The *Drosophila* homolog of the human AF10 is an HP1-interacting suppressor of position effect variegation

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In chromosomal rearrangements of acute myeloid leukaemia patients the *mixed lineage leukaemia* (*MLL*) gene, a human homolog of the *Drosophila* gene *trithorax*, is frequently fused to *AF10*. Here we describe the identification and a functional characterization of the *Drosophila* homolog *dAF10*. We show that *dAF10* functions in heterochromatin-dependent genomic silencing of position effect variegation, a phenomenon associated with chromosomal rearrangements that cause mosaic expression of euchromatic genes when relocated next to heterochromatin. We also demonstrate that *dAF10* can associate with the heterochromatin protein 1 (HP1) *in vitro* and *in vivo*. The results indicate that *dAF10* is an HP1-interacting component of the heterochromatin-dependent gene silencing pathway, which either contributes to the stability of the heterochromatin complex or to its function.

INTRODUCTION

Leukaemogenesis correlates with alterations in chromatin structure brought about by chromosomal translocations (Saha *et al.*, 1998). One gene, which is rearranged in 20% of acute myeloid leukaemias (AMLs), is termed *mixed lineage leukaemia* (*MLL*) (Bower *et al.*, 1994). It codes for a human homolog of *trithorax* (*trx*), a *Drosophila* gene encoding a member of the trithorax group (*trx-G*) proteins which are necessary to maintain the spatially correct expression of homeotic genes during both *Drosophila* and vertebrate embryogenesis (Kennison, 1995; Paro, 1995; Yu *et al.*, 1995). *Trx-G* proteins as well as their functional counterparts, the Polycomb group of proteins (*Pc-G*) act not only in the context of homeotic genes but also during oogenesis, neural development and cell proliferation (Gebuhr *et al.*, 2000). *Trx-G* and *Pc-G* proteins comprise a long-term memory system of cells to pass on a particular developmental fate through many rounds of cell division by an epigenetic mecha-

nism that probably involves chromatin remodelling (Gebuhr *et al.*, 2000). Although the *MLL* protein has not been shown to remodel chromatin, its C-terminal region was found to interact with components of the SNF/SWI complex, a chromatin remodelling system which maintains the active state of transcription (Gebuhr *et al.*, 2000). In acute leukaemia patients, the C-terminal region of *MLL* is lost due to reciprocal translocations at chromosomal locus 11q23, which result in fusion proteins composed of the N-terminal portion of *MLL* extending into structurally and functionally unrelated fusion partners (Look, 1997; Adler *et al.*, 1999).

Translocations that cause AML show remarkable specificity for hematopoietic cells blocked in defined stages of differentiation. This property suggests that the different oncogenes produced by the translocations specifically interfere with transcriptional networks which normally function in concert with growth factors to regulate hematopoiesis. This conclusion is reinforced by the results of genetically manipulated mice which showed significant effects on normal hematopoiesis in response to translocation-targeted transcription factors (reviewed in Look, 1997). To date fifteen *MLL* fusion genes that result from chromosomal translocations have been cloned and characterized (reviewed in Look, 1997; Adler *et al.*, 1999). Two of these are *CBP* and *AF10* (Chaplin *et al.*, 1995), they have been shown to carry oncogenic potential also in the context of fusion partners different from *MLL* (Borrow *et al.*, 1996; Dreyling *et al.*, 1996). *CBP* is a transcriptional coactivator that acts as a multifunctional adapter protein to regulate transcription through acetylation of chromatin and recruitment of basal transcription factors (Kitabayashi *et al.*, 1998), whereas the molecular function of the second, *AF10*, is unknown.

Here we present a functional analysis of the *Drosophila* homolog of *AF10*, referred to as *dAF10*. We show that *dAF10* contains a pentameric protein-protein interaction motif and can

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Fig. 1. Genomic organization and the spatiotemporal expression pattern of *dAF10*. (A) Polytene chromosome *in situ* hybridization showing that *dAF10* is located on the right arm of chromosome 3 in region 84C1–84C2, indicated by an arrow. (B) Schematic representation of the genomic organization of *dAF10*, the mapping of the two major *dAF10* transcripts and location of the P-element *EP3618* in the first intron. Position of fragments used as probes for northern blot analysis (C) are indicated (1,2; blue or green boxes). (C) Developmental northern blot probed with antisense RNA of fragments 1 and 2 [see blue or green boxes in (B)] and ribosomal protein *rp49* antisense RNA (top to bottom). Total RNA of 2, 4 and 18 h old embryos, first (L1), second (L2) and third (L3) instar larvae, and adult females (f) and males (m) were loaded. Transcript sizes are indicated. (D) *In situ* hybridization pattern of whole-mount preparations from embryos (stages 4, 6, 8; Campos-Ortega and Hartenstein, 1997) as revealed by antisense RNA corresponding to both the 3 and 5 kb cDNAs. Lateral views (anterior is left, dorsal up) showing that expression is restricted along the anterior–posterior axis of embryos (stage 4), and subsequently resolves into a series of stripes during cellular blastoderm and early gastrulation (stages 6 and 8).

associate *in vitro* with HP1, the *Drosophila* heterochromatin protein 1 (Paro and Hogness, 1991) which is encoded by the gene *Su(var)2-5*. HP1 takes part in a gene silencing phenomenon, termed position effect variegation (PEV), which refers to mosaic expression of euchromatic genes if they become re-located next to heterochromatin due to chromosomal rearrangements (Spofford, 1967). PEV is suppressed by HP1 mutations (Clark and Elgin, 1992). We show that *dAF10* functions like HP1 as a suppressor of PEV and interacts, by genetic means, with *Su(var)2-5*. The results suggest that *dAF10* participates in chromatin mediated gene silencing.

RESULTS AND DISCUSSION

Structure and transcript pattern of the *Drosophila AF10* homolog

Cloning of the *Drosophila* homolog *dAF10*, was initiated by a database search (Berkeley *Drosophila* Genome project, BDGP; <http://www.fruitfly.org>). This search found the corresponding annotated transcription unit (CG1070) (Adams *et al.*, 2000), which maps into region 84C1-2 on the right arm of chromosome 3 (Figure 1A). Sequence analysis of both the genomic DNA and

Fig. 2. (A) Sequence alignment of the *Drosophila* and human AF10 proteins showing the conserved N-terminal PHD- (light grey) and extended PHD-finger (dark grey) motifs and the C-terminal leucine zipper region. Sequence identities are 75 and 52%, respectively. Consensus Cys-His-residues of the PHD-fingers and Leu of the leucine zipper region are highlighted. The chromo shadow domain-binding peptide (PLVVL) is indicated. *In vitro* translated sub-fragments of dAF10 for pull down assays are shown below. (B) Schematic presentation of the protein structure of HP1 with chromo domain (CD) and chromo shadow domain (CSD) marked in grey; regions used for the protein-protein interaction experiments are indicated below. (C) GST pull-down assays showing an interaction between dAF10 and HP1-GST fusion protein. *In vitro* translated dAF10 and fragments thereof are indicated below the panel. GST fusion proteins on top. Note that the chromo shadow domain of HP1 binds to the PLVVL-containing C-terminal region of dAF10. Apparent molecular weights are indicated on the left.

various EST clones (SD04152, SD09324, LP02873 and LP08584) confirmed the chromosomal location as outlined by the BDGP (<http://www.fruitfly.org/>) and revealed that *dAF10* encodes different splicing variants of which the two major forms (see below) could be assigned unambiguously. The structure of the *dAF10* gene and the two major *dAF10* transcripts are summarized in Figure 1B.

In order to establish the temporal pattern of the *dAF10* expression, we performed developmental northern blot analysis (Figure 1C). *dAF10* codes for four different transcripts with distinct temporal expression profiles during the *Drosophila* life cycle. One of two major transcripts, ~5 kb in length, is constitutively expressed with high levels during embryogenesis and in adult females. The second major transcript of ~3 kb is restricted to embryogenesis and females. In addition, two splicing variants of the transcript (6 and 8 kb) were found from later stages of embryogenesis onwards (Figure 1C).

Whole-mount *in situ* hybridization to oocytes and embryos revealed that the 5 and 3 kb transcripts are expressed maternally. Both splicing variants were found in nurse cells and later equally distributed in oocytes (data not shown). They are maintained in fertilized eggs and decrease gradually until the preblastoderm stage. Zygotic *dAF10* expression is initiated during syncytial blastoderm (stage 4; stageing according to Campos-Ortega and Hartenstein, 1997) and develops a stripe pattern similar to pair-rule segmentation genes (Figure 1D). A detailed description of the *dAF10* expression patterns will be described elsewhere (B. Linder, N. Gerlach and H. Jäckle, in preparation) since embryonic *dAF10* function is beyond the scope of this study.

The 3 and 5 kb transcripts were recovered in two corresponding full-size cDNA clones. The 5 kb transcript encodes a 4131 bp open reading frame (ORF) corresponding to a 1377 amino acids polypeptide (DDBJ/EMBL/GenBank accession No.

Fig. 3. Phenotype of *dAF10* mutants. (A and B) Homozygous *EP3618* flies show a severe dilation of the diventricular crop (Cr). Note that due to the lack of muscles, this organ is only cuticle lined and thus unable to contract; it appears as a gas filled bubble (B). (C and D) Larval cuticle preparations of wildtype (C) and homozygous *dAF10* mutant embryos laid by heterozygous females (D). Note the loss of abdominal segment 4 (asterisk) and pattern defects in the terminal regions (head and non-segmented tail region) in mutant embryos.

AF281145) which contains a PHD-finger next to an extended PHD-finger domain and a leucine zipper motif as observed with human AF10 (Linder *et al.*, 2000) (Figure 2A). The 3 kb transcript starts with an alternative exon (Figure 1B) and codes for an 826 amino acid protein (DDBJ/EMBL/GenBank accession No. AF281146), which lacks the N-terminal PHD-finger/extended PHD-finger domains. Thus, only the larger of the two major transcripts codes for the human AF10 homolog. However, both variants contain the leucine zipper motif and a PLVVL pentamer motif (Smothers and Henikoff, 2000) found in a subset of proteins that interact with HP1 *in vitro* (Murzina *et al.*, 1999; Ryan *et al.*, 1999; Smothers and Henikoff, 2000) (Figure 2A). This observation suggests that dAF10 may bind to HP1 and function in an HP1-dependent manner.

Interaction of HP1 and dAF10 *in vitro* and *in vivo*

We examined a possible dAF10::HP1 interaction via pull-down assays involving a GST-HP1 fusion protein and *in vitro* translated, labelled dAF10. HP1 binds via its chromo shadow domain to full-size dAF10 and to the subfragment that contains the PLVVL motif (Figure 2B and C). These findings suggest that dAF10 may function in an HP1-dependent gene silencing pathway.

HP1 is encoded by the *Su(var)2-5*, a suppressor of PEV. This silencing phenomenon is seen with the inversion mutation *In(1)w^{mh4}*, which results in a relocation of the *white* (*w*) gene next to heterochromatin. In this location, *w* expression is silenced in some ommatidia of the *Drosophila* eye but not in others (Elgin, 1996). Males which contain the *Su(var)2-5* mutation in addition to the *In(1)w^{mh4}* mutant suppress PEV, causing *w* expression in a significant larger area of the eye. If the inter-

action between HP1 and dAF10 is indeed functional, the derepression effect *Su(var)2-5* should be enhanced in flies which carry a mutant *dAF10* allele in addition to *Su(var)2-5* (see below).

In order to apply the PEV assay, we generated mutations of the *dAF10* gene by taking advantage of a P-element insertion within the first intron of the gene (*EP3618*, see Figure 1B). Homozygous *EP3618* individuals are semi-lethal, meaning that up to 70% of the individuals develop into viable and fertile adults. Such individuals exert two characteristic defects: they are unable to fly and about half of them develop a severe dilation of the diventricular crop, a cuticle-lined muscular organ of the upper digestive tract (Strasburger, 1935) (Figure 3A and B). The offspring of those flies, which have a strongly reduced lifespan of about one week only, are embryonic-lethal. They develop a segmentation phenotype (see Figure 3C and D), suggesting that maternal expression of *dAF10* in females (see above) is essential for embryogenesis.

We examined *w* expression in the eyes of *In(1)w^{mh4}* males (Figure 4A) which carried the *EP3618* insertion mutant, a *dAF10* lack-of-function allele generated by imprecise excision of the *EP3618* element (B. Linder, N. Gerlach and H. Jäckle, in preparation) or the wildtype *EP3618* chromosome from which the EP element had been excised precisely. In individuals which carry the *dAF10* lack-of-function or the the *EP3618* insertion allele, the *w* silencing area in the eye was reduced as observed in *In(1)w^{mh4}* individuals bearing the *Su(var)2-5* allele (Figure 4B,C), whereas no effect on *w* silencing had been observed with males bearing the chromosome from which the EP element had been excised precisely (data not shown). These observations suggest that like HP1, dAF10 is a functional repressor component of wildtype heterochromatin, which renders the inverted *w* gene

Fig. 4. Genetic interaction of *dAF10* and HP1 as revealed by PEV suppression. (A) Eye of a male that carries the inversion mutation *In(1)w^{m4h}*. Note *white* expression in the minority of ommatidia, which escaped heterochromatic silencing. (B) Eye of a male that carries the inversion *In(1)w^{m4h}* and the *Su(var)2-5* mutation. Note suppression of silencing as reflected in an increased number of *white* expressing ommatidia. (C) Eye of a male that carries the inversion *In(1)w^{m4h}* and the *dAF10* mutation. Note that *white* expression expands over more ommatidia in a manner similar to what is observed in the *Su(var)2-5* mutation (compare B and C), indicating that *dAF10* is a suppressor of PEV. (D) Suppression of PEV in an eye of a male that carries the inversion *In(1)w^{m4h}*, the *Su(var)2-5* and the *dAF10* mutations. Note wildtype-like *white* expression, indicating that both HP1 and *dAF10* act in heterochromatic gene silencing.

inactive in most but not all ommatidia. Furthermore, in *In(1)w^{m4h}* males which are double-heterozygous for a mutant *dAF10* and *Su(var)2-5* alleles, silencing of *w* expression was completely suppressed. The resulting wildtype-like *w* expression (Figure 4D) indicates that *dAF10* and HP1 are components of the same gene silencing pathway.

Our results establish that *dAF10* and HP1 can associate *in vitro* and that both components act in heterochromatin-induced gene silencing. It has been proposed that this type of silencing results from a co-operative assembly of heterochromatin as multimeric complexes proceeding from a pre-existing block of heterochromatin (Locke *et al.*, 1988). HP1 action involves the recognition of an epigenetic methylation mark at lysine 9 of the N-terminus of histone (Lachner *et al.*, 2001) and is likely to cause chromatin remodelling involving homo- and/or heterophilic protein-protein interactions (Cavalli and Paro, 1998). Furthermore, human HP1 was found to be associated with the

lamin B receptor, a component of the inner nuclear membrane (Ye and Worman, 1996). These observations are consistent with the argument that HP1 is a constitutive component of heterochromatin, which initiates silencing at distinct sites and may also function in the subnuclear localization of heterochromatin.

SPECULATION

dAF10 exerts a spatiotemporally restricted expression profile. This finding, the *in vitro* association between HP1 and *dAF10* and their genetic interaction in suppression of PEV suggest that *dAF10* is a component of a distinct heterochromatin-dependent silencing process. *dAF10* may either contribute to the stability of the heterochromatin complex or serve as an attachment site for other proteins to join the silencing complex. Interestingly, the human AF10 is frequently fused with the trithorax homolog MLL of AML patients (Bower *et al.*, 1994). It is therefore tempting to speculate that combining *MLL* with *AF10* in a chimeric MLL-AF10 fusion protein causes a switch in cellular memory. The protein may attach to HP1 and cause gene silencing instead of maintaining target gene expression.

METHODS

Molecular analysis of *dAF10*. ESTs used for sequencing the cDNA of *dAF10* were SD04152, LP08584, SD09324, LP02873 (BDGP; <http://www.fruitfly.org/>). Total RNA was isolated using RNAzol (Gibco-BRL, Germany) according to the manufacturer's instructions. 30 µg of each RNA fraction were separated on a 1% formaldehyde gel. For synthesis of the antisense RNA probes and blotting we used the Strip-EZ RNA and Northern Max Kit from Ambion (Germany). Northern blots were hybridized with *dAF10* probes corresponding to the following regions: bp 1–1295 of the 5 kb transcript, bp1-564 (alternative exon) of the 3 kb transcript (Figure 1B) and *rp49* as a loading control. Digoxigenin-labelled antisense LP08584 RNA was used for *in situ* hybridization of chromosome squashes (Hartmann and Jäckle, 1995) and whole-mount preparations of ovaries and embryos (Hauptmann and Gerster, 1996).

Generation and analysis of *dAF10* mutants. P-element line *EP3618* was obtained from the Hungarian stock centre (BDGP; <http://www.fruitfly.org/>). In order to correlate the *EP3618*-mutant phenotype with the *dAF10* gene, we remobilized the P-element by crossing *EP3618* flies with flies containing a transposase-expressing transgene. Mobilization caused two precise excisions of the P-element, resulting in a reversion of the *EP3618*-mutant phenotype to wildtype. Thus, the P-element insertion is the cause of the mutant phenotype. In addition, we obtained three lethal lines, each carrying a deletion of the *dAF10* gene. Embryos homozygous for the *dAF10* deletions were embryonic lethal, showing the same segmentation phenotype as seen with embryos from females homozygous for the *EP3618* allele. A detailed molecular description of the alleles and their effects on embryonic development will be presented elsewhere (B. Linder, N. Gerlach and H. Jäckle, in preparation). For the PEV modifier assay the following stocks were used: *In(1)w^{m4h}*, *Su(var)2-5(5)* and *In(1)w^{m4h}*, *Su(var)2-5(4)* (Umeå stock centre). Larval cuticle were prepared according to Nüsslein-Volhard and Wieschaus (1980). **Protein-protein interaction assay.** GST protein expression, their purification and the pull-down assays were performed as

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described (Linder *et al.*, 2000). PCR fragments covering the full ORFs of dAF10 (aa 1–1377), the PHD- and extended PHD-finger (aa 1–239) and fragments of the C-terminus (aa 232–1377, aa 994–1377) were cloned into pcDNA-HisB (Invitrogen) for *in vitro* translation. PCR fragments covering the ORF of HP1 (aa 1–206), the chromo- (aa1-96) and chromo shadow domain (aa91–206) were cloned into and expressed from pGEX-4T3 (Amersham).

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