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
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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 HMGBl–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. HMGs 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 (Bustín 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 Hmgbl 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) (Morin-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°C. All alleles and stocks used are as described in FlyBase (http://flybase.bio.indiana.edu), with the following exceptions: Df(2R)ED50000 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-3525-3, and CB-4009-3 were obtained from Szeged stock center; Dsp1 is kindly provided by D. Locker, Act5C alleles by J. Kadonaga, and Nurf301 [otherwise known as E(bx)] and Iswi alleles by P. Badenhorst.

Mounting fly wing: 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 MicroFastTrack 2.0 mRNA isolation kit (Invitrogen, San Diego), according to manufacturer’s 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’-GACACAAACCAAGGAAGA CT, Act5C 5’-ACCAAGTCAAGATCCCCGGG, HMGD 5’-CCC CGGAGTGCACATCAAGG, HMGD 5’-GCCGACCTCTCCTTCTT GCCT, HMGZ 5’-AACCAAGACCCCGGAGAAG, HMGZ 3’-CT TCTTGCTGGCTTGGC, CG30403 5’-TACCACAGGAGCT AAAGAAGA, and CG30403 3’-AAATAAGGAAGAGGGTTA GCAT.

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 (Kai 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 larger deficiency spanning HmgZ and HmgD [Df(2R)ED3923, breakpoints 57F6: 57F10] was seen. However, 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 the loss of CG30403 in Df(2R)ED50000, we performed qualitative RT–PCR on mRNA isolated from these flies. As shown in Figure 1C, we observed no product for CG30403 and HMGD, yet a band for HMGZ. To examine the level of HMGZ expression in Df(2R)ED50000, we probed Western blots of mutant embryos with an antibody raised against the HMGB domain of HMGD, which also recognizes HMGZ. Figure 1B illustrates that HMGZ levels in Df(2R)ED50000 are hardly reduced compared to wild-type expression, suggesting that HMGZ expression is upregulated to compensate for the loss of HmgD.

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 provides only qualitative evidence for the absence or presence of HMGD/Z transcripts. In wild-type flies, HMGD and HMGZ were detected, as illustrated in Figure 1C. In Df(2R)ED3921 flies, HMGD was not detected, yet HMGZ transcript was present. To quantify the amount of HMGZ mRNA remaining in these flies, Northern blot analyses of larvae and adults were performed. Figure 1D shows that flies homozygous for Df(2R)ED3921 retain low levels of HMGZ transcripts (~60% of the hybridization signal in larvae and 30% in adults compared with wild type). This agrees with the results obtained from the Western blot, and we conclude that Df(2R)ED3921 can be considered a strong hypomorph for HmgD/Z.

**HMGB redundancy:** The lack of a more severe phenotype in the HmgD/Z double mutants could perhaps be due to redundancy with other HMGB proteins. Other non-sequence-specific HMGB proteins in Drosophila are Dsp1 and an uncharacterized gene pair, CG7045 and CG7046. These proteins share high sequence similarity with HMGD/Z in the HMGB domain, but this is not extended to residues flanking this domain. Dsp1 contains two HMGB domains and a C-terminal acidic tail, while both CG7045 and CG7046 contain one HMGB domain, yet the C-termini are not enriched for acidic residues.

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 Dsp11 (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,

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**Figure 1.**—(A) Schematic overview of HmgZ and HmgD gene region. Positions of RS elements are marked, with arrows signifying the orientation of the FRT sequence. Annotated transcription start sites are shown for HmgZ, CG30403, and HmgD. The solid boxes represent exons. The figure is not drawn to scale. 5-HA-1624 and CB-5352-3, and 5-SZ-3389 and CB-0090-3, were used to generate Df(2R)ED3921 and Df(2R)ED50000, respectively. (B) Western blot of Df(2R)ED50000 and Df(2R)ED3921 embryos. Increasing amounts of wild-type (wt) and of either of the deficiency (Df) embryos were probed with an antibody against the HMGB domain of HMGD/Z (rat anti-HMGD-100, 1:250). One microliter equals one embryo. (C) Qualitative RT–PCR of HmgD and HmgZ transcripts in Df(2R)ED3921 flies compared to wild type. Z, HmgZ; D, HmgD; CG, CG30403; act, Act5C. Lanes 1–4: wild-type flies. Lanes 5–8: Df(2R)ED50000. Lanes 9–12: Df(2R)ED3921. M, 1-kb marker (Roche, Indianapolis). RT–PCR primers were designed to common spliced exons to generate a single product, and RT–PCR was performed at high cycle number (30 cycles). Df(2R)ED50000 and Df(2R)ED3921 flies show loss of HmgD and CG30403 transcript but retain HmgZ transcript. (D) Northern blot analysis of HmgZ transcripts in Df(2R)ED3921 larvae and adult flies. Blots were probed with a radiolabeled cDNA clone of HMGZ. Hybridization signals of both alternatively spliced HmgZ transcripts (HMGZ RA and HMGZ RB) are ~60 and 30% in Df(2R)ED3921 larvae and flies, respectively, compared with wild-type signal intensity.
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/+ flies. 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<sup>2</sup>, but not with alleles of domino (ATPase subunit of the NuA4 complex), Isw1 (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<sup>2</sup>/ + 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 transheterozygotes (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 transheterozygous for the HmgD/Z deficiency and the strong osa<sup>2</sup> allele displayed held out wings at 65% penetrance, and a weaker allele, osa<sup>00090</sup>, 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],...
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+. 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+ 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+ flies display variation in L4 length and we observed the same range of different L4 lengths in both rho+ 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+ 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+/+ 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; 1993).

### Table 1

<table>
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<tr>
<th>Genotype</th>
<th>% held out wings</th>
<th>N</th>
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<tr>
<td>Brahma brm+/+</td>
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<td>170/170</td>
</tr>
<tr>
<td>Df(2R)ED3921; brm+/+</td>
<td>100</td>
<td>207/207</td>
</tr>
<tr>
<td>Osa osa+/+</td>
<td>0</td>
<td>135/135</td>
</tr>
<tr>
<td>Df(2R)ED3921/+; osa+/+</td>
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<td>79/225</td>
</tr>
<tr>
<td>Df(2R)/; osa+/+</td>
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<td>120/120</td>
</tr>
<tr>
<td>osa+/+</td>
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</tr>
<tr>
<td>Moira Df(2R)/; mor+/+</td>
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<td>8/279</td>
</tr>
<tr>
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<td>0/315</td>
</tr>
<tr>
<td>Bap60 Df(1)C246/+; Df(2R)/</td>
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</tr>
<tr>
<td>Snr1 Df(2R)/; snr1+/+</td>
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<td>0/285</td>
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**Figure 3.—Interactions with rhomboid.** (A) Homozygous rho+ flies show incomplete L3, L4, and L5 veins. (B) Reduction in HMGD/Z levels enhances the rho+ phenotype in veins L2 and L5 and mildly so in L3. L4 length was variable in both rho+ and Df(2R)/; rho+ flies. A is representative of the shortest length and B of the longest L4 length observed.
Vazquez 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 AntpNs; 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 AntpNs (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 comb reduced. Df(2R)ED3921 enhanced loss-of-function alleles of Scr and partially suppressed the gain-of-function allele Scr5 (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

### Table 2

<table>
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<th>Genotype</th>
<th>Transformed flies/total % penetrance</th>
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<tr>
<td>AntpNs</td>
<td>135/145 93</td>
</tr>
<tr>
<td>AntpNs/ +</td>
<td>188/195 96</td>
</tr>
<tr>
<td>Df(2R)ED3921; AntpNs</td>
<td>96/132 72</td>
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<td>osa2/ AntpNs</td>
<td>9/97 9</td>
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![Figure 4](https://academic.oup.com/genetics/article-172/2/1069/5923030)
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 Tamrun 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), Sce (Sex combs extra), ph-p (polyhomolectic proximal), and esc (extra sex combs). No interactions were observed with any PcG mutants or with ash1 (absent, small, or homeotic discs) 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 rst" (roughest) and y" (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.

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 Antip, through loss of transcription activation at the P2 promoter. Furthermore, reduction of HmgD/Z levels partially suppresses the Antip 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 Antip.

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" phenotype in a similar way to brm mutants (Marena et al. 2004). We observe loss of both L2 and L5 veins in Df(2R)ED3921; rho" 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 dalaao. 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 HMGBI and the glucocorticoid receptor (GR) found that the residence time of GR bound to chromatin was increased in the presence of HMGBI (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 nhp6a and nhp6b genes results in a temperature-sensitive growth phenotype. Synthetic lethal phenotypes 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 CHA1 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 (Giavra et al. 2005). Increased sensitivity to UV irradiation was seen in mghb1−/− primary embryonic fibroblasts and in nhp6a/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
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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 poly peptide 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.

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