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Genetic Characterization and Cloning of *Mothers against dpp*, a Gene Required for *decapentaplegic* Function in *Drosophila melanogaster*

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ABSTRACT

The *decapentaplegic* (*dpp*) gene of *Drosophila melanogaster* encodes a growth factor that belongs to the transforming growth factor-β (TGF-β) superfamily and that plays a central role in multiple cell-cell signaling events throughout development. Through genetic screens we are seeking to identify other functions that act upstream, downstream or in concert with *dpp* to mediate its signaling role. We report here the genetic characterization and cloning of *Mothers against dpp* (*Mad*), a gene identified in two such screens. *Mad* loss-of-function mutations interact with *dpp* alleles to enhance embryonic dorsal-ventral patterning defects, as well as adult appendage defects, suggesting a role for *Mad* in mediating some aspect of *dpp* function. In support of this, homozygous *Mad* mutant animals exhibit defects in midgut morphogenesis, imaginal disk development and embryonic dorsal-ventral patterning that are very reminiscent of *dpp* mutant phenotypes. We cloned the *Mad* region and identified the *Mad* transcription unit through germline transformation rescue. We sequenced a *Mad* cDNA and identified three *Mad* point mutations that alter the coding information. The predicted MAD polypeptide lacks known protein motifs, but has strong sequence similarity to three polypeptides predicted from genomic sequence from the nematode *Caenorhabditis elegans*. Hence, MAD is a member of a novel, highly conserved protein family.

THE importance of cell-cell signaling in the establishment of cell fate has been demonstrated in many developing systems. Several classes of secreted polypeptides have been shown to mediate such signaling. The transforming growth factor-β (TGF-β) superfamily encompasses one particularly important class of signaling proteins (for review, see Kingsley 1994). In vertebrates, members of this family have been implicated as being crucial for many specific developmental events: activin for mesoderm induction in early Xenopus development (Green and Smith 1990; Thomsen *et al.* 1990), MIS for regression of the Müllerian ducts in the development of the reproductive tract in mammalian fetuses (Blanchard and Josso 1974) and bone morphogenesis proteins (BMPs) in skeletal development (Wozney *et al.* 1988). TGF-β superfamily members also contribute to development in the fruit fly *Drosophila melanogaster* (Padgett *et al.* 1987), as well as the nematode *Caenorhabditis elegans* (Estevez *et al.* 1993).

The TGF-β signaling pathway has been only partially elucidated (for review see Massagué 1990, 1992). TGF-βs are synthesized and secreted as large precursor polypeptides, with the active ligand consisting of homo- or heterodimers of the C-terminal regions. For many of the family members, the ligand is thought to be associated extracellularly with proteins that regulate its activity. In vertebrates, one such protein may be BMP1, which copurifies with other BMPs, but is not related to them by sequence (Wozney *et al.* 1988). BMP1-like sequences have been identified in Drosophila (Shimell *et al.* 1991) and *C. elegans* (Wilson *et al.* 1994). The cellular effects of the TGF-βs are mediated by transmembrane receptors with cytoplasmic serine-threonine kinase activity (Matthews and Vale 1991; Lin and Wang 1992; Ebner *et al.* 1993). Similar receptors have been identified in Drosophila (Brummel *et al.* 1994; Nellen *et al.* 1994; Penton *et al.* 1994; Xie *et al.* 1994) and in *C. elegans*. (Georgi *et al.* 1990; Estevez *et al.* 1993). Most other components of the TGF-β signaling pathway, including the targets of receptor kinase activity and components of the signal transduction pathway, remain unknown.

The *D. melanogaster decapentaplegic* (*dpp*) gene encodes a homologue of vertebrate BMP2 and BMP4 (Padgett *et al.* 1987, 1993; Wozney *et al.* 1988). Extensive genetic analysis has implicated the DPP protein in many developmental events. In the early embryo, a *dpp* activity gradient establishes fates within the dorsal ectoderm (Irish and Gelbart 1987; Ferguson and Ander-

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SON 1992b; WHARTON *et al.* 1993). Later in embryonic development, *dpp* mediates the passage of positional information across germ layers during gut morphogenesis (PANGANIBAN *et al.* 1990; IMMERGLÜCK *et al.* 1990). During larval development, *dpp* is required for proper imaginal disk patterning along the proximal-distal axis of the resulting adult appendages (SPENCER *et al.* 1982). *dpp* may also be required earlier for setting aside the imaginal disk precursor nests from adjacent epidermal cells (BLACKMAN *et al.* 1991; COHEN *et al.* 1993).

The Drosophila genes *saxophone* (*sax*) and *thick veins* (*tkv*), which encode BMP receptors, and *tolloid* (*tld*), which encodes a BMP1 homologue, interact genetically with *dpp* (FERGUSON and ANDERSON 1992a; BRUMMEL *et al.* 1994; NELLEN *et al.* 1994; PENTON *et al.* 1994; XIE *et al.* 1994). Given the conservation seen thus far between BMP-related and DPP-related functions, we anticipate that other elements of the pathway will be conserved and that the corresponding genes might, like *tld*, *sax* and *tkv*, exhibit genetic interactions with *dpp*. We have therefore undertaken extensive searches for mutations in other genes that modify particularly sensitive *dpp* mutant phenotypes (RAFTERY *et al.* 1995). We report here the genetic characterization and cloning of the first of these genes, *Mothers against dpp* (*Mad*). Our genetic characterization indicates that this gene does indeed encode a product essential for *dpp* function. Our molecular analysis demonstrates that the MAD protein is a member of a novel protein family that is highly conserved throughout metazoans.

MATERIALS AND METHODS

Fly stocks and culture: All mutations and chromosomes, except where noted, are described and referenced in LINDSLEY and ZIMM (1992). Flies were reared on standard Drosophila medium (cornmeal, yeast extract, dextrose) in half-pint plastic bottles or glass shell vials at 25°.

Isolation of *Mad* alleles: Mad^1 was isolated in a screen for mutations that affect a transvection-sensitive *dpp* phenotype (GELBART 1982). Males of the genotype $z\ w^{11E4}$; $dpp^{d\text{-}ho}$ *TE52; e* were fed EMS as described in LEWIS and BACHER (1968), then mated to $z\ w^{11E4}$; dpp^{hr4} *TE52/CyO* females. Approximately 30,000 Cy^+ male progeny were screened for individuals with heldout wings. Of 782 heldout flies that were retested, 101 transmitted a heldout phenotype. Of these, 92 were shown by genetic or cytological criteria to be associated with chromosome rearrangements that are predicted to disrupt chromosome pairing at 22F and therefore complementation between $dpp^{d\text{-}ho}$ and dpp^{hr4} (GELBART 1982). Mad^1 was among the nine remaining mutations. Four *Mad* mutations were recovered in a screen for dominant maternal-effect enhancers of *dpp* (RAFTERY *et al.* 1995). Three of these are designated Mad^2, Mad^3 and Mad^4. The fourth is a small deficiency designated *Df(2L)C28* (23D1-2; 23D3-4). The alleles Mad^5-Mad^{12} were recovered in a screen for EMS-induced lethal mutations (Y.-J. KIM and A. DIANTONIO, personal communication).

Generation of deficiencies: To recover deficiencies in the *Mad* region, we took advantage of *TE52*, a transposable element that maps less than one map unit distal to *Mad*. We screened 78,000 flies of the genotype $z\ w^{11E4}$; $dpp^{d\text{-}ho}$ *TE52*, $w^+/dpp^{d\text{-}ho}$ *TE52*, w^+ *dp cn*, in which one of the chromosomes was derived from a male exposed to 4500 rads γ rays from a ^{137}Cs source. Flies of this genotype have yellow eyes, due to the repression of paired copies of w^+ by *z* (SMOLIK-UTLAUT and GELBART 1987); flies bearing a rearrangement that removes one copy of *TE52* (by deletion, translocation, inversion *etc.*) will have red eyes. From 78 red-eyed flies, we recovered six deficiencies, two duplications and numerous other rearrangements.

Whole mount *in situ* hybridization: Whole mount *in situ* hybridization was done as described in TAUTZ and PFEIFLE (1989), with a *labial* probe as described in HURSH *et al.* (1993).

DNA manipulations: General molecular biological techniques were done according the protocols in SAMBROOK *et al.* (1989). *D. melanogaster* genomic clones were isolated from a lambda DASH II (Stratagene) library constructed from a strain isogenic for *dp cl cn bw* (provided by R. W. PADGETT). Genomic rescue constructs were made by ligating fragments cut out of phage clones into pCaSpeR4 (PIROTTA 1988). Germline transformation was carried out essentially as in RUBIN and SPRADLING (1982). *P*[Mad0-11] is an 11.5-kb fragment from the *Xba*I site at coordinate 0 to the *Not*I site in the polylinker of λab7. *P*[Mad4-16] is a 13-kb fragment from the *Not*I site in the polylinker of λa22 to the *Bam*HI site at +17.

To create the cDNA rescue construct *P*[UmMad], a *Ubiquitin* promoter containing a translation start and sequences encoding an epitope recognized by anti-Myc antibodies was cut out of pWUM (provided by M. DE CUEVAS) with *Sal*I and *Eco*RI and ligated into pBlueScript KS+ (Stratagene) to create pBUM. A 2.5-kb fragment of cDNA c28, from a *Pst*I site at nucleotide 287 to the *Not*I in pNB40, was ligated into pBUM. The resulting plasmid was digested with *Not*I and partially digested with *Sal*I to release the promotor-tag-c28 fusion, which was then ligated into the *Xho*I and *Not*I sites of pCaSpeR4. The predicted translation start of c28 is at nucleotide 347; the fusion protein resulting from translation initiation within pWUM contains the sequence SCSSGSNNLNLTTAISGSSNR between the epitope tag and MAD.

Sequencing and sequence analysis: *D. melanogaster* cDNAs were isolated from a library contructed from 0-4 hour embryos isogenic for *dp cl cn bw* (BROWN and KAFATOS 1988). *C. elegans* cDNAs were isolated from a mixed-stage cDNA library (BARSTEAD and WATERSTON 1989), using oligonucleotide probes derived from the genomic sequence of ZK370. Double-strand sequencing was done by the dideoxy chain termination method (SANGER *et al.* 1977) according to the protocols in the Sequenase version 2.0 kit (U.S. Biochemicals), using cDNA subclones and gene-specific primers. DNA sequences were assembled and analyzed with the University of Wisconsin Genetics Computing Group (UWGCG) programs (DEVEREUX *et al.* 1984). Searches of the protein databases were done on the National Center for Biotechnology and Information (NCBI) network server using the Basic Local Alignment Search Tool (ALTSCHUL *et al.* 1990). Protein sequence alignments were generated with the UWGCG program PILEUP, using the BLOSUM62 amino acid substitution matrix (HENIKOFF and HENIKOFF 1992). Searches of Release 11.1 of the PROSITE database (BAIROCH 1993) were done with the UWGCG program MOTIFS.

For *Mad* mutant sequencing, the fragment from the *Xba*I site at 0 to the *Sal*I site at +5 was cloned into pBluescript KS+ (Stratagene) from *Mad b pr/CyO* and *Df(2L)Mad⁻/CyO* adults. DNA sequence was read from the *Xba*I site to distinguish clones containing sequences from the *CyO* balancer

from those containing sequences from the *Mad* chromosome. The *Sal*I site used in this cloning scheme is at base 488 in the cDNA, 141 bases 3′ of the predicted translation start. The remaining protein-coding sequences were amplified by the PCR. DNA prepared from 20 *Mad b pr/b pr* males was suspended in 50 μl dH_2O. Each PCR reaction contained 1 μl of DNA (or dH_2O, for a negative control), 2 mM $MgCl_2$, 100 μM each dATP, dCTP, dGTP and dTTP, 0.25 μM of each primer (CCGGTTATCGGGTGACTCC and TACGGCGAATTGGGATTG) and 2 units of *Taq* DNA polymerase (Promega) in 100 μl of 1× buffer supplied. Reactions were subjected to 30 cycles of 94° for 1 minute, 60° for 90 seconds and 72° for 45 seconds. The 889-bp PCR product, which contains sequences corresponding to c28 nucleotides 162 to 988, plus a 63-bp intron, was concentrated, then electrophoresed for quantification and purification from excess primers and nucleotides. About 200 ng of PCR product was sequenced with the Cyclist kit (Stratagene) according to the protocol supplied.

Northern blot analysis: Embryo staging, RNA extraction, isolation of polyadenylated RNA, denaturing electrophoresis and transfer of RNA to nylon membranes followed standard methods. Each lane contains ~2 μg polyadenylated RNA from *cn; ry506* or 40 μg of total RNA from Canton S or *y w*. ^{32}P-labeled random primed probes (FEINBERG and VOGELSTEIN 1983) or in vitro transcribed RNA probes (MELTON *et al.* 1984) were generated from the *Mad* cDNA clone pBSc28 (the *Hin*dIII–*Not*I fragment of pNB40 carrying the c28 insert, cloned into pBluescript KS+) and from a genomic *Eco*RI–*Hin*dIII clone containing most of the *ribosomal protein 49* (*rp49*) gene (O'CONNELL and ROSBASH 1984). Filters were hybridized in 50% formamide at 45° (for DNA probes) and 65° (for RNA probes) and then washed at high stringency. Sizes were determined with reference to an ethidium bromide-stained 0.24–9.5-kb RNA ladder (Bethesda Research Laboratories).

RESULTS

Isolation and mapping of *Mad*: The initial *Mad* mutation was recovered in a screen for dominant enhancers of a *dpp* imaginal disk phenotype (see MATERIALS AND METHODS) and was subsequently found to be a dominant maternal-effect enhancer of *dpp* embryonic dorsoventral (D/V) patterning mutants. A concurrent screen for dominant maternal-effect enhancers of *dpp* in embryos (RAFTERY *et al.* 1995) produced four allelic mutations, which we have shown to be weak enhancers of *dpp* in imaginal disks. Allelism is based on these similar phenotypes, map position and failure to complement recessive lethality. We named this gene *Mothers against dpp* (*Mad*) to reflect the maternal-effect enhancement of *dpp*.

TABLE 1

Deficiency mapping of *Mad*

Deletion	Region deleted	Mad^1/Df	Enhancement[a]
Df(2L)C144	23A1-2; 23C3-5	Viable	No
Df(2L)JS7	23C3-5; 23D1-2	Viable	No
Df(2L)JS17	23C1-2; 23E1-2	Lethal	Yes
Df(2L)DTD62 2^P3^D*; H7* 3^P2^D	23D1-2; 23F	Lethal	Yes
Df(2L)C28	23D1-2; 23D3-5	Lethal	Yes

[a] Yes indicates that the deficiency is a maternal-effect enhancer of *dpp*.

We mapped *Mad* by meiotic recombination to within one map unit proximal to *TE52*, an insertion of a *TE* transposable element into polytene region 23C-D (ISING and RAMEL 1976). We generated deficiencies in this region (see MATERIALS AND METHODS) and used these to show that *Mad* is in polytene chromosome division 23D (Table 1) and that the maternal-effect enhancement of *dpp* is due to loss of *Mad* function.

***Mad* interacts with *dpp* in early embryos and in imaginal disks:** *Mad* loss-of-function mutations are dominant maternal-effect enhancers of early embryonic *dpp* mutant phenotypes: dpp^{hr4}/dpp^+ embryos, which are ordinarily viable, frequently die when derived from *Mad*/+ mothers. The dead embryos have fewer cells of the most dorsally-derived ectodermal tissue, the amnioserosa (RAFTERY *et al.* 1995). This phenotype is identical to that of partial loss-of-function *dpp* mutants (WHARTON *et al.* 1993). The maternal-effect enhancement of *dpp* by *Mad* is not *dpp* allele-specific. Rather, it appears that embryos with reduced levels of *dpp* activity are inviable when the maternal *Mad* contribution is lessened. The existing *Mad* mutants can be placed into an allelic series based on the relative severities of their maternal-effect enhancement of a weak *dpp* allele (Table 2).

Mad loss-of-function mutations are also dominant zygotic enhancers of *dpp* adult appendage defects. To characterize this interaction, we examined the effects of *Mad* dosage on the appendage phenotypes of dpp^{hr4}/

TABLE 2

Maternal-effect enhancement of *dpp* by *Mad* alleles

	Survival of *dpp* progeny of *Mad*/+ mothers (% of expected)[a]													
Allele	*Df*	*1*	*7*	*12*	*10*	*5*	*3*	*2*	*11*	*6*	*4*	*8*	*9*	+
dpp^{e87}	6	1	8	7	6	36	44	23	31	33	34	74	99	100
dpp^{hr56}	1	0	0	1	4	8	18	23	31	29	30	61	57	89

[a] *Mad/CyO* females were crossed to *dpp dp cn bw/CyO* males. The number given is the number of *dpp* progeny (average of number of *dpp dp cn bw/Mad* and *dpp dp cn bw/CyO*) as a percentage of the number of control progeny (*Mad/CyO*), which in each case was ≥200.

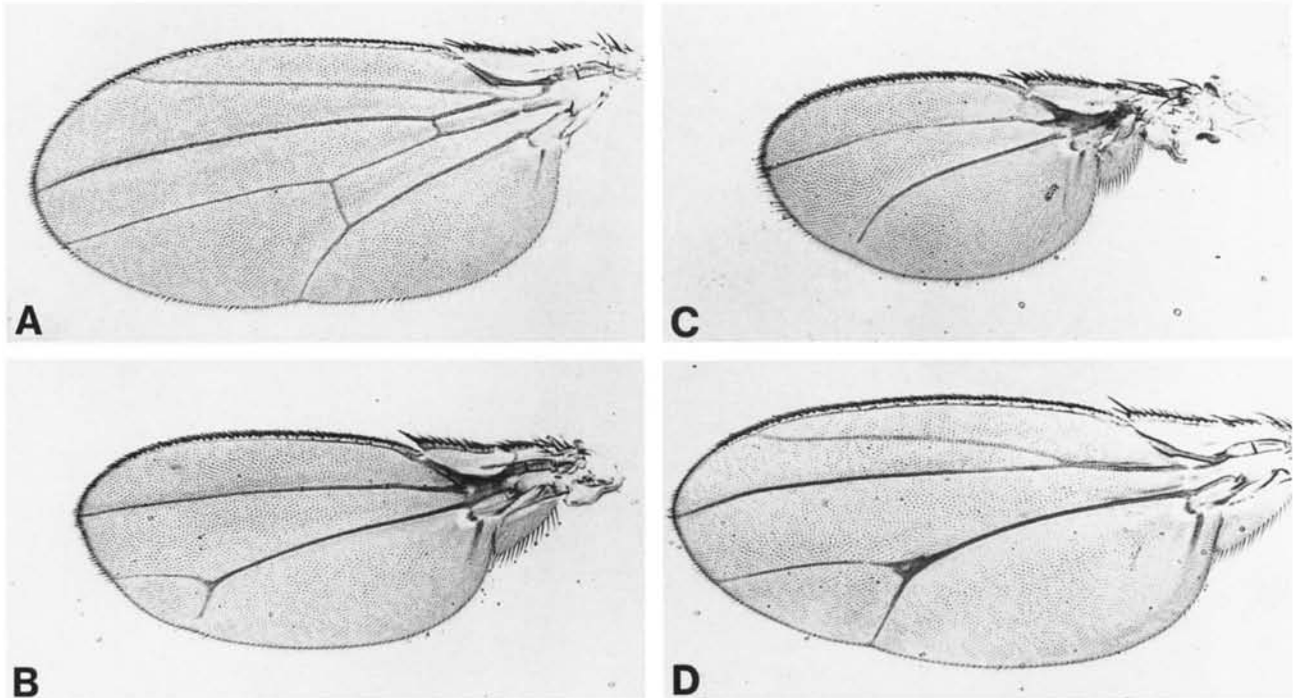

FIGURE 1.—Effects of *Mad* dosage on a *dpp* imaginal disk mutant phenotype. Adult wings from (A) wild-type, (B) dpp^{hr4}/dpp^{d6}, (C) dpp^{hr4} *Df(2L)C28*/dpp^{d6}, (D) dpp^{hr4}/dpp^{d6}; *Dp(2;3)JS20, Mad*$^+$. The dpp^{hr4}/dpp^{d6} wing (B) is approximately two-thirds normal size and has defective venation. Removal of one copy of *Mad* (C) results in a further reduction to approximately one-half normal size. Conversely, the addition of an extra copy of *Mad*$^+$ (D) partially ameliorates the phenotype, resulting in wings of normal size that show residual vein fusions. All wings at the same magnification.

dpp^{d6} flies. dpp^{d6} is associated with an inversion that has one breakpoint within the dpp^{disk} region, effectively deleting some of the regulatory sequences that direct *dpp* expression in imaginal disks (ST. JOHNSTON *et al.* 1990). The defects in flies homozygous for dpp^{d6} include greatly reduced wings and eyes and loss of tarsal claws at the tips of the legs (SPENCER *et al.* 1982). dpp^{hr4} is a point mutation that partially reduces *dpp* activity (WHARTON *et al.* 1993). dpp^{hr4}/dpp^{d6} flies have wings approximately half of normal size (Figure 1B) and normal legs and eyes. dpp^{hr4}/dpp^{d6} flies that are also either *Mad*/+ or *Df(2L)Mad*$^-$/+ show more severe phenotypes: a further reduction in wing blade (Figure 1, panel C), a slight reduction in the eye, and loss of tarsal claws (data not shown). Conversely, trisomy for *Mad*, achieved through the use of a duplication of *Mad*$^+$, partially alleviates the dpp^{hr4}/dpp^{d6} phenotype, resulting in wings that are near normal in size, though they retain vein fusions (Figure 1D). Hence, *dpp* imaginal disk mutant phenotypes are sensitive to the dosage of *Mad*.

***Mad* phenotypes are similar to *dpp* phenotypes:** The dose-dependent genetic interactions between *Mad* and *dpp* suggest that *Mad* may play an important role in mediating *dpp* signaling. If this is the case, then loss of *Mad* activity may result in phenotypes similar to those of *dpp* mutants even in dpp^+ genotypes. We found that *Mad* mutant phenotypes do indeed exhibit a close correspondence to *dpp* mutant phenotypes.

Of 12 *Mad* putative point mutations (EMS-induced and cytologically normal), nine are lethal in trans to a deficiency of *Mad* or in trans to one another, whereas three (Mad^5, Mad^6 and Mad^{11}) are hemizygous viable. The lethal alleles Mad^1, Mad^7, Mad^{10} and Mad^{12} behave as null alleles, in that each exhibits a maternal-effect enhancement of *dpp* about as severe as that of a deficiency (Table 2). In contrast, the lethal alleles Mad^8 and Mad^9 exhibit a maternal-effect enhancement of *dpp* much weaker than that of a deficiency and so are classified as hypomorphic alleles. We first examined phenotypes elicited by null alleles.

Mad zygotic null mutants exhibit several defects. *Mad* null larvae have reduced fat body, resulting in a striking mutant phenotype (Figure 2). These animals pupariate about two days after their heterozygous siblings, then die as early pupae. Dissection of mature *Mad* larvae reveals that the imaginal disks are absent or severely reduced (Figure 3). dpp^{disk} mutations also result in severe reductions of imaginal disk tissue (SPENCER *et al.* 1982; BRYANT 1988).

Homozygous *Mad* mutant larvae also have midgut defects and greatly reduced gastric caecae (Figure 3). During gut morphogenesis, *dpp* is involved in signaling from the visceral mesoderm to the presumptive midgut

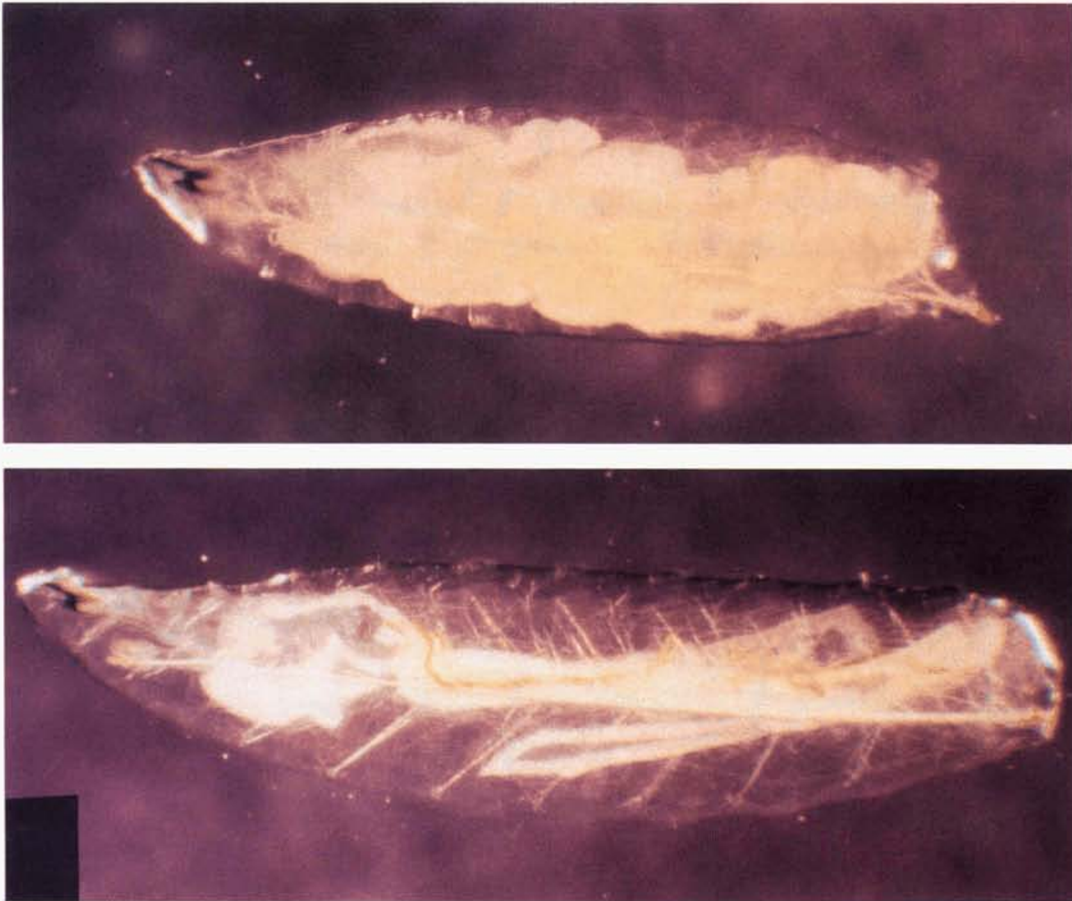

FIGURE 2.— *Mad* zygotic null phenotype. Wild-type (top) and $Mad^{12}/Df(2L)JS17$ mature third instar larvae. *Mad* larvae have greatly reduced fat body.

endoderm. *dpp* is expresssed strongly in two discrete domains of the visceral mesoderm, the tissue that underlies the developing midgut (ST. JOHNSTON and GELBART 1987). The parasegment 4 expression domain is required for the proper extension of the gastric caecae; the parasegment 7 expression domain is required for the subsequent induction of the homeotic gene *labial* (*lab*) in the adjacent endoderm and for the forma-

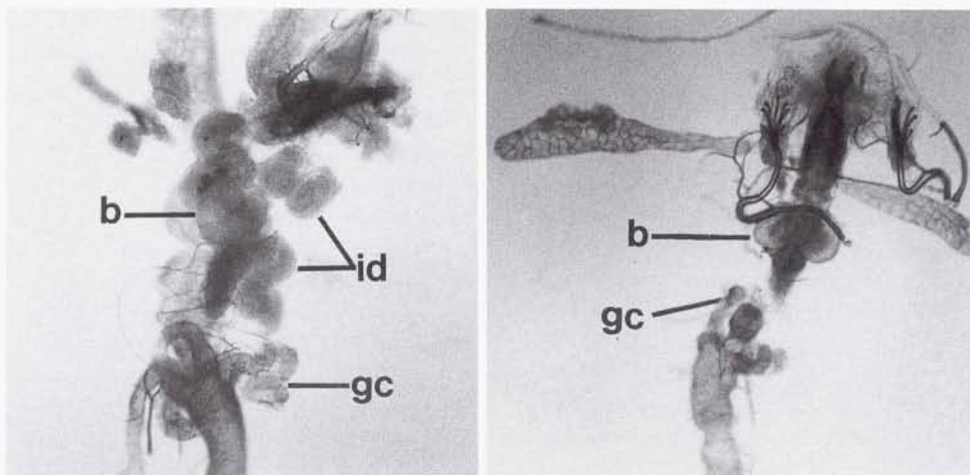

FIGURE 3.—Gut and imaginal disk defects in *Mad* mutants. Anterior region of dissected wild-type (left) and $Mad^{12}/Df(2L)JS17$ third instar larvae. The brain (b), imaginal disks (id) and gastric caecae (gc) are indicated. *Mad* larvae lack or have greatly reduced imaginal disks and shortened gastric caecae. Both larvae at the same magnification.

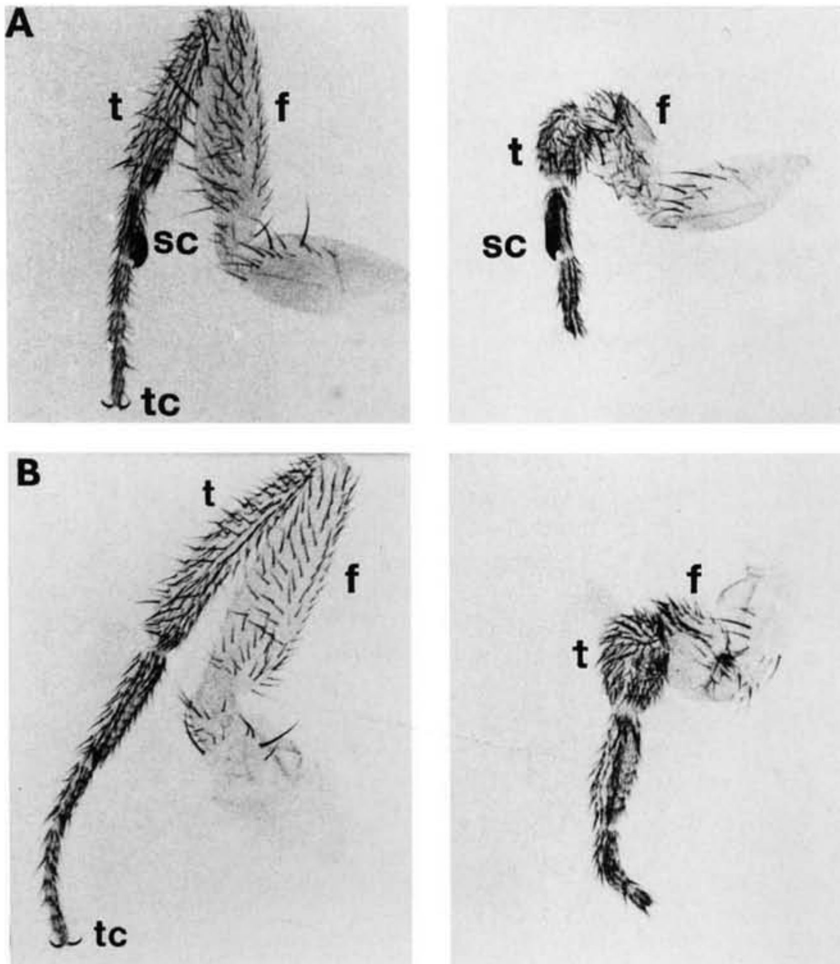

FIGURE 4.—Leg defects in adult viable *Mad* genotypes. Male prothoracic (A) and mesothoracic (B) legs from wild-type (left) and $Mad^6/Df(2L)JS17$. The femur (f), tibia (t), sex combs (sc; on the first tarsal segment of the prothoracic legs) and tarsal claws (tc; on the fifth tarsal segment) are indicated. The $Mad^6/Df(2L)JS17$ legs lack the fourth and fifth tarsal segments. The femur and tibia of these legs are also abnormal.

tion of the second midgut constriction (PANGANIBAN *et al.* 1990; IMMERGLÜCK *et al.* 1990). Homozygous *Mad* mutant embryos lack both the *dpp*-dependent *lab* expression and the corresponding midgut constriction (data not shown). Hence, *Mad* is required for *dpp*-dependent events in midgut morphogenesis.

Other (non-null) *Mad* genotypes also produce phenotypes reminiscent of *dpp* mutant phenotypes. Adults bearing any of the hemizygous viable alleles (Mad^5, Mad^6 and Mad^{11}) in trans to a deficiency or a null allele have numerous defects in imaginal disk-derived structures. These defects include gaps in the L4 wing vein, heldout wings, split notum, loss of distal leg segments, duplications of the third antennal segment and male genitalia defects (Figure 4 and data not shown), all of which are similar to defects seen in dpp^{disk} mutants (SPENCER *et al.* 1982).

Although Mad^5 and Mad^6 are lethal in trans to one another, both are viable with Mad^{11}. The viable heteroallelic adults do not display the defects in imaginal disk-derived structures described above, but exhibit female sterility, manifested either as a failure to lay eggs, or in fertilized eggs developing with a lethal, weakly ventralized phenotype similar to that of partial loss-of-function *dpp* mutants (data not shown). We do not at present know if the total absence of maternally and zygotically contributed *Mad* activity leads to a more severe D/V mutant phenotype. To address this, we have attempted to generate homozygous *Mad* null germline clones by γ ray-induced mitotic crossing over. No such clones were observed, even though Mad^+ clones were readily generated in control crosses (data not shown). The absence of *Mad* null germline clones prevented us from assessing the D/V patterning defects that might arise in embryos completely lacking *Mad* activity and raises the possibility that *Mad* is required in the germline for some aspect of oogenesis.

The hemizygous viable alleles exhibit more severe maternal-effect enhancement of *dpp* than do the hypomorphic lethal alleles Mad^8 and Mad^9. The differences in the phenotypes elicited by these two classes of alleles may stem from distinct effects of individual mutations at various developmental stages. In support of this, the hemizygous viable alleles are each viable over either of the hypomorphic lethal alleles. Adults with these combination of *Mad* alleles are morphologically normal and frequently fertile. Hence alleles from one class complement alleles from the other.

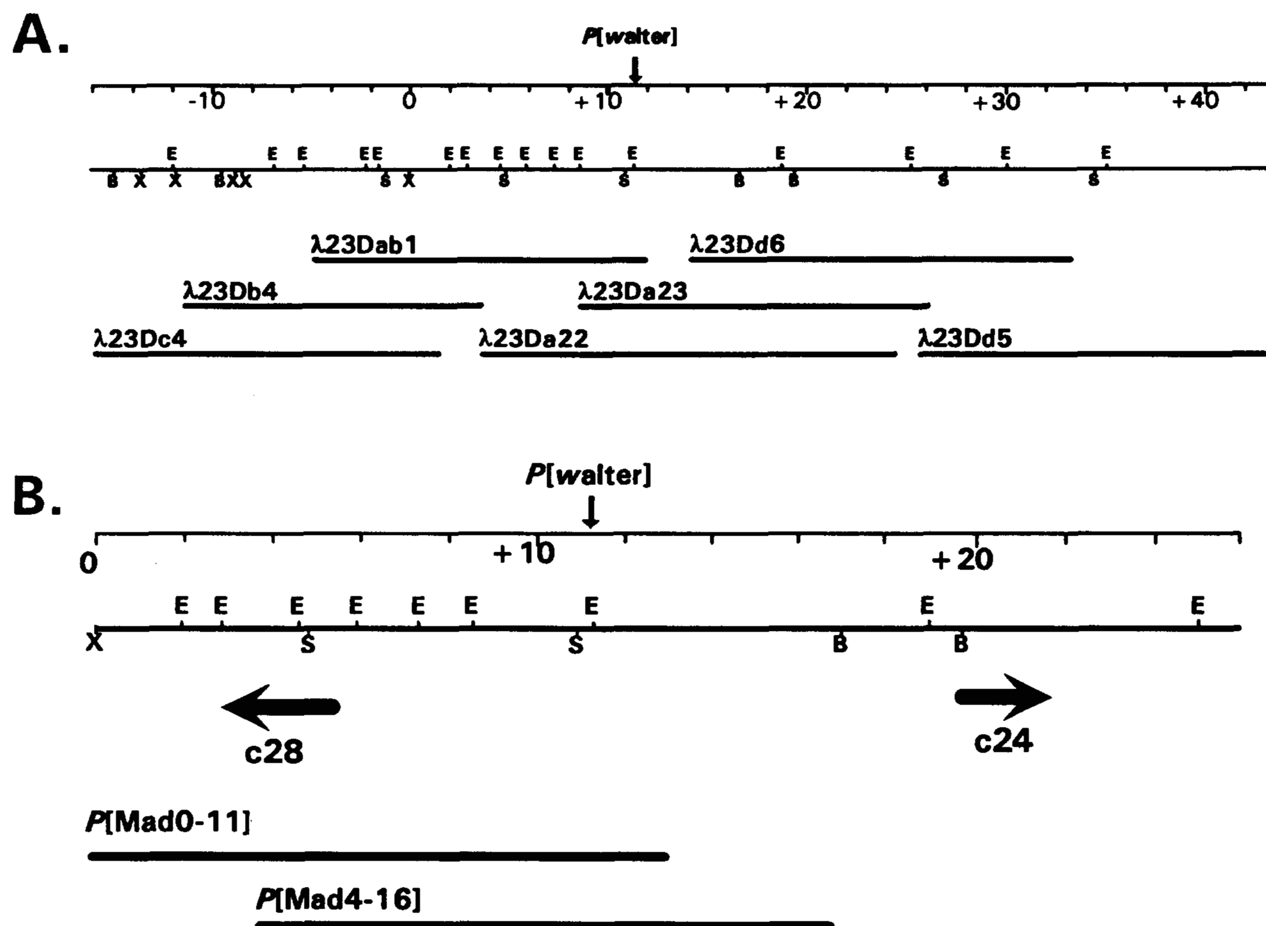

FIGURE 5.—Molecular map of the *Mad* region. (A) Molecular map of the cloned sequences flanking *P*[*walter*] (23D). The scale at the top is in kilobase pairs relative to an *Xba*I site used to clone sequences adjacent to *P*[*walter*] (23D). *P*[*walter*] (23D) contains two *P*[*walter*] elements in direct repeat, inserted at the point indicated. Inserts in lambda phages from the walk are designated by solid lines below the scale. (B) Transcripts and rescue constructs. The arrows below the scale indicate the position and direction of transcription of c28 and c24, the early embryonic cDNAs nearest the *P* element insertion point. The solid lines at the bottom of the figure represent the regions included in genomic rescue constructs. The scale and restriction map are as in (A). E, *Eco*RI; X, *Xba*I; B, *Bam*HI; S, *Sal*I.

Molecular cloning of *Mad*: To gain insight into the biochemical function of *Mad*, we molecularly analyzed the gene. Several extant *P* element insertions within polytene region 23D were examined for allelism to *Mad*. The recessive lethal insertion *P*[*walter*] (23D) (GLOOR *et al.* 1991) failed to complement *Mad* mutations. This insertion also exhibited a dominant maternal-effect enhancement of *dpp* similar to that of *Mad* partial loss-of-function mutations. We confirmed that *P*[*walter*] (23D) contains a *P* element inserted into *Mad* by inducing excisions of the element with *P* transposase. Excision of *P*[*walter*] (23D), monitored by loss of the *mini-white* marker gene, was frequently associated with reversion of the recessive lethality and of the dominant maternal-effect enhancement of *dpp* and with restored ability to complement *Mad* (data not shown).

We cloned and restriction mapped genomic sequences flanking *P*[*walter*] (23D) (Figure 5). Two transcription units in the region were detected by probing Northern blots of polyadenylated RNA from early embryos and by screening a cDNA library. These two transcripts are represented by near full-length early embryonic cDNAs of 2.4 and 2.8 kb, designated c24 and c28. Germline transformation with genomic and cDNA rescue constructs allows us to conclude that c28 corresponds to *Mad*. Germline transformants from two *P* element constructs carrying different genomic DNA fragments from the vicinity of *P*[*walter*] (23D) (Figure 5) were obtained and tested for their ability to rescue *Mad* mutant phenotypes. None of three insertions of *P*[Mad4–16], which contains a small portion of c28 and the region between c28 and c24, had detectable rescue activity (Table 3). However, of four independent insertions of *P*[Mad0–11], the construct that contains c28, one fully rescued *Mad* maternal-effect enhancement of *dpp* and *Mad* recessive lethality and a second partially rescued *Mad* maternal-effect enhancement of *dpp*. Our interpretation of these results is that

TABLE 3

Results of *Mad* rescue constructs

Insert	Rescue of *Mad*	
	Enhancement[a]	Lethality[b]
None	0	−
P[Mad4-16]1	0	−
P[Mad4-16]2	0	−
P[Mad4-16]3	0	n.d.
P[Mad0-11]1	0	−
P[Mad0-11]2	100	+
P[Mad0-11]3	0	−
P[Mad0-11]4	9	−
P[UmMad]1	89	+
P[UmMad]2	100	n.d.
P[UmMad]3a	87	n.d.
P[UmMad]3b	0	−
P[UmMad]4	100	+

[a] *y w; Mad^{7} b pr/+* virgin females carrying one copy of the rescue construct were crossed to *dpp^{e87} dp cn bw/CyO* males (44). The number given is the number of straight-winged progeny (*dpp^{e87}*-bearing) as a percentage of the number of curly-winged (*CyO*) progeny. At least 200 curly-winged progeny were counted in each cross.

[b] *y w; Mad^{7} b pr/+* males carrying one copy of the rescue construct were crossed to *Mad^{11} b pr/CyO* females. The absence of black-bodied progeny was scored as no rescue (−), whereas the presence of any such progeny was scored as rescue (+). For inserts on chromosome *2*, rescue of lethality was not determined (n.d.).

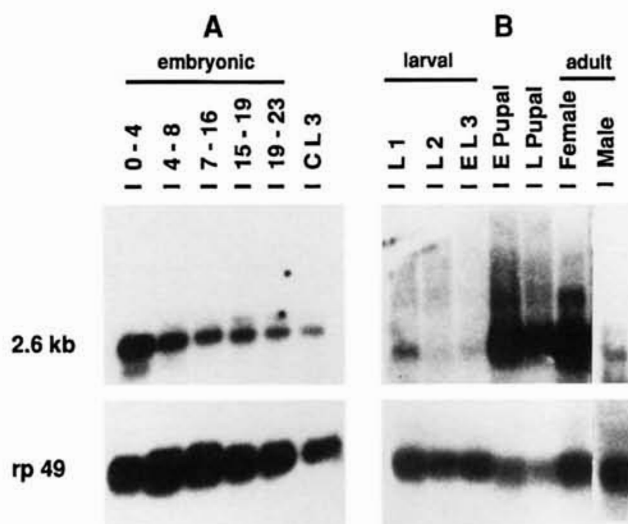

FIGURE 6.— *Mad* RNA expression profile. Filters containing polyadenylated RNA (A) or total RNA (B) were probed with a *Mad* cDNA clone. A predominant 2.6-kb transcript was identified in all stages, with the highest levels in early embryos, pupae, and adult females. The filters were probed with *rp49* as a control for loading. Embryonic stages are indicated as hour after egg deposition. L1, first instar larvae (24–30 hr); L2, second instar larvae (58–68 hr); EL3, early third instar larvae (74–78 hr); CL3, climbing third instar larvae (104–110 hr); EPupal, early pupae (162–169 hr); LPupal, late pupae (210–213 hr).

P[Mad0–11], when inserted into a favorable chromosomal location, has sufficient sequences to rescue *Mad* mutant phenotypes.

Since the *P*[Mad0–11] genomic fragment includes the c28 transcription unit but might also contain other undetected transcription units, we more critically tested the association of *Mad* with c28 through an additional rescue construct, in which c28 was fused to the constitutively expressed *Ubiquitin* promoter (LEE *et al.* 1988). Four of five insertions of this construct rescued *Mad* mutant phenotypes completely or nearly so (Table 3). Hence, c28 must represent the *Mad* transcription unit.

***Mad* is expressed throughout development:** The expression of *Mad* was examined by developmental Northern analysis. A single 2.6-kb transcript is detected (Figure 6), consistent with the idea that c28 is near full length. The transcript is detected in all developmental stages, though it appears most abundant in pupae, adult females and early embryos. In 0–4-hr embryos, which contain maternally loaded mRNA as well as early zygotic transcripts, a minor transcript that is slightly smaller than 2.6 kb is also detected. This minor transcript is not seen in any other embryonic stage. The abundance of the *Mad* transcript decreases throughout embryonic and larval development and then returns to high levels in pupae and adult females. A larger transcript is detected in total RNA from early pupal stages and from adult females.

MAD is highly similar to three putative *C. elegans* proteins: We sequenced the c28 cDNA by standard dideoxy sequencing. The 2647-bp sequence has one long open reading frame (ORF) from nucleotide 347 to 1710, conceptual translation of which predicts a polypeptide with 455 amino acid residues (Figure 7). The codon frequency in this ORF is similar to that of other Drosophila genes (ASHBURNER 1989), and the sequences surrounding the putative translation start have an excellent match to the weak consensus for translation initiation in Drosophila (CAVENER and RAY 1991).

A BLAST search (ALTSCHUL *et al.* 1992) of the protein databases revealed similarities between the putative MAD sequence and three predicted polypeptides from the nematode *C. elegans*. These sequences, which we refer to as CEM-1, CEM-2 and CEM-3 (*C. elegans* MAD homologues 1, 2 and 3), were initially identified as predicted proteins from the *C. elegans* Genome Sequencing Project (WILSON *et al.* 1994). CEM-1 was predicted as two polypeptides, ZK370.1 and ZK370.2, but because these came from adjacent regions of the same cosmid and because their physical maps aligned in colinear fashion with *Mad*, we predicted that they would, in actuality, be part of the same polypeptide. We have

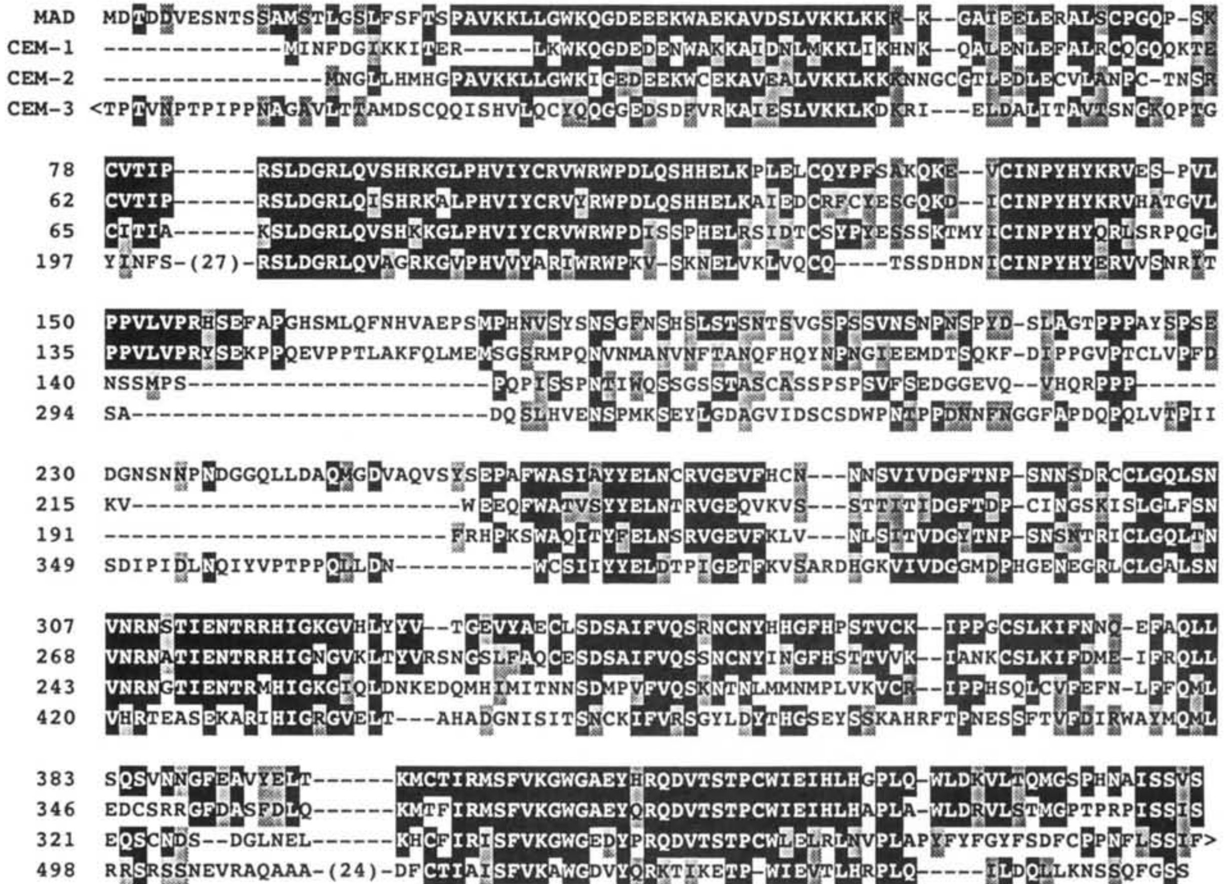

FIGURE 7.—Sequence similarity between *D. melanogaster* MAD and the *C. elegans* sequences CEM-1, CEM-2 and CEM-3. Using the MAD sequence as a reference, identical residues are indicated with a black background, and residues that score positive using the BLOSUM62 substitution matrix (HENIKOFF and HENIKOFF 1992) are indicated with a gray background. Gaps introduced for maximal alignment are represented by dashes. While it is likely that not all the indicated identities and similarities are meaningful, several conserved blocks are apparent. Some sequences have been omitted for clarity: the last 30 residues of CEM-2 (>), the first 118 residues of CEM-3 (<), and stretches of 27 and 24 residues within CEM-3 that are not present in the other sequences (-(27)- and -(24)-).

confirmed this through the isolation and sequencing of a cDNA corresponding to CEM-1, which indeed is composed of both ZK370.1 and ZK370.2 sequences, plus some sequences not contained in either predicted coding unit (the inferred amino acid sequence is shown in Figure 7). CEM-2 and CEM-3 were only recently added to the database as proteins R13F6.9 and R12B2.1, respectively, and only the predicted sequences are currently available.

An alignment of MAD with CEM-1, CEM-2 and CEM-3 (Figure 7) reveals extensive amino acid identity, including several blocks conserved between all four putative proteins. For CEM-3, the most divergent member of the family, the degree of similarity is less, though still substantial. It should be noted that CEM-2 and CEM-3 are predictions from genomic sequences, and our experience with CEM-1 suggests that the *bona fide* sequences may deviate from these predictions. The middle of each sequence contains a region of variable length that does not show extensive identity in this alignment. This region is proline-rich in all four sequences and is also serine-rich in MAD and CEM-2. A search of the PROSITE protein motif database (BAIROCH 1993) did not reveal any known protein motifs in either MAD or any of the CEM sequences. There are also no discernable signal secretion sequences or potential transmembrane domains.

While confirming that c28 encodes MAD, we sequenced genomic DNA from the MAD protein-coding region from three *Mad* point mutations. Each of these was found to contain a unique base pair alteration (two predict substitutions of conserved residues, whereas the third introduces a stop codon) in a region encoding a highly conserved block of amino acids (Figure 8).

DISCUSSION

***Mad* phenotypes resemble *dpp* phenotypes:** The observation that *Mad* loss-of-function mutations domi-

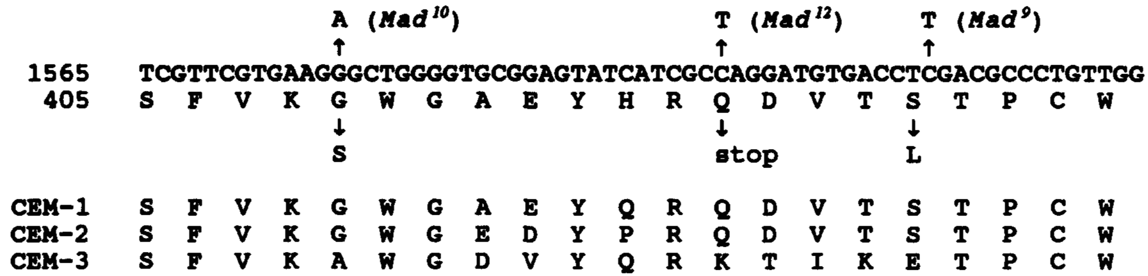

FIGURE 8.—Sequence changes in three *Mad* point mutations. The sequence of nucleotides 1565–1611 of *Mad* cDNA c28 is shown above a conceptual translation of the region. The base substitutions of three *Mad* alleles (indicated in parentheses) are shown above the sequence and their predicted translational changes are shown below. Mad^{10} and Mad^{12} are null alleles; Mad^{9} is pupal lethal but shows only a weak maternal-effect enhancement of *dpp* (Table 2). The comparable regions of *C. elegans* CEM-1, CEM-2 and CEM-3 are aligned below.

nantly interact with *dpp* in D/V patterning of the embryo and in adult appendage development suggests that *Mad* may play an important role in *dpp* function. We pursued this possibility by examining the phenotypes elicited by loss of zygotic *Mad* activity in dpp^{+} genotypes. We found evidence that, even in dpp^{+} genotypes, *Mad* mutants can engender phenotypes reminiscent of *dpp* mutations affecting all three of the best described functions of *dpp*: embryonic D/V patterning, adult appendage development and morphogenesis of the larval gut. The midgut phenotypes of homozygous *Mad* mutants are like those of homozygous dpp^{shv} alleles, including the failure to induce *labial* expression in the endoderm underlying parasegment 7 of the visceral mesoderm, the failure to form the second midgut constriction and the failure of the gastric caecae to extend properly.

The imaginal disk development defects seen in *Mad* mutants (appendage defects in hemizygous-viable alleles and loss of disk tissue in null larvae) are like those of dpp^{disk} mutants. The absence or severe reductions in imaginal disks in homozygous *Mad* larvae is perhaps more severe than the phenotypes elicited by the most severely affected class of dpp^{disk} alleles (class V), but it is quite possible that these *dpp* alleles retain a small amount of residual *dpp* activity in the developing disks.

The embryonic D/V patterning defects elicited among the progeny of adult-viable *Mad* heteroallelic combinations are clearly *dpp*-like, although milder than the phenotype of *dpp* null embryos (IRISH and GELBART 1987). As noted, we have thus far been unable to determine the severity of the phenotype resulting from complete loss of both maternal and zygotic *Mad* activity.

The fat body defect in mature *Mad* null larvae is a deviation from the concordance of *dpp* and *Mad* phenotypes. We have not investigated the ontogeny of this phenotype, however, and must consider the possibility that the fat body may develop properly but may break down precociously because of some other abnormality. With the possible exception of this phenotype, there is a very strong correlation between the phenotypes elicited by loss-of-function *dpp* and *Mad* mutations. Based on the dosage-sensitive interactions of *Mad* and *dpp* and the striking similarity of *Mad* and *dpp* recessive phenotypes, we propose that the MAD protein plays an essential role in some aspect of *dpp* signaling.

***Mad* encodes a protein belonging to a novel gene family:** The identification of three predicted proteins in the nematode *C. elegans* with strong sequence similarity to the predicted MAD polypeptide sequence suggests that MAD is an ancient, highly conserved function. The *C. elegans* genes *daf-1* and *daf-4*, which are involved in establishing the switch between normal development and dauer larva formation, encode BMP receptor-like polypeptides (GEORGI *et al.* 1990; ESTEVEZ *et al.* 1993). Thus, *C. elegans* has a DPP-like signaling system highly similar to that of other animals, including Drosophila and vertebrates. It is tempting to speculate that the three CEM proteins have roles that are similar to the role of MAD in the DPP signaling pathway. The *C. elegans* genes *sma-2*, *sma-3* and *sma-4*, which map near the CEM-1, CEM-2 and CEM-3 coding regions, respectively, share small body size and male tail ray phenotypes with *daf-4* receptor mutants. Preliminary data suggests that these *sma* genes do encode the CEM proteins (C. SAVAGE and R. PADGETT, personal communication). Remarkably, a *sma-2* mutant allele alters glycine-372 of CEM-1, which corresponds to the same glycine (residue 409) mutated in Mad^{10}.

The role of MAD in *dpp* function: MAD may be required at any of a number of steps in the *dpp* signaling pathway. Given that the MAD and CEM proteins lack any indication of secretion signal sequences or transmembrane domains, it seems likely that MAD acts either within the cells that produce DPP or within the cells that receive the DPP signal. For each of the different DPP-mediated developmental events, *dpp* RNA expression is highly localized (ST. JOHNSTON and GELBART 1987; POSAKONY *et al.* 1991), with its distributions regulated by known transcription factors (HUANG *et al.* 1993; CAPPOVILA *et al.* 1994). It is possible that MAD is a *dpp*-specific transcription factor that functions in conjunction with these known transcription factors.

This seems unlikely given that ubiquitous expression of *Mad* (from the *Ubiquitin* promoter) apparently is compatible with normal development. Preliminary experiments show that endogenous *Mad* transcripts are also ubiquitous, at least in the embryo (S. J. NEWFELD and W. M. GELBART, unpublished data).

The remaining possibility, that MAD functions in cells that receive the DPP signal, seems most plausible. Little is known about the nature of signal transduction downstream of any TGF-β receptor, so no molecular comparisons can be invoked to bolster this view. However, it is exactly for this reason, to be able to identify through genetic interaction screens functions not previously associated with DPP (or TGF-β) signaling, that we have undertaken this general approach. To test our working hypothesis, that MAD is an element of the DPP signal transduction pathway, experiments testing the epistatic relationship between MAD and DPP or the SAX and TKV receptors will be critical. Clonal analysis studies to determine if *Mad* is required in the same cells that produce *dpp* or in those that require *sax* and/or *tkv* will be extremely valuable. Further, it will be important to examine the subcellular location of MAD and to be able to identify the protein using specific antisera. We are currently pursuing these approaches.

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