·06*
Mild pupae			
Male	0·66 ± 0·06*	1·31 ± 0·04*	1·18 ± 0·07
Female	0·63 ± 0·04*	1·04 ± 0·05	0·99 ± 0·05
Late pupae			
Male	0·70 ± 0·02*	0·94 ± 0·04	0·77 ± 0·03*
Female	0·77 ± 0·02*	1·14 ± 0·05*	0·71 ± 0·04*
Adult			
Male	1·02 ± 0·09	1·24 ± 0·08	1·08 ± 0·05
Female	0·98 ± 0·06	1·08 ± 0·07	0·82 ± 0·05*

Total RNA isolated from the noted stage of development was subjected to Northern analysis. Antisense RNAs used as probes in hybridizations are indicated in the first row. After hybridization the blots were quantitiated using a Fuji Phosphorimager. The values obtained for each lane were divided by that of *rp49* used as a control. The ratio of the mRNA levels in *l(2)05208* to wild-type was calculated by dividing the value for the *l(2)05208*/+ class by the +/+ class. Ratios obtained for each of triplicate blots were used to generate the mean and the standard error presented in the table. Ratios with an asterisk are those significantly different from 1 at the 95% confidence level in a *t*-test.

enhancer interactions with the *white* gene are *spotted* alleles which are lesions in the *white* regulatory region involving the putative eye transcriptional enhancer. The *l(2)05208* interaction is eliminated when any allele from the *spotted* series, namely w^{sp}, w^{sp2}, w^{sp3} and w^{sp81d5}, was used for analysis. Unlike the majority of *white* modifiers, which act as weak suppressors of position effect variegation (Birchler *et al*., 1994; Csink *et al*., 1994; Bhadra & Birchler, 1996; Frolov & Birchler, 1998; Frolov *et al*., 1998), *l(2)05208* does not affect PEV as determined by tests with $In(1)w^{m4h}$.

(ii) *A l(2)10642 mutation affects levels of* white, scarlet *and* brown *transcripts*

WHITE, as well as its homologous proteins BROWN and SCARLET, belong to the MDR family of *P*-glycoproteins participating in pigment precursor transport into pigment cells responsible for eye colour (O'Hare *et al*., 1984, Acc. No. P10090; Dreesen *et al*., 1988). The deposition of pigments in the eye occurs during the mid–late pupal stage. We reported previously that modifiers of gene expression exert their effect at the level of transcript accumulation (see, for example, Birchler *et al*., 1994; Benevolenskaya *et al*., 1998; Bhadra *et al*., 1998). To determine the *l(2)05208* effect on the expression of *white*, *scarlet* and *brown*, total cellular RNA was isolated from early, mid and late pupae as well as adults in a segregating population, followed by Northern analysis. Blots were probed with *white*, *scarlet* and *brown* antisense RNAs in triplicate. The ratios of the steady-state mRNA levels in the mutant to wild-type animals in the segregating population were calculated for both sexes (Table 1). Consistent with the eye colour phenotype, the mRNA level of the *white* gene was decreased in the mutants at the stages of pigment deposition. *white* mRNA levels in *l(2)05208* were as low as about half of normal in early and mid pupae. In adults, *white* mRNA levels remained constant. There were significant increases in *scarlet* and *brown* mRNA levels in early pupae. However, *brown* mRNA level is significantly decreased in late pupae. Thus, there are both positive and negative effects of *l(2)05208* dosage on steady-state levels of target loci. There is a direct correlative dosage effect on *white* and *brown* mRNAs at the stages of pigment deposition in the fly eye, while there is inverse regulation of *scarlet* and *brown* mRNAs in the earlier stage. Our previous findings indicate that modifiers often exert opposite effects on the expression of different genes depending upon the developmental stage (Birchler *et al*., 1994; Benevolenskaya *et al*., 1998; Frolov *et al*., 1998).

(iii) *Sequence analysis and expression characteristics of the l(2)05208 region*

DNA flanking the *l(2)05208 P*-element insertion site was cloned by the 'plasmid rescue' method using the *Xba*I site (Fig. 1). The recovered genomic fragment was mapped to the clone DS02136 (Hartl *et al*., 1994) from which a DNA fragment of about 17 kb around the *P*-element insertion was recovered and used to determine a transcription map of the region. All

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isolated cDNA clones (estimated as approximately 0·005% of the screened library clones) corresponded to a single 1·3 kb transcription unit located almost 10 kb from the site of *P*-element insertion, but it is unaffected in *l(2)05208* mutants, implying that a lesion in another gene is responsible for the mutation.

Six lethal *P*-element insertions, two *PZ* and four *P-lacW* (which can not be tested because they carry *mini-white*), belong to the *l(2)10642* complementation group (Spradling *et al*., 1995). The *PZ* insertion *l(2)10642* also shows enhancement of the *apricot* phenotype and occurs just several hundred base pairs from *l(2)05208* (BDGP, Acc. No. G00625, unpublished). Since these data indicate that the responsible gene resides in this region, we resumed screenings between the above-described transcription unit and the *P*-element insertion sites using the genomic sequence of the DS06061 clone (Berkeley Drosophila Genome Project, unpublished). Screening the EST Database (BDGP/HHMI EST Project, unpublished) revealed that it does not contain any sequences from this region. However, a sequence of the *Kr h* mRNA (Pecasse *et al*., 1998, Acc. No. AJ005440) was found to correspond to the sequence of the rescued genomic fragment from the *l(2)10642* allele. These sequences are localized at the distal end of the DS06061 clone and correspond to only one exon of the *Kr h* gene as shown in Fig. 1. A cDNA for the *Kr h* gene has been isolated previously from a library prepared from early embryonic poly(A)$^+$ RNA (Rosenberg *et al*., 1986). The gene consists of four exons and generates a single 5·5 kb transcript present at all developmental stages as determined by Northern blots using antisense RNA generated from the *Kr h* 5′ UTR sequences. *Kr h* transcript accumulation in the embryo at the blastoderm stage and in mesoderm and ectoderm during gastrulation has been described (Schuh *et al*., 1986).

We performed enhancer trap *lacZ* staining of *l(2)05208*, which revealed almost ubiquitous expression throughout the embryos during stages 14–16 with high levels in anterior and posterior spiracles, the tip of the clypeolabrum and antennomaxillary complex. A high level of *Kr h* expression is present in embryonic brain neuromeres and the ventral nerve as well as in central brain neuroblasts and thoracic neuromeres of the larval central nervous system. Expression in *l(2)05208* larvae is moderate in eye-antennal, wing and leg discs and the strongest staining is observed in the humeral disc.

Quantitation of the *Kr h* transcript on Northern blots for the segregating genotypes was performed. A reduction in the level in the *l(2)05208* heterozygotes was found compared with wild-type. Heterozygous males and females produce $0{\cdot}70 \pm 0{\cdot}06$ and $0{\cdot}48 \pm 0{\cdot}04$, respectively, compared with normals. Thus, because *l(2)05208* decreases *Kr h* mRNA steady-state levels and both *l(2)05208* and *l(2)10642* insertions occur in the 5′ UTR of the *Kr h* gene, these mutations presumably lower *Kr h* function, partially or completely.

A search of the Swiss Prot database using the KR H sequence reveals similarity to the C2H2-type zinc finger (PROSITE, PDOC00028). *Kr h* has been identified as a *Krüppel* (*Kr*) homolog by the combination of several molecular methods (Schuh *et al*., 1986). To address the question of whether KR H is more similar to KR than to any other C2H2-type zinc finger protein, we initiated a computer study combining different analyses such as multiple sequence comparions, pairwise sequence comparisons and profile scans. This allowed us to delineate C2H2-type zinc fingers in the sequence of KR H and determine specific sequence features in the KR H and KR proteins compared with other C2H2-type zinc finger proteins. First, the C2H2-type zinc finger domain in KR H and KR is located at the same position from the N-terminus (amino acid positions 218 and 222 for KR H and KR, respectively). Secondly, KR H has Krüppel-like zinc fingers characterized by the spacing between the histidine and cysteine residues involved in coordination of a zinc atom. KR H has two more zinc finger motifs of the same type in the domain than KR. Thirdly, inter-finger sequences, namely H/C links, are identical in size and highly conserved in sequence in both proteins, which is consistent with their cross-reactivity to the anti-H/C link antibodies (Schuh *et al*., 1986). Fourthly, analysis of the percentage of overall sequence similarity among various C2H2-type zinc finger proteins demonstrates relatively high similarity between KR H and KR. Thus, these results strongly indicate that KR H is structurally similar to KR. The sequence relationships probably arise from a duplication of two C2H2-type zinc fingers in the ancestral Krüppel protein during evolution of KR H.

The *Krüppel* gene encodes a zinc finger-type transcription factor (Rosenberg *et al*. 1986), which is best known as a concentration-dependent gap gene within the segmentation gene cascade in early embryogenesis (Ingham, 1988) but which also has an essential role in several apparently unrelated events later in development (Schmucker *et al*., 1992). Krüppel-like zinc finger proteins are a large family, most of which are yet to be identified (Schuh *et al*., 1986; Tommerup & Vissing, 1995; Agata *et al*., 1998). The genetic and molecular data presented in this report demonstrate that at least another protein of the Krüppel family, KR H, is involved in dosage-dependent regulation of gene expression in *Drosophila*.

Kr h exerts both direct and inverse correlative effects on three coordinately expressed genes: *white*, *scarlet* and *brown*. The transitions from one effect to another seem to represent a regulatory switch in development because they are very common to other modifiers (Bhadra *et al*., 1998). In this regard, it is

interesting to note an analogy with *Kr*. *In vitro* binding assays and transient transfection experiments have shown that KR is able both to activate and to repress transcription from a single binding site placed in front of a promoter (Sauer & Jäckle, 1991). Most interestingly, the opposite regulatory effects of KR are concentration-dependent. Transcriptional activation is caused by a KR monomer formed at low protein concentration, while repression is caused by dimers at high concentration (Sauer & Jäckle, 1991, 1993) and involves differential interactions with the general transcriptional machinery (Sauer & Jäckle, 1993; Sauer *et al*., 1995). The concentration-dependent KR action observed might be quite similar in mechanism to the effects of dosage-dependent regulators of gene expression. Although this analogy exists between *Kr h* and *Kr*, the effect of the former on *white* could be either direct or indirect through a cascade of dosage modifiers (see discussion in Bhadra *et al*., 1998).

At least seven more genomic regions have homology to the KR finger domain in *Drosophila melanogaster* (Schuh *et al*., 1986). To seek the proteins containing KR multiple finger-like folded domains capable of binding to DNA, a rapid and efficient zinc finger gene cloning method has been developed based on RT-PCR using oligonucleotide primers corresponding to the conserved H/C link (Agata *et al*., 1998). Among the cDNAs isolated by this method are 60 different mammalian genes. Sixteen novel genes belonging to the human Krüppel family have been mapped cytologically (Tommerup & Vissing, 1995). Based on their map position, several of them were localized to regions involved in deletions and/or translocations associated with both developmental and malignant disorders. Our results add to the data indicating that this gene family is a contributor to the multiplicity of dosage-dependent modifiers of gene expression.

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