High Mobility Group Proteins HMGD and HMGZ Interact Genetically With the Brahma Chromatin Remodeling Complex in *Drosophila*

Anan Ragab,¹ Elizabeth C. Thompson and Andrew A. Travers

MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

Manuscript received August 24, 2005

Accepted for publication November 1, 2005

ABSTRACT

Many pleiotropic roles have been ascribed to small abundant HMG-Box (HMGB) proteins in higher eukaryotes but their precise function has remained enigmatic. To investigate their function genetically we have generated a defined deficiency uncovering the functionally redundant genes encoding HMGD and HMGZ, the *Drosophila* counterparts of HMGB1–3 in mammals. The resulting mutant is a strong hypomorphic allele of *HmgD/Z*. Surprisingly this allele is viable and exhibits only minor morphological defects even when homozygous. However, this allele interacts strongly with mutants of the Brahma chromatin remodeling complex, while no interaction was observed with mutant alleles of other remodeling complexes. We also observe genetic interactions between the *HmgD/Z* deficiency and some, but not all, known Brahma targets. These include the homeotic genes *Sex combs reduced* and *Antennapedia*, as well as the gene encoding the cell-signaling protein Rhomboid. In contrast to more general structural roles previously suggested for these proteins, we infer that a major function of the abundant HMGB proteins in *Drosophila* is to participate in Brahma-dependent chromatin remodeling at a specific subset of Brahma-dependent promoters.

CHROMATIN structure has important consequences for all nuclear processes, be they transcription regulation, replication, or DNA repair. In addition to histones, chromatin contains DNA-binding nonhistone proteins. One of the most abundant groups of chromatin-binding proteins are the high mobility group (HMG) proteins. These proteins make up several unrelated classes including the HMGA, HMGB, and HMGN proteins. HMGA are structurally flexible proteins that bind to A/T-rich DNA sequences and promote the binding of other transcription factors (REEVES 2003). HMGN proteins bind nucleosomes and promote chromatin decondensation (BUSTIN 2001).

The HMG-Box (HMGB) class of proteins is characterized by a DNA-binding domain or box, which binds DNA nonspecifically and induces sharp bends and consequently has a preference for distorted or prebent structures. This domain is utilized in many contexts, *e.g.*, sequence-specific transcription factors and subunits of chromatin remodeling complexes (THOMAS and TRAVERS 2001; TRAVERS 2003). However, a conserved group of small, highly abundant HMGB proteins remains, consisting of one or two HMGB domains followed by a stretch of basic residues and an acidic tail. The function of this group is poorly defined.

In vitro studies have examined the role of HMGB proteins in stimulating transcription factor binding to

their target DNA sequences (THOMAS and TRAVERS 2001). Increased rates of binding were observed, especially in the case of factors requiring prebent DNA, such as TATA-binding protein (TBP). These observations lead to the proposition that HMGB proteins act primarily as architectural chromatin factors.

The most abundant HMGB proteins in yeast are encoded by the functionally redundant genes *Nhp6a* and *Nhp6b*. Mutation of both genes results in a temperature-sensitive growth phenotype and misexpression of a small percentage of genes (MOREIRA and HOLMBERG 2000). Genetic interactions with TBP and the SAGA complex, required for activation of the *HO* gene, have been observed (YU *et al.* 2000, 2003; BISWAS *et al.* 2004). In addition, *Nhp6a/b* forms part of the yeast FACT complex that facilitates transcription elongation (FORMOSA *et al.* 2001).

Studies of HMGB proteins in mammals have been complicated by issues of redundancy. In mice three highly related HMGB proteins (HMGB1–3) exist, all ubiquitously expressed during embryogenesis, but with differing spatial expression patterns in adults. Single mutants in *Hmgb1* and *Hmgb2* show no embryonic defects, but *Hmgb1* mutants die 24 hr after birth from hypoglycemia and *Hmgb2* knockout mice exhibit reduced male fertility (CALOGERO *et al.* 1999; RONFANI *et al.* 2001). *Hmgb3* mutants are viable but exhibit erythrocythemia, with a 10% increase in the number of red blood cells (NEMETH *et al.* 2003, 2005). Thus far, to our knowledge, no double or triple mutants of HMGB1–3 have been generated and

¹Corresponding author: Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany.
E-mail: a.ragab@dkfz-heidelberg.de

therefore it has not been possible to address the issue of redundancy in mice.

In *Drosophila* small, abundant, non-sequence-specific HMGB proteins include Dorsal switch protein 1 (Dsp1) (MOSRIN-HUAMAN *et al.* 1998; DECOVILLE *et al.* 2001), HMGD and HMGZ (WAGNER *et al.* 1992; NER *et al.* 1993), and the products of two small, uncharacterized genes termed CG7045 and CG7046. Null mutants of *Dsp1* exhibit homeotic transformations relating to downregulation of *Sex combs reduced* (*Scr*) expression (DECOVILLE *et al.* 2001). Biochemical studies have observed competition between HMGD and histone H1 in binding to chromatin, implying that there may be a global role for small abundant HMGB proteins in the regulation of chromatin structure (NER and TRAVERS 1994; NER *et al.* 2001).

Using rearrangement screen (RS) *P*-elements available from the DrosDel consortium, we have produced a defined deficiency of *HmgD* and *HmgZ* in flies, resulting in a strong hypomorphic allele of these genes. In conflict with the hypothesized role in chromatin architecture, this *HmgD/Z* mutant is viable and displays no defects in global chromatin organization. Instead we observe strong genetic interactions with members of the Brahma remodeling complex, providing evidence for a role in activation of two Hox genes, *Antennapedia* (*Antp*) and *Sex combs reduced* (*Scr*), and also of *rhomboid* (*rho*), which is involved in EGFR signaling.

MATERIALS AND METHODS

Fly stocks and crosses: All stocks were maintained on standard medium at 25°. All alleles and stocks used are as described in FlyBase (<http://flybase.bio.indiana.edu>), with the following exceptions: *Df(2R)ED3921* and *Df(2R)ED50000* (this work) and *Df(2R)ED3923* (DrosDel consortium, <http://www.drosdel.org.uk>). Stocks containing RS elements 5-HA-1624, 5-SZ-3389, CB-5352-3, and CB-0090-3 were obtained from Szeged stock center; *Dsp1*⁺ was kindly provided by D. Locker, *Acf1* alleles by J. Kadonaga, and *Nurf301* [otherwise known as *E(bx)*] and *Iswi* alleles by P. Badenhorst.

Mounting fly wings: Wings from adult flies were dissected in ethanol and mounted in a 6:5 lactic acid:ethanol mixture.

Northern blot analysis and RT-PCR: Northern blot analysis was carried out using standard procedures. Total RNA was extracted using Trizol and mRNA was purified using the Micro-FastTrack 2.0 mRNA isolation kit (Invitrogen, San Diego), according to manufacturers' instructions. Blots were hybridized with a radiolabeled full-length HMGZ cDNA clone, in ULTRAhyb hybridization buffer (Ambion, Austin, TX), according to manufacturer's instructions, and bands were visualized using a phosphorimager. For RT-PCR, exon-flanking primers were designed for regions common to splice variants of *HmgD* and *HmgZ*, and reactions were performed using the SuperScript III One-Step RT-PCR System (Invitrogen), according to manufacturer's instructions. RT-PCR primer sequences are as follows: Act5C 5'-GACACCAAACCGAAAGACCT, Act5C 3'-ACCCACGTACGAGTCCTTCT, HMGD 5'-CCCGCTGCTCGATCAAGC, HMGD 3'-CGGACTCCTCCTTCTTCTCT, HMGZ 5'-AACGAGACCCGCGAACAG, HMGZ 3'-CTTCTTGCTGGCTTGGC, CG30403 5'-TACCACCAGGAGCT

AAAGAAGA, and CG30403 3'-AAATAAGGAAGAGGGTGTATGCAT.

Glycerol gradients: Nuclear extract was prepared from an overnight collection of embryos as previously described (KAMAKAKA *et al.* 1991). Nuclear extract (200 µl) was fractionated using 30–5% glycerol gradients, by centrifugation at 48,000 rpm for 16 hr in a Beckman ultracentrifuge using a SW-60 rotor. Fractions (100 µl) were collected and separated by SDS-PAGE. HMGD and HMGZ were detected by an antibody raised against the HMGB domain of HMGD (rat anti-HMGD-100, 1:250). Brahma was detected with a rabbit polyclonal antibody (gift from C. P. Verrijzer) described elsewhere (KAL *et al.* 2000) (rabbit anti-Brm 1:2000).

RESULTS

Generation of *HmgD* and *HmgZ* deficiency: The *HmgD* and *HmgZ* genes are located on chromosome 2R, at 57F9 and 57F10, ~20 kbp apart. Both *HmgD* and *HmgZ* produce alternatively spliced mRNA products (WAGNER *et al.* 1992; NER *et al.* 1993). Between the two genes lies an uncharacterized ORF, CG30403. DrosDel RS elements within the *HmgZ* and *HmgD* genes were selected to produce two deficiencies, as shown in Figure 1A. The first, *Df(2R)ED3921*, removes the first exon of *HmgZ*, the CG30403 gene, and the first exon of *HmgD*. This was generated using RS elements 5-HA-1624 and CB-5352-3.

The second deficiency, *Df(2R)ED50000*, removes CG30403 and the first exon of *HmgD*. This was made using RS elements 5-SZ-3389 and CB-0090-3 and was used as a control for all further experiments to rule out an effect of CG30403 in genetic interactions.

Flies homozygous for *Df(2R)ED50000* are viable and display no observable phenotype. No interaction with *Df(2R)ED3921* or a with larger deficiency spanning *HmgZ* and *HmgD* [*Df(2R)ED3923*, breakpoints 57F6: 57F10] was seen. Moreover, no genetic interactions between *Df(2R)ED50000* and other alleles were observed in any subsequent crosses.

Even though *Df(2R)ED3921* removes the promoter and first exon of both *HmgD* and *HmgZ* the *HmgD/Z* deficiency is viable and exhibits no apparent growth impairment at either high or low temperatures. This phenotype would not be expected if HMGD/Z were involved in the organization of chromatin structure, since gross changes in chromatin would be expected to cause lethality. However, we observed that flies homozygous for *Df(2R)ED3921* have a distinctive phenotype, exhibiting loss of the distal portion of the L5 longitudinal vein (Figure 2B), with a penetrance of 80% in females and 50% in males. Full penetrance was seen in flies crossed to the larger deficiency [*Df(2R)ED3923*]. These observations imply that the deficiency is not a null allele of *HmgD/Z*, but do verify that the phenotype is due to altered levels of HMGD/Z protein and not the CG30403 gene product.

Deficiencies were confirmed using PCR and DNA sequencing (GOLIC and GOLIC 1996; RYDER *et al.* 2004).

To verify that *Df(2R)ED3921* resulted in mutation of the *HmgD* and *HmgZ* loci Western blots of embryo extract were probed with the HMGD antibody. As shown in Figure 1B, very low levels of HMGD/Z were detected, accounting for <5% of the ECL signal in wild-type embryos. Given that the antibody may recognize other uncharacterized HMGB proteins, we performed RT-PCR on adult flies. It should be stressed that this technique

Mutation of *Dsp1* results in homeotic transformation of the first leg to the third at a low frequency (DECOVILLE *et al.* 2001). Flies homozygous for *Dsp1^l* (a null allele) and *Df(2R)ED3921* showed no enhancement of homeotic transformations of first leg to third or of an effect on the wing phenotype observed in *Df(2R)ED3921*. Null mutants do not exist for CG7045/6; therefore,

FIGURE 2.—Wing phenotypes of genetic interaction of *HmgD/Z* with the Brahma complex. (A) Wild-type wing showing longitudinal veins (L2, L3, L4, L5, and L6), anterior cross-vein (acv), and posterior cross-vein (pcv). (B) Homozygous *Df(2R)ED3921* wings show loss of the distal portion of L5. (C) *brm*^{2/+} flies display normal wing vein patterning. (D–E) Enhanced loss of veins is seen in *Df(2R)ED3921* flies crossed to *brm* and *osa* alleles. Severity of the phenotype is increased with the strong *osa*² allele (E) and by reducing HMGD/Z levels further in *Df(2R)ED3921/Df(2R)ED3923* flies heterozygous for *brm* or weak *osa* mutations (data not shown). (F) *Df(2R)ED3921*. (G) Flies homozygous for *Df(2R)ED3921* and heterozygous for *brm*² show a held out wings phenotype, which is also observed in flies trans-heterozygous for *Df(2R)ED3921* and *osa* alleles (see Table 1).

Df(2R)ED3921 flies were crossed to *Df(3R)Exel6191* (breakpoints 94A9; 94B2), a deficiency encompassing CG7045/6. No genetic interaction was observed in *Df(2R)ED3921; Df(3R)Exel6191/+* flies. This was also true for the triple mutant *dsp1¹; Df(2R)ED3921; Df(3R)Exel6191/+*. It was not feasible to generate a triple null for all four genes and therefore the possibility of redundancy cannot be excluded. Yet, if the hypothesis of a role for HMGB proteins in determining global chromatin architecture were valid, depletion of a substantial proportion of HMGB proteins would be expected to result in a more pronounced phenotype than that observed.

HMGD/Z interact specifically with the Brahma complex: Small abundant HMGB proteins have been implicated in chromatin remodeling *in vitro* with the ISWI-containing CHRAC complex (BONALDI *et al.* 2002). To test the biological relevance of this observation, *Df(2R)ED3921* flies were crossed to available mutants of chromatin remodeling factors. We observed a strong interaction with the *brahma* amorphic allele, *brm*², but not with alleles of *domino* (ATPase subunit of the NuA4 complex), *Iswi* (ATPase subunit of NURF, CHRAC, and ACF complexes), *Nurf301* (member of the NURF complex), *Acf1* (member of the ACF complex), *dMi-2* (ATPase subunit of the NURD complex), *kismet* (ATPase subunit, uncharacterized complex), or *lodestar* (ATPase

subunit, uncharacterized complex). Flies of genotype *Df(2R)ED3921; brm*^{2/+} have held out wings and loss of vein L5 up to the posterior cross-vein with 100% penetrance (Figure 2, D and G).

Since held out wings have also been observed in flies heterozygous for partially complementing *brm* alleles and in *brm/osa* trans-heterozygotes (TAMKUN *et al.* 1992; VAZQUEZ *et al.* 1999), we tested the interaction of *Df(2R)ED3921* with other members of the Brahma remodeling complex, summarized in Table 1. The same phenotypes were seen with alleles of *osa*. Notably flies trans-heterozygous for the *HmgD/Z* deficiency and the strong *osa*² allele displayed held out wings at 65% penetrance, and a weaker allele, *osa*⁰⁰⁰⁹⁰, at 35%. This provides evidence of a function for *HmgD/Z* in the same pathway as the Brahma complex. No significant frequency of held out wings was observed for selected alleles of Brahma complex members *moira*, *Snr1*, and deficiencies covering *dalao* (encodes Bap111) and *Bap60*.

Interestingly the vein phenotype seen in *Df(2R)ED3921* was also enhanced by *brm* and *osa* alleles, resulting in loss of vein L5 up to the posterior cross-vein, but this was not observed with other members of the Brahma complex (Figure 2, C–E). When strong alleles of *osa* were used and/or the levels of *HmgD/Z* were reduced further by crossing to a larger defined deficiency [*Df(2R)ED3923*],

TABLE 1
Interactions with the Brahma remodeling complex

	Genotype	% held out wings	N
Brahma	<i>brm</i> ² / +	0	170/170
	<i>Df(2R)ED3921; brm</i> ² / +	100	207/207
Osa	<i>osa</i> ⁰⁰⁰⁰⁹⁰ / +	0	135/135
	<i>Df(2R)ED3921/ +; osa</i> ⁰⁰⁰⁰⁹⁰ / +	35	79/225
	<i>Df(2R)ED3921; osa</i> ⁰⁰⁰⁰⁹⁰ / +	100	120/120
	<i>osa</i> ² / +	2	5/247
	<i>Df(2R)ED3921/ +; osa</i> ² / +	65	166/256
	<i>Df(2R)ED3921; osa</i> ² / +	100	160/160
Moirai	<i>Df(2R)ED3921; mor</i> ¹ / +	3	8/279
Bap111	<i>Df(1)18.1.15/ +; Df(2R)ED3921</i>	0	0/315
Bap60	<i>Df(1)C246/ +; Df(2R)ED3921</i>	0	0/327
Snr1	<i>Df(2R)ED3921; snr1</i> ¹³⁰⁹ / +	0	0/285

partial loss of vein L2 was also seen (Figure 2E and data not shown).

HMGD/Z in vein specification: The phenotype obtained in *HmgD/Z* and *brm* double mutants is reminiscent of the loss-of-function *rhomboid* allele, *rho*^{ve}. This allele results in loss of the distal portions of veins L2–L5, with L3 and L4 displaying the greatest variation in the extent of vein shortening (STURTEVANT *et al.* 1993). Previous studies have examined Brahma complex function in wing vein formation. The complex was found to be required for the correct specification of both vein and nonvein cells. Overexpression of dominant negative Brahma resulted in loss of Rhomboid expression and loss of vein tissue, while mutation of the negatively regulating Snr1 subunit resulted in ectopic vein formation (MARENDA *et al.* 2003, 2004; ZRALY *et al.* 2003). Mutations in *brm* were shown to enhance the loss of vein phenotype of *rho*^{ve} mutants, specifically in the L5 region. As shown in Figure 3B, reducing the expression of HMGD/Z also enhances this phenotype and there is also a partial loss of L2. We also observed a mild enhancement of L3 vein loss and variation in the length of L4. It should be noted that *rho*^{ve} flies display variation in L4 length and we observed the same range of different L4 lengths in both *rho*^{ve} and *Df(2R)ED3921* flies, as shown in Figure 3, A and B. We interpret this to mean that *Df(2R)ED3921* does not significantly enhance the loss of L4 and L3, but affects L2 and L5. The interaction with *rho*^{ve} provides additional evidence of a role for HMGD/Z involvement in gene activation, in specific contexts, as an accessory factor to the Brahma remodeling complex.

Regulation of homeotic genes: To examine HMGD/Z function in transcription regulation, we have made use of the visible phenotypes associated with Hox gene misregulation. This allowed us to ask whether HMGD/Z act as general transcription factors, since many Hox gene alleles show dominant phenotypes. We reasoned that if HMGD/Z acted as general regulators of transcription, we would see modification of the dominant

Hox gene phenotypes since these provide highly sensitized backgrounds. In addition, Brahma was originally isolated in a screen for proteins involved in Hox gene expression and we wanted to examine the effect of loss of HMGD/Z on Brahma-regulated promoters (KENNISON and TAMKUN 1988; TAMKUN *et al.* 1992).

The held out wings phenotype observed in *Df(2R)ED3921; brm*²/+ is not uncommon, but notably has been observed in loss-of-function alleles of *Antennapedia*. Previous studies have linked Brahma complex function to correct expression of *Antp* (TAMKUN *et al.* 1992;

FIGURE 3.—Interactions with *rhomboid*. (A) Homozygous *rho*^{ve} flies show incomplete L3, L4, and L5 veins. (B) Reduction in HMGD/Z levels enhances the *rho*^{ve} phenotype in veins L2 and L5 and mildly so in L3. L4 length was variable in both *rho*^{ve} and *Df(2R)ED3921; rho*^{ve} flies. A is representative of the shortest length and B of the longest L4 length observed.

TABLE 2
Interactions with *Antennapedia*

Genotype	Transformed flies/total	% penetrance
<i>Antp^{Ns}</i>	135/145	93
<i>Antp^{Ns}/ +</i>	188/195	96
<i>Df(2R)ED3921; Antp^{Ns}</i>	96/132	72
<i>osa²/ Antp^{Ns}</i>	9/97	9

VAZQUEZ *et al.* 1999). Vasquez *et al.* observed that *osa* and *brm* alleles interacted with promoter-specific *Antp* mutants, both proteins being required for activation of *Antp* from the P2 promoter specifically (VAZQUEZ *et al.* 1999). We decided to test whether *HmgD/Z* were needed for this specific activation, by crossing *Df(2R)ED3921* to the gain-of-function allele *Antp^{Ns}*; this allele contains an internal partial duplication of the *Antp* gene plus insertion of two transposable elements and additional sequence (TALBERT and GARBER 1994). This results in ectopic activation of *Antp* in eye-antennal discs, inducing transformation of antennae into leg tissue. The *HmgD/Z* deficiency was shown to partially suppress *Antp^{Ns}* (see Table 2). The degree of suppression

was not as great as that observed with *brm* and *osa* mutations [21% for *Df(2R)ED3921* vs. 87% for *osa²*] and underscores the supporting role of HMGD/Z in gene activation. We have also examined the role of HMGD/Z with other Brahma-regulated homeotic genes. We observed an interaction with *Sex combs reduced*. *Df(2R)ED3921* enhanced loss-of-function alleles of *Scr* and partially suppressed the gain-of-function allele *Scr^S* (see Figure 4). This is similar to the interactions observed between mutations in the Brahma complex and *Scr*, where overexpression of dominant negative Brahma (DN-brm) in leg discs results in a reduced number of sex comb teeth (ELFRING *et al.* 1998). The Brahma complex has also been shown to be required for activation of *Ultrabithorax*, as *brm*, *osa*, and *mor* mutations enhance loss-of-function *Ubx* alleles (TAMKUN *et al.* 1992). We did not observe an increase in haltere-to-wing transformation when *Df(2R)ED3921* was crossed to *Ubx* alleles (see supplemental Table 1 at <http://www.genetics.org/supplemental/>). This result suggests that HMGD and HMGZ do not function as ubiquitous general transcriptional activators that promote gene activation and also that the interaction of *HmgD/Z* with

Brahma is required only at certain Brahma-regulated promoters.

Trithorax (*trxG*) and polycomb (*PcG*) group genes were originally characterized as opposing effectors of homeotic gene expression (see KENNISON and TAMKUN 1988 and references therein). Subsequent studies showed that many *PcG* and *trxG* proteins have roles in chromatin modulation. To further assess the role of HMGD/Z in chromatin regulation and to look for interactions with this group of genes we crossed *Df(2R)ED3921* to alleles of *Pc* (*Polycomb*), *Psc* (*Posterior sex combs*), *See* (*Sex combs extra*), *ph-p* (*polyhomeotic proximal*), and *esc* (*extra sex combs*). No interactions were observed with any *PcG* mutants or with *ash1* (*absent, small, or homeotic discs 1*) and *trx* (*trithorax*) of the trithorax group of proteins (see supplemental Table 1 at <http://www.genetics.org/supplemental/>). This result indicates that HMGD/Z do not act as typical *trxG* or *PcG* proteins, but interact specifically with *Brahma*.

Proteins that modulate chromatin structure have also been isolated in screens for genes that affect position effect variegation (PEV). If the *HmgD/Z* deficiency had an effect on PEV it could imply a role in global modulation of chromatin structure, as seen, for example, in *Su(var)3-9* or *Su(var)205* (heterochromatin protein 1) mutations. HMGB proteins have been hypothesized to promote a more open chromatin conformation and would therefore be expected to act as enhancers of PEV. *Df(2R)ED3921* did not suppress or enhance the variegated phenotype of *rst³* (*roughest*) and *y^{3p}* (*yellow*) inversions, providing further evidence against the proposed role of HMGD/Z in global chromatin architecture (see supplemental Table 1 at <http://www.genetics.org/supplemental/>).

HMGD and HMGZ are not integral components of the Brahma complex: We next wanted to examine whether HMGD and HMGZ are directly associated with the Brahma complex *in vivo*. Nuclear extract prepared from *Drosophila* embryos was separated on 15–35% glycerol gradients. Both purified Brahma complexes are ~2 MDa in size and sediment in the bottom fractions of the gradient (MOHRMANN *et al.* 2004); see Figure 5. As shown in Figure 5, there was no overlap between the Brahma-containing fractions and HMGD/Z, which was located in fractions ranging from ~1 MDa (estimated from markers) to the top fractions, corresponding to HMGD/Z bound to sheared chromatin.

DISCUSSION

The HMGB domain is found in many proteins, from sequence-specific transcription factors to members of chromatin remodeling complexes. The DNA-binding and bending properties of HMGB proteins are clearly important, yet defining a function for small, abundant chromatin-associated HMGB proteins has proven difficult.

FIGURE 5.—HMGD and HMGZ are not integral components of the Brahma complex. Nuclear extract derived from 0- to 12-hr wild-type (*yw*) embryos was fractionated on 15–35% glycerol gradients and probed with antibodies against *Drosophila* Brahma (rabbit anti-Brm, 1:2000) and HMGD/Z (rat anti HMGD-100, 1:250). HMGD/Z is associated with sheared chromatin but does not cofractionate with the Brahma complex. L, input fraction.

We have investigated the role of HMGD and HMGZ in *Drosophila* and have observed a strong genetic interaction with the Brahma remodeling complex, the first genetic link observed in multicellular organisms. We have also provided evidence for a role of HMGD and HMGZ in aiding the Brahma complex in activation of certain target genes. The genetic interaction of *HmgD/Z* with *brm* and *osa* alleles results in held out wings and loss of wing veins. The held out wings phenotype is attributed to decreased levels of *Antp*, through loss of transcription activation at the P2 promoter. Furthermore, reduction of *HmgD/Z* levels partially suppresses the *Antp^{Ns}* gain-of-function mutation, caused by ectopic activation of the P2 promoter (VAZQUEZ *et al.* 1999). These results indicate that *HmgD* and *HmgZ* cooperate with the Brahma complex in activating transcription of *Antp*.

Another gene regulated by the Brahma complex is *rhomboid*, a gene required for vein specification in the *Drosophila* wing. Loss of HMGD/Z enhances the *rho^{ve}* phenotype in a similar way to *brm* mutants (MAREDA *et al.* 2004). We observe loss of both L2 and L5 veins in *Df(2R)ED3921; rho^{ve}* flies; a similar phenotype is observed for combinations of the *HmgD/Z* deficiency with *brm* and *osa*. This strengthens the concept that HMGD/Z aid the Brahma complex in activation of its targets.

In examining whether HMGD/Z were involved in chromatin remodeling *in vivo*, we have observed a genetic interaction of these proteins with a SWI/SNF-like remodeler (Brahma) but not with an ISWI type (NURF, CHRAC, and ACF) although *in vitro* experiments have previously shown that HMGB1 can facilitate remodeling by the ACF complex (BONALDI *et al.* 2002). The modes of nucleosome remodeling effected by these two types of complex are thought to be different, where SWI/SNF, but not ISWI, remodelers are able to alter DNaseI cleavage periodicity in nucleosomes and release supercoils (FLAUS and OWEN-HUGHES 2004).

It is interesting to note that the Brahma complex contains a protein, Bap111 (encoded by *dalao*), with an

HMGB domain (PAPOULAS *et al.* 2001). One might therefore expect that loss of HMGD/Z would not affect Brahma complex function, since it already contains an HMGB protein. We did not observe an interaction in flies heterozygous for a deficiency covering *dalao*. However, in *brm* mutants, reduced amounts of functional Brahma complex may render certain promoters more dependent on small HMGB proteins such as HMGD/Z, and it is possible that HMGD/Z and Bap111 have different functions in Brahma-mediated transcriptional activation.

We observed stronger genetic interactions between *osa* mutants and *Df(2R)ED3921* than with other Brahma complex members. *Osa* is able to bind DNA, and HMGD/Z could possibly facilitate *Osa* binding to nucleosomes, thereby enhancing the rate of association of the remodeling complex to nucleosomes. A recent study of the interaction between HMGB1 and the glucocorticoid receptor (GR) found that the residence time of GR bound to chromatin was increased in the presence of HMGB1 (AGRESTI *et al.* 2005).

We have attempted to look for redundancy between *Drosophila* HMGB proteins, but are limited by the lack of available mutants for CG7045/6. The developing wing is particularly sensitive to perturbation. If there was any functional redundancy between HMGD/Z and CG7045/6 or Dsp1, modification of the phenotype would have most likely been observed in this tissue. However, until CG7045/6 mutants are made the issue of redundancy cannot be completely discarded. In examining *HmgD/Z* function in transcription regulation, we made use of the visible phenotypes associated with Hox gene misregulation. These phenotypes are known to be sensitive to changes in the activity of chromatin regulators such as Brahma and Polycomb (KENNISON and TAMKUN 1988; TAMKUN *et al.* 1992). In a manner similar to that of *brahma* HmgD/Z were found to facilitate activation of *Antp* and *Scr*. In contrast, *brm* mutants enhance loss-of-function *Ubx* mutations, yet we observe no effect for the *HmgD/Z* deficiency. We infer that *HmgD/Z* may be functionally necessary only at some Brahma-regulated promoters.

Interestingly both Dsp1 and HmgD/Z play a role in the activation of *Scr*. *Dsp1* suppresses the extra sex combs phenotype of *Pc*, but shows no interactions with *brm* (DECOVILLE *et al.* 2001). The opposite is true for the *HmgD/Z* deficiency, suggesting that these proteins act in independent pathways to activate *Scr*. Dsp1 has recently also been shown to promote the association of PcG group proteins to PREs (PcG response elements) by binding to a G(A) sequence motif, suggesting a role in transcription repression for this HMGB protein (DEJARDIN *et al.* 2005).

Our observations in *Drosophila* have interesting parallels to studies in *Saccharomyces cerevisiae*. Mutation of both *nbp6a* and *nbp6b* genes results in a temperature-sensitive growth phenotype. Synthetic lethal pheno-

types with SWI/SNF mutants have been observed for *snf5* and more recently for *swi2* (YU *et al.* 2000; BISWAS *et al.* 2004). In addition *Nhp6a/b* has been shown to be required for repression of the *CHAI* locus, and an interaction with the RSC remodeling complex has been inferred (MOREIRA and HOLMBERG 2000). Similarly Nhp6a/b have been connected with the INO80 remodeling complex; overexpression of Nhp6a rescues the phenotypes associated with mutations in *arp6* and *arp7* (nuclear actin-related protein, Arp), which are components of the INO80 complex (SZERLONG *et al.* 2003).

HMGB proteins are widely thought of as architectural chromatin proteins. This implies a global chromatin role, and mutation of such proteins should have dramatic effects on chromatin structure if removed.

Previous work by NER and TRAVERS (1994) hypothesized a role for HMGD in chromatin structure in the syncytial blastoderm. HMGD was proposed to act as an early embryonic linker binding protein, producing a more open chromatin conformation to facilitate rapid nuclear divisions in the absence of H1. Embryos containing the *HmgD/Z* deficiency have normal nuclear morphology and no abnormal nuclear divisions were observed (A. RAGAB, unpublished observations). This argues against an "early embryonic linker" role for HMGD/Z and suggests that other factors, or the lack or histone H1, are responsible for the decondensed chromatin state during these stages. In addition, no alteration either in micrococcal nuclease digestion kinetics of embryonic chromatin or in the morphology of mitotic and polytene chromosomes of these mutants was seen (E. C. THOMPSON and A. RAGAB, unpublished observations).

Recently an additional role for yeast Nhp6a/b and mammalian HMGB1 in genome stability has been proposed (GIAVARA *et al.* 2005). Increased sensitivity to UV irradiation was seen in *hmgbl1*—/— primary embryonic fibroblasts and in *nbp6a/b* double-mutant yeast. In addition, sensitivity to hydrogen peroxide and methyl methanesulphonate (MMS) was also observed in the yeast mutants. We have not observed any enhanced sensitivity to UV irradiation, X irradiation, or MMS in the *HmgD/Z* deficiency (E. C. THOMPSON, unpublished observations). We therefore infer that a major role for the abundant HMGB proteins in *Drosophila* is cooperation with the Brahma complex.

Previous biochemical studies are able to provide a model for how HMGD/Z assists chromatin remodeling by exposing DNA at the nucleosome surface. HMGB1 has been shown to bind to DNA at the entry site to nucleosomes (AN *et al.* 1998). We have previously shown that HMGD binding induces an area of DNA accessibility at the entry site and dyad of nucleosomes *in vitro*, presumably by bending the DNA (RAGAB and TRAVERS 2003). The exposed DNA could promote association of remodeling complexes to nucleosomes and be used

by the complex to propagate nucleosome sliding (BONALDI *et al.* 2002) or promote association of transcription factors (AGRESTI *et al.* 2005). Significantly the region where DNA accessibility is increased is immediately adjacent to the binding site for the motor polypeptide of the SWI/SNF complex (SAHA *et al.* 2005). HMGB proteins are known to bind chromatin with high on/off rates, which would result in rapid transient exposure of target sites and perhaps in more readily recognized and remodeled nucleosomes. This may afford organisms an advantage in the control of gene transcription and would explain the conservation of these small HMGB proteins from yeast to man.

We acknowledge D. Locker, P. Badenhurst, and J. Kadonaga for providing fly stocks; C. P. Verrijzer for the Brm antibody; and J. Brennecke, K. Brown, and A. Buscaino for valuable discussions and critical reading of the manuscript. A. Ragab was supported by a Medical Research Council (MRC) studentship. E. C. Thompson is supported by an MRC studentship.

LITERATURE CITED

- AGRESTI, A., P. SCAFFIDI, A. RIVA, V. R. CAIOLFA and M. E. BIANCHI, 2005 GR and HMGB1 interact only within chromatin and influence each other's residence time. *Mol. Cell* **18**: 109–121.
- AN, W., K. VAN HOLDE and J. ZLATANOVA, 1998 The non-histone chromatin protein HMG1 protects linker DNA on the side opposite to that protected by linker histones. *J. Biol. Chem.* **273**: 26289–26291.
- BISWAS, D., A. N. IMBALZANO, P. ERIKSSON, Y. YU and D. J. STILLMAN, 2004 Role for Nhp6, Gcn5, and the Swi/Snf complex in stimulating formation of the TATA-binding protein-TFIIA-DNA complex. *Mol. Cell. Biol.* **24**: 8312–8321.
- BONALDI, T., G. LANGST, R. STROHNER, P. B. BECKER and M. E. BIANCHI, 2002 The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding. *EMBO J.* **21**: 6865–6873.
- BUSTIN, M., 2001 Chromatin unfolding and activation by HMGN chromosomal proteins. *Trends Biochem. Sci.* **26**: 431–437.
- CALOGERO, S., F. GRASSI, A. AGUZZI, T. VOIGTLANDER, P. FERRIER *et al.*, 1999 The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat. Genet.* **22**: 276–280.
- DECOVILLE, M., E. GIACOMELLO, M. LENG and D. LOCKER, 2001 DSP1, an HMG-like protein, is involved in the regulation of homeotic genes. *Genetics* **157**: 237–244.
- DEJARDIN, J., A. RAPPAILLES, O. CUVIER, C. GRIMAUD, M. DECOVILLE *et al.*, 2005 Recruitment of Drosophila Polycomb group proteins to chromatin by DSP1. *Nature* **434**: 533–538.
- ELFRING, L. K., C. DANIEL, O. PAPOULAS, R. DEURING, M. SARTE *et al.*, 1998 Genetic analysis of *brahma*: the Drosophila homolog of the yeast chromatin remodeling factor SWI2/SNF2. *Genetics* **148**: 251–265.
- FLAUS, A., and T. OWEN-HUGHES, 2004 Mechanisms for ATP-dependent chromatin remodelling: farewell to the tuna-can octamer? *Curr. Opin. Genet. Dev.* **14**: 165–173.
- FORMOSA, T., P. ERIKSSON, J. WITTMAYER, J. GINN, Y. YU *et al.*, 2001 Spt16-Pob3 and the HMG protein Nhp6 combine to form the nucleosome-binding factor SPN. *EMBO J.* **20**: 3506–3517.
- GIAVARA, S., E. KOSMIDOU, M. P. HANDE, M. E. BIANCHI, A. MORGAN *et al.*, 2005 Yeast Nhp6A/B and mammalian Hmgbl facilitate the maintenance of genome stability. *Curr. Biol.* **15**: 68–72.
- GOLIC, K. G., and M. M. GOLIC, 1996 Engineering the Drosophila genome: chromosome rearrangements by design. *Genetics* **144**: 1693–1711.
- KAL, A. J., T. MAHMOUDI, N. B. ZAK and C. P. VERRIJZER, 2000 The Drosophila Brahma complex is an essential coactivator for the trithorax group protein Zeste. *Genes Dev.* **14**: 1058–1071.
- KAMAKAKA, R. T., C. M. TYREE and J. T. KADONAGA, 1991 Accurate and efficient RNA polymerase II transcription with a soluble nuclear fraction derived from Drosophila embryos. *Proc. Natl. Acad. Sci. USA* **88**: 1024–1028.
- KENNISON, J. A., and J. W. TAMKUN, 1988 Dosage-dependent modifiers of polycomb and antennapedia mutations in Drosophila. *Proc. Natl. Acad. Sci. USA* **85**: 8136–8140.
- MAREDA, D. R., C. B. ZRALY, Y. FENG, S. EGAN and A. K. DINGWALL, 2003 The Drosophila SNR1 (SNF5/INI1) subunit directs essential developmental functions of the Brahma chromatin remodeling complex. *Mol. Cell. Biol.* **23**: 289–305.
- MAREDA, D. R., C. B. ZRALY and A. K. DINGWALL, 2004 The Drosophila Brahma (SWI/SNF) chromatin remodeling complex exhibits cell-type specific activation and repression functions. *Dev. Biol.* **267**: 279–293.
- MOHRMANN, L., K. LANGENBERG, J. KRIJGSVELD, A. J. KAL, A. J. HECK *et al.*, 2004 Differential targeting of two distinct SWI/SNF-related Drosophila chromatin-remodeling complexes. *Mol. Cell. Biol.* **24**: 3077–3088.
- MOREIRA, J. M., and S. HOLMBERG, 2000 Chromatin-mediated transcriptional regulation by the yeast architectural factors NHP6A and NHP6B. *EMBO J.* **19**: 6804–6813.
- MOSRIN-HUAMAN, C., L. CANAPLE, D. LOCKER and M. DECOVILLE, 1998 DSP1 gene of *Drosophila melanogaster* encodes an HMG-domain protein that plays multiple roles in development. *Dev. Genet.* **23**: 324–334.
- NEMETH, M. J., D. J. CURTIS, M. R. KIRBY, L. J. GARRETT-BEAL, N. E. SEIDEL *et al.*, 2003 Hmgb3: an HMG-box family member expressed in primitive hematopoietic cells that inhibits myeloid and B-cell differentiation. *Blood* **102**: 1298–1306.
- NEMETH, M. J., A. P. CLINE, S. M. ANDERSON, L. J. GARRETT-BEAL and D. M. BODINE, 2005 Hmgb3 deficiency deregulates proliferation and differentiation of common lymphoid and myeloid progenitors. *Blood* **105**: 627–634.
- NER, S. S., and A. A. TRAVERS, 1994 HMG-D, the Drosophila melanogaster homologue of HMG 1 protein, is associated with early embryonic chromatin in the absence of histone H1. *EMBO J.* **13**: 1817–1822.
- NER, S. S., M. E. A. CHURCHILL, M. A. SEARLES and A. A. TRAVERS, 1993 dHMG-Z, a second HMG-1-related protein in Drosophila melanogaster. *Nucleic Acids Res.* **21**: 4369–4371.
- NER, S. S., T. BLANK, M. L. PEREZ-PARALLE, T. A. GRIGLIATTI, P. B. BECKER *et al.*, 2001 HMG-D and histone H1 interplay during chromatin assembly and early embryogenesis. *J. Biol. Chem.* **276**: 37569–37576.
- PAPOULAS, O., G. DAUBRESSE, J. A. ARMSTRONG, J. JIN, M. P. SCOTT *et al.*, 2001 The HMG-domain protein BAP111 is important for the function of the BRM chromatin-remodeling complex in vivo. *Proc. Natl. Acad. Sci. USA* **98**: 5728–5733.
- RAGAB, A., and A. TRAVERS, 2003 HMG-D and histone H1 alter the local accessibility of nucleosomal DNA. *Nucleic Acids Res.* **31**: 7083–7089.
- REEVES, R., 2003 HMGA proteins: Flexibility finds a nuclear niche? *Biochem. Cell Biol.* **81**: 185–195.
- RONFANI, L., M. FERRAGUTI, L. CROCI, C. E. OVITT, H. R. SCHOLER *et al.*, 2001 Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2. *Development* **128**: 1265–1273.
- RYDER, E., F. BLOWS, M. ASHBURNER, R. BAUTISTA-LLACER, D. COULSON *et al.*, 2004 The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster*. *Genetics* **167**: 797–813.
- SAHA, A., J. WITTMAYER and B. R. CAIRNS, 2005 Chromatin remodeling through directional DNA translocation from an internal nucleosomal site. *Nat. Struct. Mol. Biol.* **12**: 747–755.
- STURTEVANT, M. A., M. ROARK and E. BIER, 1993 The Drosophila rhomboid gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**: 961–973.
- SZERLONG, H., A. SAHA and B. R. CAIRNS, 2003 The nuclear actin-related proteins Arp7 and Arp9: a dimeric module that cooperates with architectural proteins for chromatin remodeling. *EMBO J.* **22**: 3175–3187.
- TALBERT, P. B., and R. L. GARBER, 1994 The Drosophila homeotic mutation *Nasobemia* (*AntpNs*) and its revertants: an analysis of mutational reversion. *Genetics* **138**: 709–720.
- TAMKUN, J. W., R. DEURING, M. P. SCOTT, M. KISSINGER, A. M. PATTATUCCI *et al.*, 1992 *brahma*: a regulator of Drosophila homeotic genes

- structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell* **68**: 561–572.
- THOMAS, J. O., and A. A. TRAVERS, 2001 HMG1 and 2, and related 'architectural' DNA-binding proteins. *Trends Biochem. Sci.* **26**: 167–174.
- TRAVERS, A. A., 2003 Priming the nucleosome: A role for HMGB proteins? *EMBO Rep.* **4**: 131–136.
- VAZQUEZ, M., L. MOORE and J. A. KENNISON, 1999 The trithorax group gene *osa* encodes an ARID-domain protein that genetically interacts with the brahma chromatin-remodeling factor to regulate transcription. *Development* **126**: 733–742.
- WAGNER, C. R., K. HAMANA and S. C. ELGIN, 1992 A high-mobility-group protein and its cDNAs from *Drosophila melanogaster*. *Mol. Cell. Biol.* **12**: 1915–1923.
- YU, Y., P. ERIKSSON and D. J. STILLMAN, 2000 Architectural transcription factors and the SAGA complex function in parallel pathways to activate transcription. *Mol. Cell. Biol.* **20**: 2350–2357.
- YU, Y., P. ERIKSSON, L. T. BHOITE and D. J. STILLMAN, 2003 Regulation of TATA-binding protein binding by the SAGA complex and the Nhp6 high-mobility group protein. *Mol. Cell. Biol.* **23**: 1910–1921.
- ZRALY, C. B., D. R. MAREDA, R. NANCHAL, G. CAVALLI, C. MUCHARDT *et al.*, 2003 SNR1 is an essential subunit in a subset of *Drosophila* brm complexes, targeting specific functions during development. *Dev. Biol.* **253**: 291–308.

Communicating editor: J. TAMKUN