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The Multi-AT-Hook Chromosomal Protein of *Drosophila melanogaster*, D1, Is Dispensable for Viability

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ABSTRACT

The D1 protein is a high mobility group A (HMGA)-like nonhistone chromosomal protein with primary localization to certain AT-rich satellite DNA sequences within heterochromatin. The binding of D1 to euchromatic sequences is less studied and the functional significance of its chromosomal associations is unclear. By taking advantage of existing *P*-insertion alleles of the *D1* gene, I generated *D1* null mutations to investigate the phenotypic effect of loss of the *D1* gene. In contrast to a previous report, I determined that the *D1* gene is not essential for viability of *Drosophila melanogaster*, and moreover, that loss of *D1* has no obvious phenotypic effects. My tests for an effect of *D1* mutations on PEV revealed that it is not a suppressor of variegation, as concluded by other investigators. In fact, the consequence of loss of *D1* on one of six variegating rearrangements tested, $T(2;3)Sb^{V}$, was dominant enhancement of PEV, suggesting a role for the protein in euchromatic chromatin structure and/or transcription. A study of D1 protein sequence conservation highlighted features shared with mammalian HMGA proteins, which function as architectural transcription factors.

THE Drosophila genome, like that of other eukaryotes, exists in the form of chromatin, a complex of DNA and an assortment of DNA-binding proteins. Histone proteins facilitate the organization of DNA into nucleosomal fibers, and together with nonhistone chromosomal proteins compact, organize, and regulate the activity of the genome. Differential targeting of nonhistone chromosomal proteins is important for generating distinct chromatin domains, and both the genomic distribution and functions of such proteins continue to be a rich area of investigation. In Drosophila, genetic and biochemical studies have successfully identified proteins specific for or highly enriched in the heterochromatic regions of the genome (James and Elgin 1986; reviewed by Grigliatti 1991; Cortes *et al.* 1999; De Felice *et al.* 1999; reviewed by Schotta *et al.* 2003). For example, both methodologies converged in the identification of HP1 (heterochromatin protein 1, encoded by the *Su(var)205* gene; Sinclair *et al.* 1983; James and Elgin 1986). This protein, found in animals, plants, and fungi, associates with nucleosomes having lysine 9 of histone H3 methylated, a characteristic of heterochromatin (Bannister *et al.* 2001). However, the activities of many heterochromatin-associated proteins have yet to be defined. An example of this is the D1 (Drosophila protein 1) protein, which binds to a subset of highly repetitive DNAs, called satellite DNAs, that are found in heterochromatin.

The D1 protein was first identified >30 years ago (Alfageme *et al.* 1974), but its function remains elusive. It is a nonhistone chromosomal protein that shares many structural similarities with high mobility group (HMG) proteins (Rodriguez Alfageme *et al.* 1980). These include its solubility in 5% perchloric acid, high fraction of charged amino acids, properties for extraction from chromatin, and relative nuclear abundance. The cloning and sequence analysis of the *D1* gene showed that it is most similar to the high mobility group A (HMGA) family of proteins (Ashley *et al.* 1989). Both D1 and the HMGA proteins possess several copies of the AT-hook DNA binding motif, which confers upon them the ability to bind to short uninterrupted AT tracts (Levinger 1985a; Ashley *et al.* 1989; Reeves and Nissen 1990). While HMGA proteins have only three copies of this motif, there are 10 AT hooks in the D1 protein, which is more than three times larger. Biochemical studies have shown that D1 associates with two AT-rich satellite DNAs, *in vivo* and *in vitro* (Levinger and Varshavsky 1982a; Levinger 1985a,b). It shows greatest affinity for the simple 1.672 g/cm^3 satellite, which has the pentamer AATAT as its primary repeat. It also binds to the complex 1.688 g/cm^3 satellite, which is chiefly a 359-bp repeat and 69% AT in composition. In agreement with this work, a heterochromatic localization was observed for D1 in both mitotic and interphase diploid cells by immunostaining (Renner *et al.* 2000;

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Aulner *et al.* 2002). As revealed by immunostaining to salivary gland polytene chromosomes, D1 shows a less predominant localization to euchromatic sites, which could reflect its binding to interspersed AT tracts (Alfageme *et al.* 1976; Rodriguez Alfageme *et al.* 1980).

Mutant alleles of the *D1* gene have not been isolated in phenotype-based genetic screens. However, two *P*-insertion alleles of *D1* were recently obtained in *P*-element mutagenesis experiments conducted as part of the Berkeley Drosophila Genome Project (BDGP) functional annotation of the Drosophila genome (Rorth 1996; Bellen *et al.* 2004). Chromosomes bearing these *P* insertions were reported to be homozygous lethal (http://flystocks.bio.indiana.edu), suggesting that the *D1* gene was essential for viability. These strains could thus represent a starting point for the genetic and molecular characterization of *D1* function. To this end, I examined the lethality of the *P*-insertion lines, carried out genetic screens to isolate *D1* null mutants, and performed genetic tests to study the effects of loss of D1 protein. Although other investigators reported that the *D1* gene is essential (Aulner *et al.* 2002), the studies described herein demonstrated that *D1* is not required for viability or fertility. In addition, it is not a suppressor of position effect variegation.

MATERIALS AND METHODS

Drosophila stocks and culture conditions: Stocks were maintained at 25° on cornmeal-malt medium described as standard medium by the Bloomington Drosophila Stock Center (BDSC) (http://flystocks.bio.indiana.edu). The *P{EP}-D1^{EP473}* stock was obtained from the BDSC, although it is no longer available through that facility. The *w^{m51b}*, *w^{mmcT} w^{m4f}*, and *w^{m4Ta}* stocks were a gift of P. Talbert and S. Henikoff (Talbert and Henikoff 2000). K. Ahmad generously provided the *bw^{D}* and *Byron* stocks. The *Df(3R)BSC24* deletion was created at the BDSC using the hybrid element insertion (HEI) strategy (Gray *et al.* 1996; Preston *et al.* 1996) with *P*-element insertions *P{EP}EP3243* (3R:4,757,601) and *P{EP}EP707* (3R:5,220,293) (Parks *et al.* 2004). The Exelixis deletion *Df(3R)Exel6152* was synthesized using *FLP* recombinase and the FRT-bearing transposon insertions *P{XP}d04033* (3R:4,983,798) and *P{XP}CG8420^{d04746}* (3R:5,073,203) (Golic and Golic 1996; Parks *et al.* 2004). Other mutations and strains utilized in this study are described in FlyBase (Tweedie *et al.* 2009).

***P*-element excision:** The *P{EP}D1^{EP473}* and *P{EPgy2}D1^{EY05004}* insertions within the *D1* gene were mobilized using *P* transposase to determine if excision of the *P* element restored homozygous viability to the chromosome. Males bearing the *P* insertion heterozygous with the *TMS, Sb P{ry^{+} Δ2-3}99B* P-transposase chromosome were backcrossed to *w; P{EP}D1^{EP473}/TM3, Sb* or *y^{1} w^{67c35}; D1^{EY05004}/TM3, Sb Ser* females as appropriate. The progeny were screened for either Sb^{+} individuals, produced by reversion of the lethal mutation, or white-eyed males, produced by loss of expression of the *P*-element marker gene, *w^{+}*. The same process was carried out for a *P{EP}D1^{EP473}* chromosome that had undergone *P*-element-mediated male recombination to replace the third chromosome left arm and DNA proximal to the *P* insertion on the right arm (recombinant 70).

The *P* element that remained at the deletion site on the *Df(3R)D1^{C12}* chromosome was mobilized by crossing *st^{1} Df(3R)D1^{C12}/TM3, Sb st^{AP1} e* females to *w/Y; T(2:3)lt^{x13}, Sp lt^{x13}/CyO, H{PDelta2-3}Hop2.1; TM3, Sb st^{AP1} e* males to produce *+/CyO, H{PDelta2-3}Hop2.1; st^{1} Df(3R)D1^{C12}/TM3, Sb st^{AP1} e* dysgenic males, which were then mated to *w; TM3, Sb st^{AP1} e/TM6B, Tb Hu e* females. The *Df(3R)D1^{C12w-}* chromosome was isolated among the w^{-} e^{+} Cy^{+} male progeny, and stocked.

PCR analysis: The presence of *P*-element sequence in the *D1* gene following exposure of the chromosome to *P* transposase was assessed by isolating genomic DNA and performing PCR as follows. Single fly DNA was isolated according to Gloor *et al.* (1993). For the *P{EP}D1^{EP473}* mobilization experiments, the *D1* proximal primer (D1 2171F; 5′-GCGCTTCTTTACCGCAACTT-3′) was used in combination with primer Pry4 (5′-CAATCATATCGCTGTCTCACTCA-3′; BDGP) to assess the presence of the 3′ *P* end and integrity of the flanking sequence. The *D1* distal primer (D1 2965R; 5′-GGCCAGCCGTCTCATGTAGT-3′) was used in combination with primer Plac1 (5′-CACCCAAGGCTCTGCTCCCA CAAT-3′; BDGP) to assess the presence of the 5′ *P* end and integrity of the flanking sequence. For the *P{EPgy2}D1^{EY05004}* mobilization experiments, primer D1 2171F was used in combination with primer Plac1 to assess the presence of the 5′ *P* end and integrity of the flanking sequence. Primer D1 2965R was used in combination with primer Pry2 (5′-CTTGCCGACGGGACCACCTTATGTTATT-3′; BDGP) to assess the presence of the 3′ *P* end and integrity of the flanking sequence. The D1 2171F and D1 2965R primer combination generated a 794-bp fragment from wild-type *D1* sequence.

For w^{-} excision line 2A, the extent of the residual *P*-element sequence and the integrity of the flanking *D1* gene sequence was assessed by PCR amplification using primers Pwht1 (5′-GTAACGCTAATCACTCCGAACAGGTCACA-3′) and D1 2171F, followed by DNA sequencing using the same primers.

The *D1* mutant third chromosomes of lines 1A, 4A, and 70-7 were balanced with *TM3, P{w^{+} GAL4-twi.G}2.3, P{ w^{+} UAS-2xEGFP}AH2.3, Sb^{1} Ser^{1}* and these flies were crossed to *Df(3R)BSC24/TM3, P{w^{+} GAL4-twi.G}2.3, P{ w^{+} UAS-2xEGFP}AH2.3, Sb^{1} Ser^{1}* flies. The *D1* mutant/*Df(3R)BSC24* progeny were identified as EGFP-negative first instar larvae, and DNA was isolated from single larvae in a 10-μl volume as per Gloor *et al.* (1993). The *D1* PCR primers were as follows: D1 21F (5′-CGAAGCGCACTGAGAAACAC-3′), D1 853F (5′-CATAACCG TCGTTGGCATCA-3′), D1 1605F (5′-TGGTTGCGGAATGTT GAAAT-3′), D1 2171F, D1 3374F (5′-GTGCATCGAGCAGC GATAA-3′), D1 3688F (5′-TGCGTGAACAACCAAGTTAAGC-3′), D1 3941F (5′-CGCTCACTTCCACAGCTTGA-3′), D1 921R (5′-GGACACCAACCAAAGGAGATG-3′), D1 1699R (5′-TGCT TCCACCAAACTTGCAC-3′), D1 2305R (5′-TGAGCGTGTG TTCGTGAGAG-3′), D1 3285R (5′GCAAGTAATTCCCTTTC GGATCT-3′), D1 2965R, D1 4320R (5′-GGACATCACCAACC CAAAGAA-3′). The *pumilio* primer sets were pum 9494F (5′-TCCCTTTCGGTCCTTTCGT-3′) and pum 9835R (5′-TGT GTGTGCTCTCTCGCTCTT-3′), and pum 6925F (5′-CTCAA CATGTTACTACAATGGCTCT-3′) and pum 7624R (5′-CGT GTGGTTCTTTGTGCTG-3′). The DNA integrity of each larval DNA sample was verified by successful amplification using a primer set specific for the *BSC24* deficiency chromosome, BSC24 5′ (5′-CAACTCGTCCGCTCCGCACAAC-3′) and Plac1. Positive control DNA was isolated from *TM3/Df(3R)BSC24* first instar larvae, identified as EGFP positive and giving rise to the *BSC24*-specific PCR fragment.

The inversion breakpoint of *In(3R)D1^{1A}* was isolated by inverse PCR according to the protocol of E. J. Rhem, BDGP (http://www.fruitfly.org/about/methods/inverse.pcr.html). Genomic DNA isolated from the line 1A was digested with *Bam*HI, ligated and PCR amplified using primers D1 3941F

and D1 2965R, which directed synthesis away from each other. The PCR product was gel purified using the QIAquick Gel Extraction kit (QIAGEN, Valencia, CA) prior to sequencing.

For identification of the *pum–D1* deletion chromosome, *Df(3R)D1^{C12}*, DNA isolated from each recombinant line was analyzed by PCR to show that sequences proximal to the *pum^{KG02259}* insertion and distal to the *D1^{EY05004}* insertion were present and abutting *P*-element ends, but that the *pum^{KG02259}* distal and *D1^{EY05004}* proximal sequences were not detectable. Both transposon insertions were oriented with the 5′ *P* end centromere proximal. Primer combinations were pum 9494F and Plac1, Pry2 and pum 9835R, D1 2171F and Plac1, and Pry2 and D1 2965R. Subsequent to mobilization of the *P* element marking the deletion, primers pum 9494F and D1 2965R were used to amplify across the deletion breakpoint. This PCR product was sequenced.

Primers were designed using Primer 3 (http://primer3.sourceforge.net/). For DNA sequence analysis, PCR products were treated with ExoSAP-IT (USB, Cleveland) and used directly for sequence determination at the ISU Molecular Research Core Facility.

Southern analysis: Genomic DNA was isolated using the DNeasy Blood and Tissue kit (QIAGEN). For each strain, DNA derived from ~10 mg of whole flies was digested with *Nco*I (Figure 2) or *Bam*HI (Figure 4) and fractionated on an agarose gel. The DNA was transferred to a positively charged nylon membrane and processed using the DIG Luminescent system according to the manufacturer's instructions (Roche Applied Science, Indianapolis). For analysis of the deficiency lines (Figure 2), the PCR DIG probe consisted of the entire *D1* coding region, using a cloned *D1* cDNA as a template. For analysis of the *D1^{EP473}* excision lines (Figure 4), the PCR DIG probes were synthesized using the D1 2171F and D1 2965R primer combination (Part A) or a primer set that amplified *CG17360* genomic DNA (5′-TGATGGTTGCTGCTGGTGTT-3′ and 5′-GAGCCCAATATCGGAGATGC-3′; Part B) and a fly genomic DNA template.

***P*-element-mediated male recombination:** The technique of *P*-element-mediated male recombination was used to induce the exchange of DNA flanking the *P{EP}D1^{EP473}* insertion site (*i.e.*, 3L and proximal 3R, or distal 3R) with that of the homolog, with the potential of recovering deletion alleles among the recombinants. The *Gl^{1}* mutation was recombined onto the left arm of the *P{EP}D1^{EP473}* chromosome to serve as a dominant marker for detecting recombinants. *w^{1118}* females were crossed to *P* transposase-expressing *w/Y; +/CyO, H{PDelta2-3}Hop2.1; Gl^{1} P{EP}D1^{EP473}/Bsb* males, and Gl^{+} Bsb^{+} or Gl^{-} Bsb^{-} male recombinant progeny were recovered. Recombinant 70, which retained the *P* insertion and did not suffer a flanking deletion, was identified within the Gl^{+} Bsb^{+} class.

As a means to isolate a deletion between the *P{EPgy2}-D1^{EY05004}* and *P{SUPor-P}pum^{KG02259}* elements, *P* transposase was expressed in male flies carrying the *P* insertions in *trans*. In preparation, a *st^{1} P{SUPor-P}pum^{KG02259} ca^{1}* chromosome was produced by meiotic recombination to facilitate the subsequent identification of recombinants. The *y^{1} w^{67c35}/Y; +/CyO, H{PDelta2-3}Hop2.1; st^{1} P{SUPor-P}pum^{KG02259} ca^{1}/P{EPgy2}D1^{EY05004}* males were obtained by crossing *y^{1} w^{67c35}; P{EPgy2}D1^{EY05004}/TM3, Sb Ser* females to *+/CyO, H{PDelta2-3}Hop2.1; st^{1} P{SUPor-P}pum^{KG02259} ca^{1}/TM3, Ser* males and selecting Cy^{-} Sb^{+} Ser^{+} progeny. These males were then crossed to *st^{1} Sb^{sbd-1} e^{s} ro^{1} ca^{1}* females and st^{-} ca^{+} recombinant progeny, which were expected to include the desired deletion class, were recovered and stocked.

RT–PCR analysis: Total RNA was isolated from ovaries dissected from females of each genotype, using UltraSpec RNA (Biotecx Laboratories, Houston). Random-hexamer primed cDNA was synthesized from 1 μg of total RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. One-twentieth of the synthesis reaction was used as a template for a 25-μl PCR reaction, unless otherwise indicated. The primers D1 1261F (5′-CCGACTTGTTCTGTGGTGGA-3′) and D1 1803R (5′-CCAGCGATAGCGAGAATGAA-3′) were used to amplify a 224-bp segment of the *D1* mRNA. The primers *E(var)3-9* 2261F (5′-GCCGAACTGCTCCTGTGTCT-3′) and *E(var)3-9* 2660R (5′-GTCGCTTTGTGTGGAACGGATT-3′) were used to amplify a 351-bp segment of the *E(var)3-9* mRNA, as a control (Weiler 2007). RT–PCR was performed using two sets of independently isolated RNA samples, with identical results.

***Stubble* variegation assay:** A reciprocal translocation between the second and third chromosomes places the *Stubble* (*Sb*) gene, having the *Sb^{1}* mutation, under the repressive influence of the chromosome 2 heterochromatin in the *T(2;3)Sb^{V}* strain. Silencing of *Sb^{1}* effects a wild-type bristle, while its expression results in the Stubble phenotype. Crosses were performed at 25° between *T(2;3)Sb^{V}, In(3R)Mo, Sb^{1} sr^{1}/+; TM3, Ser e* females and males heterozygous for the *D1* mutant or wild-type control chromosome and the *ru^{1} h^{1} th^{1} st^{1} cu^{1} sr^{1} e^{s} Pr^{1} ca^{1}* chromosome. The *D1/T(2;3)Sb^{V}* progeny were identified as Ser^{+} Pr^{+}. To eliminate the potential influence of sex on variegation, only female progeny were scored. Fourteen bristles: the anterior and posterior sternopleurals, the upper and lower humerals, the anterior and posterior scutellars, and the posterior dorsocentrals, were scored for a Sb^{-} or wild-type phenotype.

D1 protein comparison: The predicted protein sequences of D1 orthologs in other Drosophila species were obtained from FlyBase (Tweedie *et al.* 2009) with the exception of that of *D. simulans*, which was not present. The partial gene sequence of the *D. simulans D1* gene was identified by tBLASTn of *D. simulans* genomic sequence using the *D. melanogaster* protein as a query (http://insects.eugenes.org/species/blast/). FGENESH+ (http://www.softberry.com) was used to predict the partial protein sequence. The missing N terminus of the protein (33 amino acids) was constructed by translation of the adjoining genomic sequence, assuming two DNA sequencing errors that affected the reading frame and using the *D. melanogaster* D1 protein sequence as a guide. Prediction of protein motifs was performed against the Pfam database (Finn *et al.* 2006). Amino acid similarity to *D. melanogaster* D1 was determined using BLAST2 (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi).

RESULTS

***P*-element alleles of the *D1* gene do not revert to viability:** A genetic approach toward elucidating the function of the *D1* gene was undertaken using two *P*-insertion alleles of the *D1* gene, which were recovered in the *P{EP}* and *P{EPgy2}* mutagenesis experiments that contributed to the BDGP Gene Disruption Project (Rorth 1996; Rorth *et al.* 1998; Bellen *et al.* 2004). Both insertions map to the 5′-untranslated region (UTR) of *D1* (Bellen *et al.* 2004). For both stocks, the third chromosome bearing the *P* insertion was homozygous lethal, suggesting that the insertions disrupted the *D1* gene and that the *D1* gene was essential for viability. If true, it should have been possible to mobilize the *P* elements and revert the lethal phenotype. However, I was unable to recover any homozygous viable

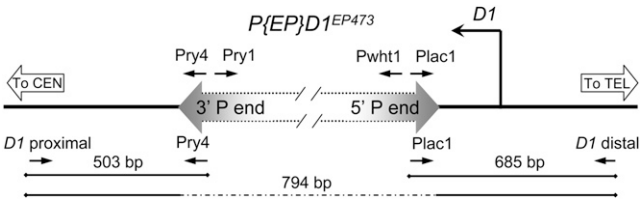

Figure 1.—PCR analysis of *D1 P*-insertion alleles. The left–right block arrow represents the *P{EP}D1*^{EP473} element inserted within the 5′-UTR of the *D1* genomic locus. The relative locations of the PCR primers used to analyze the insertion and its excision derivatives and PCR product sizes are shown. The distance between the *D1* proximal and *D1* distal primers was too large for PCR amplification with standard Taq polymerase when the entire *P{EP}* element was present, but yielded a 794-bp product in its absence. The *P{EPgy2}D1*^{EY05004} insertion is similarly located in the 5′-UTR but in the opposite orientation. PCR analyses of this element typically utilized the Pry2 primer (adjacent to the Pry4 primer; not shown) instead of the Pry4 primer.

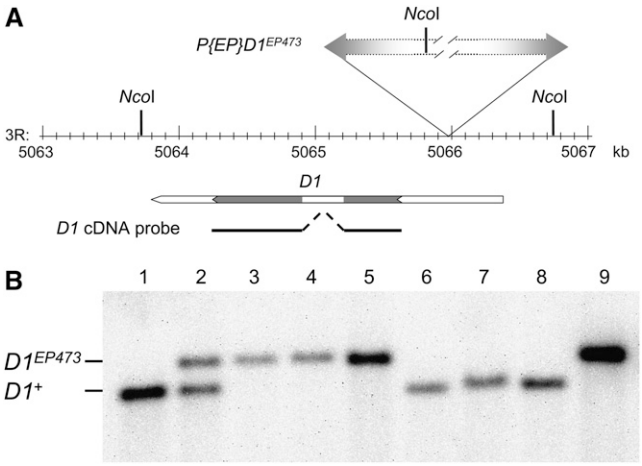

Figure 2.—Southern analysis of deletion strains. (A) The *Nco*I restriction map of the chromosome 3R genomic region surrounding the *D1*^{EP473} insertion site is illustrated. The gene span of *D1*, with coding region in gray, and position of the *D1* cDNA probe used for Southern analysis (solid line) are shown below the map. (B) Genomic DNA was isolated from *w*^{1118} (lane 1), *D1*^{EP473}/+ (lane 2), *D1*^{EP473}/*Df(3R)Exel6152* (lane 3), *D1*^{EP473}/*Df(3R)BSC24* (lane 4), *D1*^{EP473}/*Df(3R)D1*$^{C12w-}$ (lane 5), +/*Df(3R)Exel6152* (lane 6), +/*Df(3R)BSC24* (lane 7), +/*Df(3R)D1*$^{C12w-}$ (lane 8), and *D1*^{EP473} (lane 9), and digested with *Nco*I. A wild-type *D1* locus was expected to yield a 3041-bp genomic fragment whereas the *D1*^{EP473} insertion was expected to yield a 3481-bp genomic fragment. Only the *D1*^{EP473} allele was observed when the flies were heterozygous for *D1*^{EP473} and deletions *Df(3R)Exel6152*, *Df(3R)BSC24*, or *Df(3R)D1*$^{C12w-}$. In contrast, only the wild-type allele was observed when the flies were heterozygous for *D1*$^{+}$ and deletions *Df(3R)Exel6152*, *Df(3R)BSC24*, or *Df(3R)D1*$^{C12w-}$.

chromosomes following the introduction of a *P*-transposase source. For example, none of ~1500 progeny produced from a cross between *w; P{EP}D1*^{EP473}*/TM3, Sb* females and *P{EP}D1*^{EP473}*/TMS, Sb P{Δ2-3}99B* males was found to be Sb^{+}, the phenotype expected for *D1*$^{+}$*/P{EP}D1*^{EP473} flies. Similarly, none of ~850 progeny produced from a cross between *y*^{1} *w*^{67c35}*; P{EPgy2}-D1*^{EY05004}*/TM3, Sb Ser* females and *y*^{1} *w*^{67c35}*/Y; P{EPgy2}-D1*^{EY05004}*/TMS, Sb P{Δ2-3}99B* males were Sb^{+}. In contrast, a high frequency of white-eyed progeny resulting from loss of expression of the *w*$^{+}$ gene carried by either *P* element was observed. This result indicated that the *P* element was being mobilized in both experiments.

Chromosomes isolated upon precise excision of the *P{EP}D1*^{EP473} (hereafter referred to as *D1*^{EP473}) and *P{EPgy2}D1*^{EY05004} (hereafter referred to as *D1*^{EY05004}) elements remained homozygous lethal, revealing the presence of extraneous lethal mutations. Presumptive precise excision lines were identified among the w^{-} progeny following *P* mobilization, by PCR analysis of the genomic DNA encompassing the insertion site (see Figure 1 and materials and methods). DNA sequencing confirmed that the wild-type gene sequence was restored upon excision of *D1*^{EP473}, for isolate *D1*^{Rev1B} (see below). Nevertheless, the chromosome bearing the *D1*^{Rev1B} "revertant" allele was homozygous lethal, as were excision lines derived from the *D1*^{EY05004} insertion chromosome. These results indicated that lethal mutations were present on the *D1*^{EP473} and *D1*^{EY05004} chromosomes, but did not reveal if the insertions themselves conferred lethality.

***P*-element alleles of the *D1* gene are not lethal:** The presence of lethal mutations on the *D1*^{EP473} and *D1*^{EY05004} chromosomes left open the question of whether the *P* insertions themselves were lethal. To address this issue, the two mutants were crossed to each other as well as to strains bearing deficiencies of the *D1* gene. When *w; D1*^{EP473}*/TM3, Sb* and *y*^{1} *w*^{67c35}*/Y; D1*^{EY05004}*/TM3, Sb Ser* flies were crossed, the expected frequency of *D1*^{EP473}*/D1*^{EY05004} progeny flies was observed (32.1%; $n = 134$), indicating that the two *P*-insertion chromosomes did not share lethal mutations. Outcrossing of the *D1*^{EP473} strain eventually resulted in the isolation of a homozygous viable *D1*^{EP473} chromosome, confirming the viable nature of this allele. Moreover, both *D1 P*-insertion alleles were hemizygous viable in combination with the large deficiencies *Df(3R)BSC24* and *Df(3R)Exel6152*. The *Df(3R)BSC24* chromosome is reported to possess a deletion of ~463 kb (from *pyd* to *Fsp85D*) that includes the *D1* gene (Parks *et al.* 2004; Tweedie *et al.* 2009). The ~89-kb *Df(3R)Exel6152* deletion has breakpoints proximal (in *pumilio*) and distal (in *CG8420*) to *D1* (Parks *et al.* 2004). The *D1* gene was not detectable in the deficiency chromosomes by Southern analysis using the entire *D1* coding sequence as a probe (Figure 2) or by PCR (data not shown). These results clearly demonstrated that the two *D1 P*-insertion alleles were not lethal.

Generation of new *D1* alleles: Although the two *D1 P*-insertion alleles were determined to be hemizygous viable, the possibility existed that, being located within the 5′-UTR of the gene, neither precluded *D1* expression.

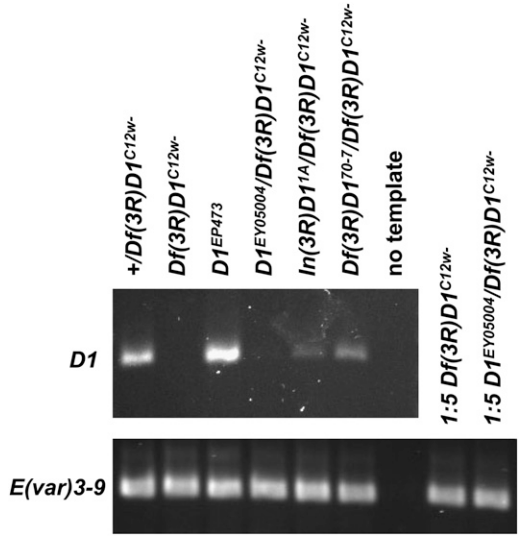

Figure 3.—RT–PCR analysis of *D1* mutant alleles. cDNA samples prepared from equivalent amounts of ovary RNA, for the indicated strains, were used as templates for PCR of the *D1* mRNA (primers D1 1261F and D1 1803R) and control *E(var)3-9* mRNA (primers *E(var)3-9* 2261F and *E(var)3-9* 2260R). Both primer sets spanned an intron, enabling products from potential contaminating genomic DNA to be distinguished. However, no genomic DNA products were observed. Where no ($Df(3R)D1^{C12w-}$) or very little ($D1^{EY05004}/Df(3R)D1^{C12w-}$) *D1* RT–PCR product was observed, the control *E(var)3-9* PCR reaction was performed using fivefold diluted cDNA template, as a means to confirm the integrity of the cDNA template. *E(var)3-9* mRNA has been quantified at ~69% of the level of *D1* mRNA in the ovary, using microarray analysis (Chintapalli *et al.* 2007).

Therefore, the results described above did not rule out that the *D1* gene could be essential and that a level of gene product sufficient for viability was produced by the *P*-insertion alleles. Indeed, both the D1 protein and *D1* RNA were detectable in ovarian tissue of $D1^{EP473}$ flies (Figure 3 and data not shown). To generate an unequivocal *D1* null allele, one that lacked *D1* coding sequence, two approaches were undertaken (as described in more detail below). The $D1^{EP473}$ insertion was mobilized with the intention of recovering imprecise excision events that would delete some or all of the *D1* gene. Second, *P*-element-mediated male recombination was performed in flies possessing both a *P* insertion into the 5′-UTR of the *D1* gene and a *P* insertion downstream of the *D1* coding sequence with the aim of recovering a deletion mediated and demarcated by the two *P* insertions (Parks *et al.* 2004).

Imprecise excision strategy: As a means to isolate imprecise excision derivatives of $D1^{EP473}$, the male progeny of a cross between *w;* $D1^{EP473}$*/TM3, Sb* females and $D1^{EP473}$*/TMS, Sb P{ry*$^{+}$ *Δ2-3}99B* males were screened for loss of expression of the w^{+} *P{EP}* element marker gene. The results of two experiments are described here, the second using a derivative of the original $D1^{EP473}$ chromosome, recombinant 70, which had been recovered following *P*-element-mediated male recombination (see materials and methods). A combined total of 18 w^{-} exceptions were stocked. The PCR strategy illustrated in Figure 1 was employed for initial molecular analyses of the exceptional lines. To ascertain if either *P*-element end did not excise, primers that hybridized to *D1* genomic sequence proximal and distal to the insertion site were used in combination with primers that hybridized to the 3′ and 5′ *P*-element ends, respectively. To detect small deletions of genomic DNA (extending no more than ~400 bp in either direction from the insertion site), the proximal and distal *D1* genomic primer combination was used. Southern analysis was performed using genomic DNA extracted from balanced stocks of each w^{-} line to further investigate the nature of each *P*-excision event. A probe was synthesized by PCR using the *D1* proximal and distal genomic primers (Figure 1). This probe should recognize an ~1.7-kb fragment for a wild-type *D1* allele, such as was present on the *TM3, Sb* balancer chromosome of each stock, and an ~9.7-kb fragment for the $D1^{EP473}$ allele.

The results of the molecular analyses, as shown in Figure 4 and Table 1, suggested that eight w^{-} isolates likely resulted from precise excision, seven w^{-} chromosomes retained some or all of the *P{EP}* element, and three w^{-} chromosomes possessed *D1* deletions or other rearrangements.

Precise excision lines: The PCR data (Table 1) and Southern data (Figure 4 and Table 1) strongly suggested that the $D1^{EP473}$ element had precisely excised in lines 1B, 1C, 1D, 1F, 70-1, 70-2, 70-3, and 70-9. The PCR assays showed no evidence of *P*-element sequence at the *D1* locus nor the existence of a small deletion. This was consistent with the results of Southern analysis, which revealed a single band of 1.7 kb for each line. DNA was extracted from allele 1B/*Df(3R)BSC24* and allele 70-1/*Df(3R)Exel6152* flies, and the region encompassing the original insertion site of the *P{EP}* element was PCR amplified using the *D1* proximal and distal genomic primers. Given the absence of *D1* gene sequence on the deficiency chromosome, the only PCR template was the revertant allele. Sequence analysis of this PCR fragment confirmed that line 1B (allele $D1^{Rev1B}$) and line 70-1 (allele $D1^{Rev70\text{-}1}$) resulted from precise excision of the $D1^{EP473}$ insertion; the *D1* gene sequence was restored to wild type.

D1 insertion mutants: For lines 1E, 2B, 2C, 3A, and 70-6, the *D1* locus appeared to possess an extra ~50 bp upon *P*-element excision, observed as an additional PCR fragment using the *D1* genomic primer set and as a doublet band by Southern (Figure 4 and Table 1). To determine the nature of this insertion, DNA was extracted from line 1E/*Df(3R)BSC24* flies and the region encompassing the original insertion site of the *P{EP}* element was PCR amplified and sequenced using the *D1* proximal and distal primers (Figure 1). Consistent with the PCR results, an 8-bp *P*-target site repeat and 33 bp of additional

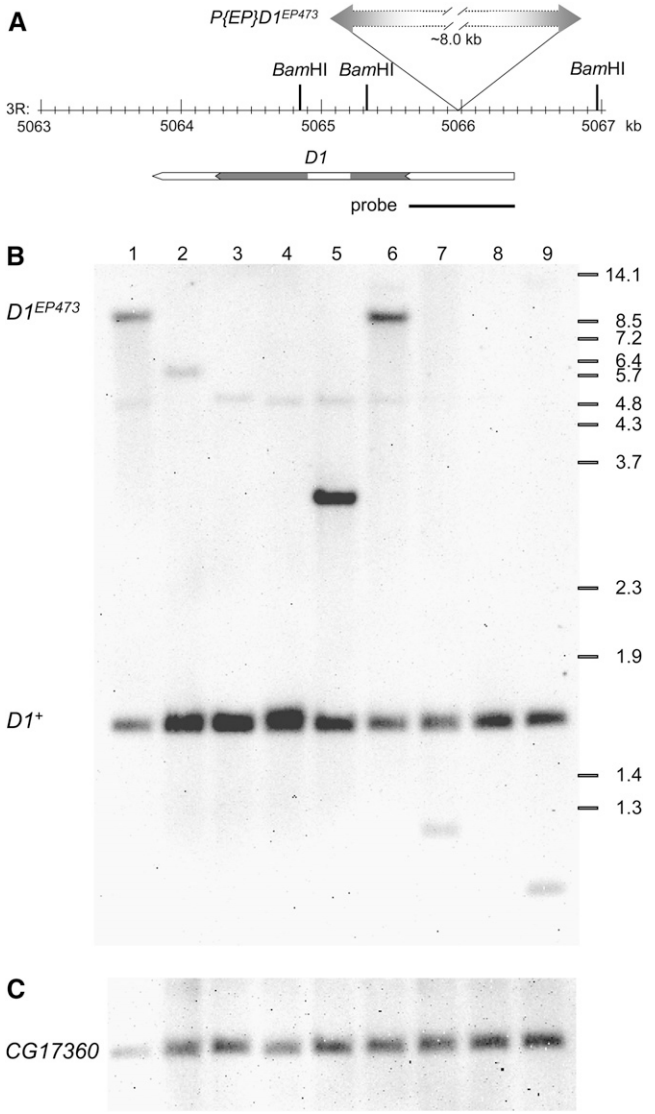

FIGURE 4.—Southern analysis of $D1^{EP473}$ excision lines. (A) The *Bam*HI restriction map of the chromosome 3R genomic region surrounding the $D1^{EP473}$ insertion site is illustrated. The gene span of *D1*, with coding region in gray, and position of the region amplified by the D1 proximal and D1 distal primer pair (Figure 1), which was used as a probe for Southern analysis, are shown below the map. (B and C) A representative line from each class of w⁻ excision lines (see Table 1) is included in the Southern blot shown here. Genomic DNA was isolated from $D1^{EP473}$/*TM3, Sb* (lane 1), w^{1118} (lane 2), $D1^{Rev1B}$/*TM3, Sb* (lane 3), $D1^{1E}$/*TM3, Sb* (lane 4), $D1^{2A}$/*TM6B, Tb* (lane 5), $D1^{70\text{-}5}$/*TM6B, Tb* (lane 6), $In(3R)D1^{1A}$/*TM3, Sb* (lane 7), $Df(3R)1^{4A}$/*TM3, Sb* (lane 8), and $Df(3R)D1^{70\text{-}7}$/*TM3, Sb* (lane 9), and digested with *Bam*HI. (B) The *D1* probe hybridizes to a ~1.7-kb genomic fragment for wild-type flies, and an ~9.7-kb genomic fragment for the $D1^{EP473}$ allele. A faint cross-hybridizing fragment, polymorphic in w^{1118}, can be detected at 5–6 kb. The approximate positions of the *Bst*EII-digested λ-size markers are shown at right. (C) The blot was rehybridized with a *CG17360* probe that recognizes an ~2.2-kb genomic fragment, to serve as a normalization (loading) control.

P-element sequence were found to remain at the insertion site.

Line 2A also retained extra DNA sequence upon *P* excision, as indicated by the novel band observed by Southern analysis and revealed by PCR to include the *P*-element 5′ end (Figure 4 and Table 1). As this *P*-transposase-induced lesion might include a deletion of *D1* sequence, sequence analysis of the genomic DNA was carried out. A PCR primer that hybridized to the 5′-*P* end and directed synthesis proximally (Pwht1; see Figure 1) was used in combination with the *D1* proximal primer to amplify across the genomic DNA-*P{EP}*-element junction. Sequence analysis of the resulting PCR fragment revealed the presence of *P*-element sequence, but no deletion of *D1* coding sequence, associated with the imprecise *P* excision.

Line 70-5 appeared to retain the *P{EP}* element despite loss of expression of the w^+ marker gene. Both *P*-element ends were retained, as indicated by PCR analysis, and the electrophoretic migation of the *D1* band observed by Southern analysis was consistent with the *P{EP}* element being intact (Figure 4 and Table 1).

D1 structural mutants: Lines 1A, 4A, and 70-7 distinguished themselves by yielding PCR results that appeared wild type (*i.e.*, no *P*-element sequence) together with Southern analysis results, suggesting that the w⁻ chromosomes of lines 1A, 4A, and 70-7 were disrupted for the *D1* gene (Figure 4 and Table 1). The signal intensity of the 1.7-kb band was about half that of the precise excision lines, and lines 1A and 70-7, but not 4A, exhibited a band of altered size that was recognized by the probe. These results suggested that line 4A possessed a deletion of *D1* coding sequence, and that lines 1A and 70-7 were partial deletions or other aberrations. To explore this possibility, PCR was performed on individuals heterozygous for the mutant chromosome and a deficiency for the region, *Df(3R)BSC24*, using overlapping primer sets that spanned the *D1* locus (Figure 5). The inability to amplify a genomic segment, in the context of proper controls, suggested a deletion of some or all of the region. The DNA from single first instar larvae was used, as adult flies were not viable. Sample data are presented in Figure 5B and the results from the most informative of the PCR assays are presented in Table 2. For line 4A, none of the primer sets corresponding to the *D1* coding region yielded a product, confirming that line 4A represented a deletion of the *D1* gene. The deletion extended into the distal *CG9746* gene, disrupting it as well. The deletion did not extend proximally into the *pumilio* (*pum*) coding region, but, as the endpoint was not precisely mapped, it could affect expression of three *pum* transcripts. The DNA sequence proximal to the former $D1^{EP473}$ insertion site was amplifiable for line 70-7, although distal sequence was not. Similar to line 4A, the deletion extended into the distal *CG9746* gene. Figure 5A illustrates how these deletions map to the genomic region. In contrast to the

TABLE 1

Molecular analyses of *D1*^{EP473} w^{-} excision lines

Line[a]	*D1* proximal + Pry4 PCR	*D1* distal + Plac1 PCR	*D1* proximal + *D1* distal PCR[b]	Southern[c]
D1^{EP473}	685 bp	503 bp	794 bp	~9.7 kb/~1.7 kb
1A	−	−	+	~1.3 kb/~1.7 kb
1B, 1C, 1D, 1F	−	−	+	~1.7 kb
1E	−	−	+/~0.85 kb	~1.7 kb doublet
2A	+	−	+	~3.2 kb/~1.7 kb
2B, 2C	−	−	+/~0.85 kb	~1.7 kb doublet
3A	−	−	+/~0.85 kb	~1.7 kb doublet
4A	−	−	+	~1.7 kb
70-1, 70-2, 70-3	−	−	+	~1.7 kb
70-5	+	+	+	~9.7 kb/~1.7 kb
70-6	−	−	+/~0.85 kb	~1.7 kb doublet
70-7	−	−	+	~1.1 kb/~1.7 kb
70-9	−	−	+	~1.7 kb

The PCR primer sets are as illustrated in Figure 1. The presence of a PCR product of size expected for the original *D1*^{EP473}*/TM3, Sb* strain (first row) is denoted by a +, the absence of a product by a −, and a product of other size by the estimated size.

[a] Lines that may not be independent isolates are grouped together in a row. Analyses were performed on balanced lines, due to extraneous lethal mutations.

[b] The PCR products derived from one or both homologs. When the intact *P{EP}* element is present, size limitations preclude amplification of a product from that homolog. However, the wild-type *D1* locus of the balancer chromosome yielded a 794-bp product.

[c] The *D1* proximal + *D1* distal PCR product was used as a probe of *Bam*HI-digested genomic DNA, as illustrated for representative lines in Figure 4. The band(s) derived from one or both homologs. The intensity of the 1.7-kb band for lines 1A, 2A, 4A, 70-5, and 70-7 appeared approximately half that of the 1.7-kb band for all other w^{-} lines.

results with lines 4A and 70-7, *D1* coding sequence was amplifiable to either side of the former *D1*^{EP473} insertion site for line 1A. However, no PCR products were obtained when the predicted amplimer spanned the former insertion site, suggesting the existence of an inversion. To test this hypothesis, inverse PCR was employed to amplify the genomic sequence spanning the putative inversion breakpoint for line 1A (see MATERIALS AND METHODS). Sequence analysis of the inverse PCR product confirmed the existence of a small inversion with breakpoints at the site of the *D1*^{EP473} insertion and within the *CG9746* gene (Figure 5).

P-element-mediated male recombination strategy: As demonstrated by PARKS *et al.* (2004), deletion of the genomic sequence between two *P* elements on homologous chromosomes can be induced by expressing *P* transposase. The rare deletion events are recovered among progeny that exhibit recombinant flanking markers. To delete *D1* coding sequence, the *D1*^{EY05004} insertion within the 5′-UTR of *D1* and the *P{SUPor-P}pum*^{KG02259} insertion within the first intron of the *pum* gene were selected. The region separating the two elements is 3871 bp long and includes the entire *D1* coding sequence as well as the noncoding first exons of the *pum* A, D, and C transcripts (Figure 5). The *scarlet*^{1} (*st*^{1}) and *claret*^{1} (*ca*^{1}) mutations were recombined onto the *P{SUPor-P}pum*^{KG02259} (hereafter referred to as *pum*^{KG02259}) chromosome for selection of recombinants. *P* transposase was expressed in *D1*^{EY05004}*/ st*^{1} *pum*^{KG02259} *ca*^{1} male flies and their progeny screened for the st^{-} ca^{+} recombinant class that would include the desired deletion events. PCR was used to assess the presence of either or both parental *P* elements on the recombinant chromosome of progeny flies. Of 22 st^{-} ca^{+} recombinants, six were determined to have retained a 5′-*P* end within the *pum* gene and a 3′-*P* end within the *D1* gene, but have lost the 3′-*P* end adjacent to *pum* sequence and 5′-*P* end adjacent to *D1* sequence. This result was consistent with the six recombinant chromosomes possessing a deletion marked by a single, hybrid *P* element. However, I desired further proof that the desired deletion, rather than another anomalous recombination event, had occurred. The anticipated large size of the hybrid *P* element made it impractical to show by standard PCR using *pum* and *D1* primers that the two *P* ends were part of a single transposon that joined distant *pum* and *D1* genomic sequence. Therefore, the hybrid *P* element was mobilized by expressing *P* transposase in a putative deletion line, recombinant C12, and progeny showing loss of the *w*$^{+}$ marker were isolated. A small PCR product was generated using a primer proximal to the *pum*^{KG02259} insertion site and the *D1* distal primer for one w^{-} isolate, named C12w^{-} (see Figure 5A). Sequence analysis of this product confirmed that genomic sequence normally separated by almost 4 kb had been juxtaposed by deletion, for chromosome *Df(3R)D1*$^{C12w-}$. In addition, no coding sequence was detectable on this

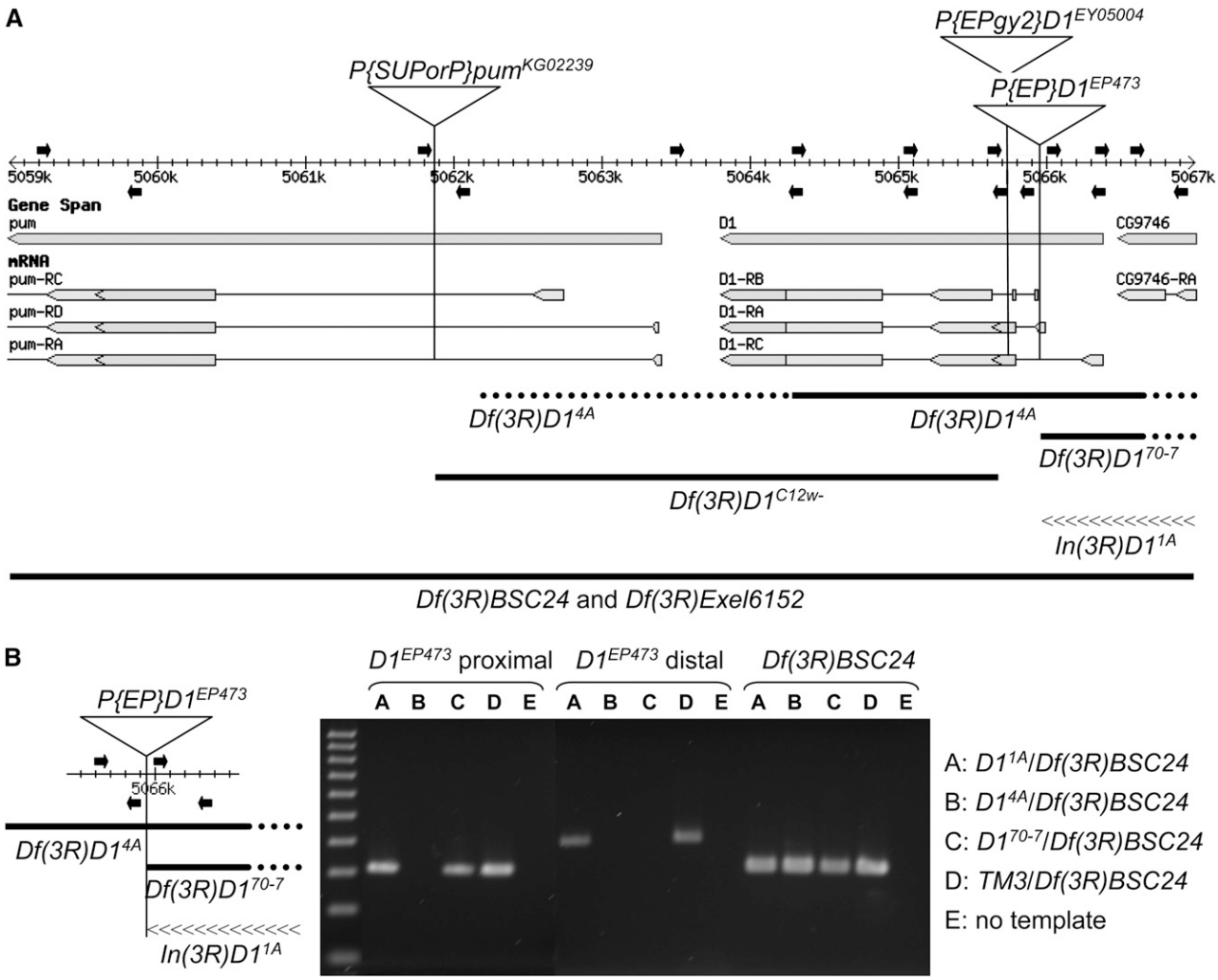

Figure 5.—A map of *D1*-mutant alleles. (A) The *D1* gene and portions of the flanking *pumilio* and *CG9746* genes and their transcripts are illustrated as they map to the 3R genomic sequence (adapted from FlyBase Release 5.1, http://www.flybase.org). Proximal is to the left and distal is to the right. The insertion sites of the three *P* elements utilized in this study are indicated by vertical lines on the map. Both the $pum^{KG02239}$ and the $D1^{EY05004}$ *P* insertions are oriented with the 5′-*P* end proximal and the 3′-*P* end distal. The $D1^{EP473}$ insertion is oriented with the 3′-*P* end proximal and the 5′-*P* end distal. Primers used for PCR analyses of *D1* mutants isolated in this study are shown above (forward primers) and below (reverse primers) the map as solid arrows. The precise locations are listed in Table 2. The extents of the deletions are shown below as thick solid lines, with the dotted portions reflecting the uncertainty of the endpoints. The *Df(3R)BSC24* (3R:4,757,601-5,220,293) and *Df(3R)Exel6152* (3R:4,983,798-5,073,203) deletions extend well beyond this ~8-kb region. The inverted region of $In(3R)D1^{1A}$ (distal breakpoint at position 5,067,087) is illustrated by a linear array of "<" symbols. (B) The PCR data for two primer sets, which amplify genomic segments immediately proximal and distal to the $D1^{EP473}$ insertion as illustrated at left, is shown for the three $D1^{EP473}$ excision alleles associated with chromosome rearrangements that were isolated in this study. Genomic DNA was isolated from single first instar larvae of genotypes $In(3R)D1^{1A}/Df(3R)BSC24$, $Df(3R)1^{4A}/Df(3R)BSC24$, and $Df(3R)D1^{70-7}/Df(3R)BSC24$, as hemizygous adults were inviable. A sibling *TM3/Df(3R)BSC24* larva served as a positive control for the PCR reaction. A third PCR primer set that hybridized to the *P* element marking the *Df(3R)BSC24* deficiency and the flanking genomic DNA, thus specific for the *Df(3R)BSC24* chromosome, was used to confirm the integrity of the DNA preparation, as well as the genotypes. A 100-bp ladder (100–1000 bp) is shown in the first lane.

chromosome by Southern analysis (Figure 2). Thus, $Df(3R)D1^{C12w-}$ represents a second definitive *D1*-null allele.

***D1*-null flies are fully viable and fertile:** As described above, both $Df(3R)D1^{4A}$ and $Df(3R)D1^{C12w-}$ were deleted for the *D1* coding region and were therefore clearly null alleles. Although the $In(3R)D1^{1A}$ and $Df(3R)D1^{70-7}$ alleles suffered inversion or deletion of the *D1* regulatory region, respectively, they could conceivably still be expressed. To address this possibility and ascertain if these two alleles were also *D1*-null, ovarian RNA was isolated from $In(3R)D1^{1A}/Df(3R)D1^{C12w-}$ and $Df(3R)D1^{70-7}/Df(3R)D1^{C12w-}$ females, and qualitatively assessed by RT–PCR. Ovarian tissue was selected due to the high expression level of *D1* in this tissue in wild-type flies (Renner *et al.* 2000; Aulner *et al.* 2002). As shown in Figure 3, *D1* RNA was detected for both alleles, suggesting that they are hypomorphs. This assay also revealed gene product for the $D1^{EY05004}$ *P*-insertion allele. As

TABLE 2

PCR analysis of putative *D1* deletions generated by imprecise *P* excision

Primer set[a]	Genomic coordinates[b]	Line 1A	Line 4A	Line 70-7
pum 6925F + pum 7624R	5,059,192–5,059,891	+	+	+
pum 9494F + pum 9835R	5,061,761–5,062,102	+	+	+
D1 21F + D1 921R	5,063,450–5,064,350	+	−	+
D1 853F + D1 1699R	5,064,282–5,065,128	+	−	+
D1 1605F + D1 2305R	5,065,034–5,065,734	+	−	+
D1 2171F + D1 3285R	5,065,600–5,065,914	+	−	+
D1 2171F + D1 2965R	5,065,600–5,066,394	−	−	−
D1 3374F + D1 2965R	5,066,003–5,066,394	+	−	−
D1 3688F + D1 4320R	5,066,317–5,066,949	+	−	−
D1 3941F + D1 4320R	5,066,570–5,066,949	+	−	−

The generation of a PCR product is indicated by a plus and the absence of a product by a minus.

[a] The results of overlapping PCR amplifications that did not yield additional information are omitted.

[b] The genomic coordinates of the amplified regions correspond to Flybase Release 5.1 of chromosome 3R. The insertion site for the $D1^{EP473}$ element is 5,065,965–5,065,972 (8-bp duplication). The *D1* gene coding region extends between 5,064,241 and 5,065,626 and the *pum* coding regions extend between 4,896,667 and 5,059,583.

expected, no *D1* RNA was detectable for $Df(3R)D1^{C12w-}$ ovaries.

The generation of two new null alleles of the *D1* gene enabled a test of the requirement for the *D1* gene product for viability. Flies bearing the targeted *D1* deletion, of genotype *w; st*^{1} $Df(3R)D1^{C12w-}$*/TM3, Sb*, were crossed to *w/Y;* $Df(3R)D1^{4A}$*/TM3, Sb* flies for complementation analysis. As shown in Table 3, *D1*-deficient flies were obtained at expected frequency. The same result was obtained when *w; st*^{1} $Df(3R)D1^{C12w-}$*/TM3, Sb* flies were crossed to *w/Y; Df(3R)Exel6152/TM3, Sb* flies or to *Df(3R)BSC24/TM3, Sb* flies, the two large deficiencies that span the *D1* gene (Table 3). These data clearly proved that the *D1* gene was not essential for viability.

D1-null flies did not exhibit any obvious phenotypic abnormalities. In addition, females of genotype $Df(3R)D1^{C12w-}$*/Df(3R)Exel6152* and $Df(3R)D1^{C12w-}$ homozygotes were tested and found to be fertile.

***D1* is not required for heterochromatin-mediated repression:** As the *D1* gene encodes a nonhistone chromosomal protein that localizes to the heterochromatin, the dominant effect of a *D1* loss-of-function mutation on position effect variegation (PEV) was assessed. Modification of the severity of PEV due to a decrease in gene dosage of an unlinked locus has been used to implicate that locus in the determination of chromosome structure (reviewed by Weiler and Wakimoto 1995; Schotta *et al.* 2003). The $D1^{EP473}$ insertion was tested for a modifying effect on PEV of three different rearrangements that induce variegation of the *white* (*w*) gene. To avoid the potentially confounding effects of the w^{+} marker gene present within the *P{EP}* transposon on an assessment of *w* variegation, the w^{-} $D1^{EP473}$ derivative allele 70-5 was used for the experiments. The isogenic $D1^{+}$ third chromosome, $D1^{Rev70\text{-}1}$ served as the control. In the first experiment, $In(1)w^{m4}$ females were crossed to *w/Y;* $D1^{EP473w-}$*/TM3, Ser* and *w/Y;* $D1^{+}$*/TM3, Ser* males. A visual examination of the male and female progeny of the two crosses revealed no difference in eye pigmentation among the genotypes (when sorted by age and sex; data not shown). As this result differed from that in the published literature (see discussion), the experiment was repeated using two $In(1)w^{m4}$ stocks from another source, designated $In(1)w^{m4Ta}$ and $In(1)w^{m4f}$ (Talbert and Henikoff 2000). These stocks were molecularly verified as having the w^{m4} inversion (Talbert and Henikoff 2000). In addition, two other *w*-variegating alleles, $In(1)w^{m51b}$ and $In(1)w^{mMc}$, were tested in case there might be rearrangement-specific

TABLE 3

Complementation analysis of *D1* null mutants

Cross	Trial	No. of progeny	Sb^{+} progeny (%)
w; st^{1} $Df(3R)D1^{C12w-}$*/TM3, Sb* x *w/Y;* $Df(3R)D1^{4A}$*/TM3, Sb*	1	189	69 (36.5)
	2	353	110 (31.2)
w; st^{1} $Df(3R)D1^{C12w-}$*/TM3, Sb* x *w/Y; Df(3R)BSC24, st*^{1} *ca*^{1} */TM3, Sb*	1	463	162 (35.0)
w; st^{1} $Df(3R)D1^{C12w-}$*/TM3, Sb* x *w/Y; Df(3R)Exel6152/TM3, Sb*	1	555	194 (35.0)
	2	601	200 (33.3)
	3	705	249 (35.3)

TABLE 4

Loss of *D1* enhances *Stubble* variegation

Genotype	Trial	No. of flies	Average no. Sb$^-$ bristles (± SD)[a]	*P*-value[b]
+; *P{EP}D1^{EP473}/ T(2:3)Sb^V*	1	21	9.3 ± 1.6	0.015
+; *Df(3R)D1^{4A}/ T(2:3)Sb^V*	1	40	9.5 ± 1.6	0.011
+; *D1^{Rev1B}/ T(2:3)Sb^V*	1	31	10.5 ± 1.7	
+; *P{EP}D1^{EP473}/ T(2:3)Sb^V*	2	50	8.5 ± 2.1	<0.001
+; *Df(3R)D1^{4A}/ T(2:3)Sb^V*	2	46	8.8 ± 2.1	0.001
+; *D1^{Rev1B}/ T(2:3)Sb^V*	2	40	10.2 ± 1.7	

[a] As described in MATERIALS AND METHODS, 14 bristles were scored per fly.
[b] For each trial, the mean number of Sb$^-$ bristles for the two *D1* mutants was compared with that of the *D1^{Rev1B}* control using a Student's' *t*-test.

effects. An additional advantage to testing these four *w*-variegating alleles was their generally higher level of eye pigmentation in comparison with the lab *In(1)w^{m4}* stock used initially, a more extreme variant. Thus, either suppression or enhancement of PEV should have been readily detectable using these alleles. The results of this experiment confirmed and extended those of the first study. Neither suppression nor enhancement of PEV of the *w* gene by the *D1$^{EP473w-}$ P*-insertion allele was observed for any of the rearrangements (data not shown).

D1 mutant alleles were similarly tested for the recessive modification of PEV, again using the *In(1)w^{m4}* variegating rearrangement. *w; Df(3R)D1^{C12w-}* females were crossed to *In(1)w^{m4}* males bearing the w$^-$ *D1^{EP473}* derivative allele 70-5, the isogenic *Df(3R)D1$^{70-7}$* allele, or the isogenic *D1$^+$* allele, *D1$^{Rev70-1}$*, each heterozygous with *TM3, Sb*. Comparative visual examination of the *w/ In(1)w^{m4}* progeny females revealed no difference in pigmentation between D1$^+$ and D1$^-$ genotypes (data not shown). The experiment was repeated by crossing *w; Df(3R)D1^{C12w-}* females to *In(1)w^{m4}/Y; Df(3R)D1^{4A}/TM3, Sb* and isogenic *D1$^+$ In(1)w^{m4}/Y; D1^{Rev1B}/TM3, Sb* males, to generate and assess *D1*-null flies. Consistent with prior results, loss of both *D1* alleles did not significantly affect variegation of *In(1)w^{m4}* (data not shown).

Assays for modification of PEV were expanded to include rearrangements variegating for the *brown* (*bw*) or *Stubble* (*Sb*) genes. *Sb* variegation in the adult bristles is associated with the *T(2:3)Sb^V* translocation, which exhibits variable inactivation of the dominant *Sb^1* allele due to its juxtaposition near heterochromatin (SINCLAIR *et al.* 1983). For this experiment, the *D1^{EP473}* allele was tested as was the deletion derivative *Df(3R)D1^{4A}*. The precise excision allele *D1^{Rev1B}* was used as a control. As the third chromosomes of the three strains should only differ at the *D1* locus, any differential effect on PEV would be attributable to loss of *D1*. Enhancement of PEV is viewed as a decrease in Sb$^-$ (abnormal) bristles while suppression of PEV is observed as an increase in Sb$^-$ bristles. As shown in Table 4, the *D1^{EP473}* allele and *Df(3R)D1^{4A}* allele similarly enhanced *Sb^V* variegation.

A potential role for *D1* in *trans*-inactivation or *para*-inactivation was assessed using the variegating rearrangements *bw^D* and *Dp(2;2)Byron* (HENIKOFF *et al.* 1995). The *bw^D* allele is a large insertion of heterochromatin into the *bw* coding region, which can variably repress expression of a wild-type *bw* allele on the homolog by chromosome pairing (SLATIS 1955; HENIKOFF and DREESEN 1989). In the case of the *Byron bw^D bw$^+$* duplication, the heterochromatic block causes variegation of the *bw$^+$* genes in *cis*

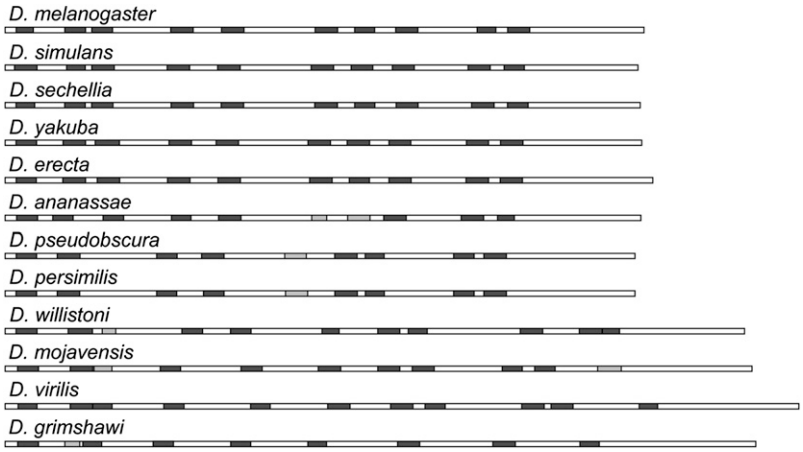

FIGURE 6.—AT-hook organization of the D1 proteins of 12 Drosophila species. The D1 protein sequences are drawn to relative scale as rectangles. The AT-hook motifs predicted by Pfam (http://pfam.janelia.org/) are illustrated as shaded boxes, with light shading indicating matches of lower confidence. The proteins are ordered to reflect the evolutionary relatedness of the species (http://insects.eugenes.org/species/).

TABLE 5

Comparison of D1 proteins of Drosophila

Species[a]	Length (aa)	% identity	% similarity	% Asp + Glu	% Arg + Lys
D. melanogaster	355	—	—	18.3	17.7
D. simulans	352	88	92	18.2	17.6
D. sechellia	353	90	94	18.7	18.1
D. yakuba	354	81	86	18.6	17.2
D. erecta	360	74	81	18.6	16.9
D. ananassae	353	47	56	17.3	17.6
D. pseudoobscura	350	41	51	18.9	18.6
D. persimilis	350	41	51	18.9	18.6
D. willistoni	411	35	48	19.5	16.1
D. mojavensis	415	40	51	19.3	17.3
D. virilis	441	42	51	19.3	16.6
D. grimshawi	417	35	45	19.2	18.0

[a] The order of species reflects the phylogeny (http://insects.eugenes.org/species/).

and in *trans*. Females of genotype bw^{D}; *st* or *Byron sp/CyO; st* were concurrently crossed to st^{1} *Df(3R)*$D1^{C12w-}$*/TM3, Sb st* and st^{1} *P{SUPorP}*$pum^{KG02239}$*/TM3, Sb st* males. The st^{1} *P{SUPorP}*$pum^{KG02239}$ chromosome is the progenitor for *Df(3R)*$D1^{C12w-}$. The male and female (Cy^{+}) progeny of each pair of crosses were compared between genotypes, with the Sb^{-} progeny serving as an internal control. No differences in eye pigmentation were observed in the progeny due to haploinsufficiency for *D1* (when sorted by age and sex; data not shown).

Conservation of the D1 protein: As described above, no noticeable phenotypes were manifest by *D1*-null flies that might hint at the function of the D1 protein. Furthermore, the results indicated that *D1* is not a modifier of PEV. To potentially gain insight into *D1* function, in the absence of phenotypic data, a comparative genomics approach was applied. Homologs of the *D. melanogaster* D1 protein appeared limited to the Drosophila genus, using standard protein similarity search tools. The *D1* homolog was identified within the genomes of the other 11 sequenced Drosophila species (see MATERIALS AND METHODS; RICHARDS *et al.* 2005; CLARK *et al.* 2007). The amino acid identity and similarity to *D. melanogaster* D1, as shown in Table 5, was low as compared to the median identity for *D. melanogaster* homologs in each species (HEGER and PONTING 2007). Multiple sequence alignment of the 12 proteins revealed that similar amino acids almost exclusively localized to the AT-hook DNA-binding motifs. Each protein had 10 ± 1 predicted AT hooks similarly distributed throughout the protein, as illustrated in Figure 6. There were no additional functional motifs predicted for any of the proteins, a characteristic of a subset of AT-hook proteins including the HMGA family (ARAVIND and LANDSMAN 1998). While the amino acid sequence was not well conserved, per se, the preponderance of charged amino acids was. Positively and negatively charged residues accounted for between 34.7 and 37.4% of the total (Table 5).

DISCUSSION

The experiments described herein revealed that the *D1* gene of *D. melanogaster* was not required for viability. The homozygous lethality of the original chromosomes bearing either of two different *P* insertions in the *D1* gene was determined to be due to other lethal mutations on the chromosomes. Indeed, the $D1^{EP473}$ and $D1^{EY05004}$ *P*-insertion alleles were found to be hemizygous viable. However, neither *P*-element insertion disrupted the *D1* coding region, and both *D1* protein and RNA were readily detected for the $D1^{EP473}$ allele (Figure 3 and data not shown). Consequently, to determine if the *D1* gene were essential, it was necessary to generate a null allele. Several genetic approaches were undertaken, including the imprecise *P*-element excision strategy and *P*-element-mediated male recombination HEI strategy described herein, to isolate *D1* mutant alleles that removed the *D1* coding region, as such alleles would be unarguably null for function. These approaches were successful in generating two small deletion alleles. Thus, the requirement for *D1* for viability could be unequivocally assessed. The results of complementation analysis demonstrated that flies heterozygous for the two *D1*-null alleles were fully viable, being recovered from a cross at expected frequency (Table 3). The different genetic backgrounds of the two *D1*-null alleles effectively eliminated any phenotypic contribution by second-site mutations. As the *D1* gene product is maternally loaded into the oocyte during oogenesis (RENNER *et al.* 2000; AULNER *et al.* 2002), it was formally possible that the maternal contribution was sufficient for embryogenesis in the absence of zygotic *D1* expression in null embryos. However, I observed that *D1*-null females were fertile, indicating that maternal expression of *D1* was not required for oogenesis or early embryogenesis. In support of these conclusions, both *Df(3R)*$D1^{C12w-}$ and *Df(3R)*$D1^{C12w-}$*/Df(3R)Exel6152* flies were maintained as stocks for many generations.

It is difficult to reconcile these results with those of Aulner *et al.* (2002), who reported that excision of the *P{EP}D1*^{EP473} transposon restores homozygous viability to the chromosome. In case there might be differences between *P{EP}D1*^{EP473} fly cultures, I obtained a copy of the stock from the Szeged Drosophila Stock Center, the likely source for this group. The reversion studies were repeated using this stock with the same results. Precise excision of the transposon did not revert the lethality, and the Szeged *P{EP}D1*^{EP473} insertion was lethal in combination with the *D1*^{Rev1B} allele, a precise excision of the Bloomington Stock Center *P{EP}D1*^{EP473} insertion (data not shown). Thus, copies of the stock derived from both locations shared chromosome 3 lethal mutations. The additional observation of Aulner *et al.* (2002) that a heat-shock promoter-driven *D1* cDNA transgene could partially rescue the lethality of *D1*^{EP473} homozygotes could potentially be explained by changes in gene expression mediated by ectopic *D1* expression (see below).

It is likely that multiple lethal mutations were present on the original *P{EP}D1*^{EP473} chromosome. The viability of *P{EP}D1*^{EP473}*/Df(3R)BSC24* flies indicated that the mutations were not closely linked to the *P* insertion. *P*-element-mediated male recombination was employed to replace the chromosomal regions to either side of the insertion with that of the homolog. However, homozygous lethality persisted for both classes of single recombinants—those that had replaced 3L and proximal 3R and those that had replaced distal 3R (data not shown). By maintaining the *P{EP}D1*^{EP473} insertion heterozygous with the *Df(3R)Exel6152* chromosome for many generations, a homozygous viable *P{EP}D1*^{EP473} chromosome was eventually recovered.

The dispensability of the *D1* gene for development suggests that it has overlapping function(s) with other genes. Functional redundancy was observed for the products of the HMGB genes of Drosophila, *HMGZ* and *HMGD*. Although these two proteins do not share sequence similarity with D1, they share some biochemical properties and have similarly been proposed to play an architectural role in chromatin (Grosschedl *et al.* 1994; Renner *et al.* 2000; Aleporou-Marinou *et al.* 2003). The *HMGZ HMGD* double mutant has only minor phenotypic defects and, surprisingly, no severe phenotypes were revealed in combination with null alleles of one or more other HMGB genes (although these studies were limited by available mutant alleles; Ragab *et al.* 2006). The mammalian HMGA genes, *HMGA1* and *HMGA2*, are similarly not essential for viability. Developmental abnormalities are associated with loss of either gene, although the null phenotypes are distinct (Zhou *et al.* 1995; Foti *et al.* 2005; Fedele *et al.* 2006). Whereas there are five HMGB genes in Drosophila and two HMGA genes in mammals, *D1* appears to be the only HMGA-like gene of Drosophila. At the level of protein architecture, the D1 protein appears unique in having 10 predicted copies of the AT-hook motif. Most of the AT-hook proteins of *D. melanogaster* have 1 or 2 copies of this motif, with only ASH1 protein having 3. Unlike the D1 protein, these proteins (including ASH1 protein) typically possess additional functional motifs. If the function(s) of the D1 protein rely on its ability to bind to AT tracts, then perhaps proteins with similar DNA binding properties mediated by other motifs share in its activities.

HMGA-like features of D1 proteins: The D1 proteins of other Drosophila species having sequenced genomes were identified on the basis of protein sequence homology, but sequence similarity rapidly declined with increasing evolutionary distance (Table 5). It was consequently not surprising that D1 protein homologs were not identified in other genera using sequence homology. However, the comparison and alignment of the drosophilid D1 proteins suggested that other features of the protein, such as the density of AT-hook motifs and/or the frequency of charged amino acids, might more appropriately be the defining criteria for a D1 protein family. Perhaps not coincidentally, these are among properties shared with mammalian HMGA proteins. In this regard, it is relevant that the HMGA1 and HMGA2 proteins are only ~50% similar to each other (in both mouse and human) and that this similarity is primarily in the three AT-hook regions and acidic C terminus (Reeves and Beckerbauer 2001). Indeed, the numerous similarities between the D1 and HMGA proteins suggest that they might share functional, rather than evolutionary, relatedness. In addition to the shared biochemical properties already noted, the D1 protein is predicted to have extensive intrinsic protein disorder (Uversky *et al.* 2005; data not shown), a demonstrated attribute of HMGA proteins (Lehn *et al.* 1988; Huth *et al.* 1997). Both D1 and HMGA proteins are highly post-translationally modified (Zhai *et al.* 2008; Zhang and Wang 2008). The primary distinction, increased size for D1, is accompanied by a proportional increase in number of AT-hook motifs. Although the HMGA proteins do not have intrinsic transcriptional regulatory activity, they have been shown to regulate the activity of many genes as architectural proteins (Reeves and Beckerbauer 2001). A potential similar gene regulatory role for the D1 protein is supported by the finding, in this study, of decreased *Sb*^{1} expression (enhancement of *Sb* variegation) in a *D1*-mutant background.

***CG9746* is essential:** This work revealed that predicted gene *CG9746* is essential for viability. The three *D1* mutants obtained through imprecise excision of the *P{EP}D1*^{EP473} insertion, two deletions and an inversion, disrupted the neighboring *CG9746* gene, as well. All were lethal in combination with the ~89-kb deficiency *Df(3R)Exel6152*. In contrast, these *D1* mutants were viable in combination with the small *Df(3R)D1*$^{C12-}$ deficiency, which deleted the *D1* gene and noncoding sequences of the *pum* gene, but not *CG9746*. The

lethality of the *In(3R)D1^{1A}/Df(3R)Exel6152* mutant flies in particular indicated that *CG9746* is an essential gene, as no other gene was affected by this inversion. The sequence of the *CG9746* gene predicts that it encodes a protein serine/threonine kinase.

D1 and PEV: As the D1 protein is enriched in the heterochromatin, I sought to test the hypothesis that mutant alleles of the *D1* gene might be haplosuppressors of PEV (reviewed by Weiler and Wakimoto 1995; Schotta *et al.* 2003). However, I realized that a potential effect of *D1* mutations on PEV of *In(1)w^{m4}*, the variegating rearrangement most commonly used to assess potential PEV modifiers, could reflect the local influence of the block of 359-bp satellite repeat sequence normally present at the heterochromatic base of the X chromosome (Hilliker and Appels 1982). This repeat is a high-affinity binding site for the D1 protein (Levinger and Varshavsky 1982b). For this reason, this study included several *w*-variegating alleles that were molecularly and cytologically characterized by Talbert and Henikoff (2000). The *w* locus of the *w^{m51b}* rearranagement is juxtaposed to the 359-bp satellite block, whereas it is separated from it by rDNA for the *In(1)w^{m4}* chromosomes (Tartof *et al.* 1984; Talbert and Henikoff 2000). In contrast, the 359-bp satellite block was determined to be absent from the *In(1)w^{mMcT}* inversion chromosome (Talbert and Henikoff 2000). The tests failed to show any dominant effect of mutations in the *D1* gene on variegation of *w* associated with *In(1)w^{m4}*, *In(1)w^{mMcT}*, or *In(1)w^{m51b}*. Hence, my findings contradict those of Aulner *et al.* (2002) who reported suppression of *w^{m4}* variegation by the *P{EP}D1^{EP473}* insertion. One difference between experiments of the two laboratories is that the *P{EP}-D1^{EP473}* insertion tested by Aulner *et al.* (2002) expressed the *w* marker gene, whereas both *D1* mutant alleles tested herein were w$^-$. My approach of assessing *w^{m4}* variegation in a w$^-$ background obviates the need for methods to subtract the effects of extraneous *w*-gene activity and thus makes data interpretation straightforward. A second difference is the possibility that their results were influenced by a maternal effect, as their tests for modification of PEV involved at least one strain bearing the *TM6B* balancer chromosome, which harbors a mutation in the *E(var)3-9* gene (Weiler 2007). Although I was unable to deduce the details of the crosses that were performed, the enhancing effect of an *E(var)3-9* mutation could make a wild-type chromosome appear to have a suppressor phenotype by comparison. A third difference is that the reported suppression of PEV attributed to the *P{EP}D1^{EP473}* insertion might actually map elsewhere on the chromosome. In this report, the *P{EP}D1$^{EP473w-}$* insertion is compared to an isogenic *D1$^+$* control.

The isolation of new *D1* mutant alleles that were viable in combination (this report) enabled a test for potential recessive effects of *D1* mutations on PEV. To this end, *D1*-null females were crossed to *w^{m4}* males bearing isogenic D1$^+$ or D1$^-$ chromosomes, and the *w* variegation of the D1$^+$ and D1$^-$ female progeny was compared. Neither *D1$^{EP473w-}$/Df(3R)D1^{C12w-}* nor *Df(3R)D1^{4A}/Df(3R)D1^{C12w-}* females showed suppression (or enhancement) of *w^{m4}* variegation in comparison to their respective controls (data not shown). Hence, *D1* is not a recessive modifier of *w^{m4}*.

Additional tests for dominant modification of PEV by *D1* mutant alleles were performed using the *brown*-variegating rearrangements *bw^D* and *Byron*, and the *Stubble*-variegating rearrangement, *T(2;3)Sb^V*. The former set of crosses assess for the potential requirement for *D1* in *trans*-inactivation and both *trans*-inactivation and *para*-inactivation of *bw*, respectively. For these tests, flies bearing the *D1*-null chromosome *Df(3R)D1^{C12w-}* were compared to those bearing the D1$^+$ progenitor chromosome. No effect was observed on *bw* variegation for either rearrangement (data not shown). In contrast, both the *P{EP}D1^{EP473}* and the *Df(3R)D1^{4A}* chromosomes were found to enhance *Sb* variegation in comparison to the isogenic *D1^{Revu1B}* wild-type control chromosome (Table 4).

Collectively, the assays for an effect on PEV by *D1* mutant alleles indicated that *D1* is not a modifier of PEV. Variegation associated with five of the six rearrangements tested was not affected by mutation of *D1*. Several of these assays included *D1*-null alleles. In addition, Aulner *et al.* (2002) noted seeing no significant effect of *D1^{EP473}* on PEV for two other rearrangements that they tested, not included in this study. Enhancement of *Sb* variegation, the only effect observed in these studies, most likely reflects a role for the D1 protein in promoting transcription of the *Sb* gene. It has previously been postulated that D1 could regulate gene expression via binding to AT-rich promoter elements (Levinger 1985b). As noted above, a gene regulatory function has been clearly demonstrated for HMGA proteins (Reeves and Beckerbauer 2001). The number and spacing of AT tracts required for D1 binding has not been established. However, there are 25 AT tracts extending five or more bases within 1 kb 5′ of the transcription start of the *Sb* gene. Thus, positive regulation of *Sb* gene expression by the D1 protein is a formal possibility.

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