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Molecular Characterization of the Key Switch *F* Provides a Basis for Understanding the Rapid Divergence of the Sex-Determining Pathway in the Housefly

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ABSTRACT

The housefly, *Musca domestica*, is an excellent model system to study the diversification of the pathway that specifies the sexual fate. A number of different mechanisms have been described in the housefly, which reflects in part the broad diversity of sex-determining strategies used in insects. In this study we present the molecular identification and characterization of *F*, which acts as the master switch in the housefly pathway. We provide evidence that *F* corresponds to the *transformer* ortholog in Musca (*Mdtra*), which, as a result of alternative processing, expresses functional products only in individuals committed to the female fate. We demonstrate that, once activated, a self-sustaining feedback loop will maintain the female-promoting functions of *Mdtra*. Absence of *Mdtra* transcripts in eggs of *Arrhenogenic* (*Ag*) mutant females suggests that maternally deployed *Mdtra* activity initiates this self-sustaining loop in the zygote. When an *M* factor is paternally transmitted to the zygote, the establishment of the loop is prevented at an early stage before cellularization and splicing of *Mdtra* shifts irreversibly to the male nonproductive mode. On the basis of the analysis of two mutant alleles we can explain the different sex-determining systems in the housefly largely as deviations at the level of *Mdtra* regulation. This plasticity in the housefly pathway may provide a suitable framework to understand the evolution of sex-determining mechanisms in other insect species. For instance, while sex determination in a close relative, the tsetse fly *Glossina morsitans*, differs at the level of the instructive signal, we find that its *tra* ortholog, *Gmtra*, is regulated in a mode similar to that of *Mdtra*.

PROPER sexual development is based on a binary decision between two alternative developmental programs. In insects, the genetic system underlying this decision has been most extensively studied in *Drosophila melanogaster*, providing profound insights in the molecular mechanisms that determine the sexual fate (Cline and Meyer 1996; Schutt and Nothiger 2000). Nevertheless, it appears that in many other insect species the primary instructive signal that specifies the sexual fate has diversified extensively. Alone in the housefly, *Musca domestica*, several types have been described in natural populations, ranging from dominant male determiners to female determiners and even the use of maternal signals (Dubendorfer *et al.* 2002). Since this spectrum, to a certain extent, reflects the variety of sex-determining signals found in insects, the Musca system appears particularly suited for studying evolutionary diversification of this key developmental process.

The "standard" type of sex determination in the housefly employs a dominant male-determining factor, *M*, which is located on the Y chromosome (Hiroyoshi 1964). In addition, naturally occurring strains exist where the *M* factor can be located on any of the five autosomes or even on the X chromosome (Denholm *et al.* 1983; Inoue *et al.* 1983). In some populations, all individuals are homozygous for the *M* factor, and the female fate is determined by the presence of a dominant female determiner F^D (McDonald *et al.* 1978). Even more remarkably, the sex of the housefly can be determined by the maternal genotype. Such strains consist of arrhenogenic females (*Ag*/+) that produce only sons and of thelygenic females (+/+) that give rise to daughters only (Inoue and Hiroyoshi 1981). Our genetic analysis revealed that all these different systems have a common genetic basis and led us to propose the following model: In *M. domestica* the gene *F* acts as the key switch in sex determination. An active *F* is conceived as a female signal, whereas male development follows when *F* is inactive. Zygotic activation of *F* requires its own

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maternal activity, suggesting that this gene relies on a self-sustaining feedback loop to maintain its female-promoting function (Dubendorfer *et al.* 2002). The presence of a dominant male-determining factor *M* in the zygote prevents the activation of *F*, thereby promoting male development. The *F* factor is genetically defined by two alleles. The naturally occurring dominant female-determining allele F^D is thought to act as a gain-of-function mutation, which is resistant to repression by *M* and no longer relies on the autoregulatory function (Dubendorfer and Hediger 1998). The recessive allele F^{man}, which spontaneously arose in a laboratory strain, acts as a strong hypomorphic mutation resulting in male development of homozygous F^{man} animals in the absence of the male-promoting *M* factor (Schmidt *et al.* 1997a).

The pivotal position of *F* at the top of the sex-determining hierarchy in Musca, and its function in selecting and maintaining the female fate through a positive feedback loop, resembles that of *Sex-lethal* (*Sxl*) in Drosophila. However, we previously showed that the Musca homolog of *Sxl*, *MdSxl*, is not sex-specifically expressed and thus an unlikely candidate for *F* (Meise *et al.* 1998). Studies in the fruit fly *Ceratitis capitata* identified the *transformer* ortholog, *Cctra*, as the main switch in the pathway that determines the sexual fate (Pane *et al.* 2002). Selection and maintenance of the female fate is based on a positive autoregulatory function of *Cctra*. The same mechanism might also be operational in other members of the Tephritidae (Lagos *et al.* 2007; Ruiz *et al.* 2007) and in *Lucilia cuprina*, a member of the Calliphoridae family (Concha and Scott 2009). Since Musca is phylogenetically more closely related to Tephritidae and Calliphoridae, *transformer* seems to be a more likely candidate for *F*.

In a previous study we showed that the Musca homolog of *doublesex*, *Mddsx*, acts as a main effector in the pathway downstream of *F* (Hediger *et al.* 2004). It produces a set of sex-specific protein isoforms that functionally correspond to the *dsx* variants in Drosophila. Sex-specific regulation of *Mddsx* is achieved at the level of splicing by a mechanism similar to that of *dsx* in Drosophila. The Musca homolog of Drosophila *transformer2*, *Mdtra2*, was proposed not only to participate as an essential cofactor in the regulation of *Mddsx*, but also to act as an upstream regulator of *F* on the basis of the finding that the dominant F^D allele is largely resistant to *Mdtra2* silencing (Burghardt *et al.* 2005). In F^+/F^+ females, on the other hand, transient silencing of *Mdtra2* causes a complete and irreversible shift to male development, indicating that *Mdtra2* is required for upholding the self-sustaining feedback loop of *F* (Burghardt *et al.* 2005). This situation is reminiscent of that in Ceratitis, where Salvemini and co-workers (Salvemini *et al.* 2009) recently reported that *Cctra2* is required to maintain the productive female-specific splicing mode of *Cctra*. The presence of multiple clusters of putative TRA/TRA2 binding sites, intronic splice silencers (ISS), and RBP1 binding sites in the sex-specifically processed region suggested that CcTRA and CcTRA2 form a complex that directly associates with *Cctra* pre-mRNA to impose the female splice (Salvemini *et al.* 2009). Clustering of such putative binding sites was also previously observed in *tra* orthologs of other tephritids (Ruiz *et al.* 2007).

Here we present the molecular identification of the Musca *transformer* ortholog, *Mdtra*, and present evidence that this gene indeed corresponds to the key switch *F*. In addition, we isolated the *tra* ortholog in the close relative, the tsetse fly *Glossina morsitans* (*Gmtra*). In *G. morsitans* the instructive signal appears to be different from that in *M. domestica*. Although standard sex determination in the tsetse fly is also based on XX–XY male heterogamety, the Y chromosome does not have a sex-determining function. From the study of aneuploid sets of sex chromosomes it has been inferred that the sexual phenotype is based on the ratio of X to autosomes, as in Drosophila (Maudlin 1979). Nevertheless, sex-specific processing of *Gmtra* shares several features with *Mdtra* that are not found in Drosophila *tra*. Transgenic expression of the female variant of *Mdtra* is sufficient to activate and maintain female expression of endogenous *Mdtra*, invoking an autoregulatory function that serves to maintain the female-promoting functions of this gene. We show that the establishment of this feedback loop in the early zygote requires maternally deployed activity of *Mdtra* and that presence of *M* causes this loop to collapse shortly after fertilization. On the basis of small lesions found in the two known alleles of *F* we propose a model of how splicing regulation of *Mdtra* is achieved at the molecular level.

MATERIALS AND METHODS

Isolation of *Mdtra*: Genomic DNA was isolated from two to three adult flies according to standard procedures. A "touchdown" PCR was performed using the degenerate primer pair Mddsx-70 (5′-NNN NTC ATC AAT CAA CA-3′) and Mddsx-69 (5′-NNN NTG TTG ATT GTT GT-3′). These primers were designed according to putative TRA/TRA2 binding sites found in *Mddsx*. The following concentrations and conditions were used for the PCR: 500 ng genomic DNA, 300 μM primer each, 10 mM dNTP each, and 25 mM Mg^{2+} in a total volume of 50 μl; denaturation at 94° for 2 min, followed by 16 cycles of 94° denaturation for 50 sec, annealing for 90 sec starting from 57° and then decreasing 1° every cycle to a touchdown of 41°, and extension at 72° for 2.5 min; and the subsequent 10 cycles were denaturation at 94° for 50 sec, annealing at 41° for 50 sec, and extension at 72° for 2.5 min; and finally extension at 72° for 5 min. Subcloning and sequencing of the candidate fragments were carried out by standard procedures.

To retrieve full-length transcripts 5′ and 3′ RACEs were performed using the BD SMART RACE cDNA amplification kit of Clontech or the 5′/3′ RACE kit from Boehringer Mannheim (Mannheim, Germany). The Expand long template system from Roche was used to isolate long genomic fragments according to the manufacturer's protocols. The

genomic sequence has been submitted to GenBank (accession no. GU070694).

Isolation of *Gmtra*: Searching the *G. morsitans* database (http://www.sanger.ac.uk/Projects/G_morsitans/) with the TRA-CAM domain identified a fragment (GMsg-2558) that showed sequence similarity in the 3′ part of the TRA-CAM domain and contained one putative TRA/TRA2 binding site. PCR on genomic DNA and 5′/3′ RACEs on RNA isolated from adult flies led to the identification of additional sequences spanning the entire *Gmtra* region. The *Gmtra* splice pattern was defined using standard PCR conditions and the gene-specific primers Gmtra-5 (5′-ACAGGTACATTGCAGTA GCTG-3′), Gmtra-13 (5′-CTTTACACAACAACGTGCCC-3′), Gmtra-3B (5′-TTTGCGCCAACGCATTCTG-3′), and Gmtra-18B (5′-TTAGCTTATAATTAGGTTTGGGG-3′). The genomic sequence has been submitted to GenBank (accession no. GU070695).

Isolation of *Md-l(3)73Ah*: The degenerate primer pair F2 and R2, designed according to the *D. melanogaster* sequence of *l(3)73Ah*, were used to isolate part of the Musca homolog *Md-l(3)73Ah*. The sense primer F2 (5′-GAR TGS STS CAY ACS TTY TG-3′) is located at the end of exon 2, whereas the antisense primer R2 (5′-TTS ARS GTR TGS TCY TTS CC-3′) is located near the end of exon 5, which enabled us to isolate a 500-bp cDNA fragment of the 3′ part of the *Musca-l(3)73Ah* using standard PCR conditions.

RNA expression analysis: Total RNA was either extracted from single adult flies (Musca or Glossina) according to the RNeasy Mini protocol of QIAGEN (Valencia, CA) or from 200 mg of adult flies, larvae, or embryos using the TRI REAGENT (Sigma, St. Louis) protocol. RT–PCR analysis was performed using the Enhanced Avian HS RT–PCR kit of Sigma and standard protocols for PCR reactions. The *Mdtra* splice pattern was defined using standard PCR conditions and the gene-specific primers Mdtra-16s, Mdtra-34as, and Mdtra-35as. Analyses for male-specific transcripts of the *Mdtra* gene were made using the primer pair Mdtra-16s and Mdtra-36as or Mdtra-12Bs and Mdtra-20as. Analyses for female-specific transcripts of the *Mdtra* gene were made using the primer pair Mdtra-16s and Mdtra-33as or Mdtra-9s and Mdtra-24as. For more information about all primers used to analyze the *Mdtra* splice pattern see Table S1.

Transcripts of the *Mddsx* gene were amplified using primers in the common exon (Mddsx-6s, 5′-CTAAAAGATGCCGGTG TTGAC-3′) and in the female-specific (Mddsx-11as, 5′-TGCA AGCACATTCATGTTTTG-3′) or the male-specific (Mddsx-46as, 5′-CCGCTGCACTTGCCGAC-3′) exon, respectively. Control transcripts of the *Mdtra2* gene were amplified using the primer pair Mdtra2-16 (5′-TTGCTTGAGTTGCCTGCTGC ATA-3′) and Mdtra2-9 (5′-CGTCCCCTGTAAACACCTGGG-3′). Control transcripts of the *CYP6D3* gene were amplified using the primer pair CYP6D3-1 (5′-GTTCGGTAATATTTGGCT TGG-3′) and CYP6D3-2 (5′-CCCGTATTCCGTAGTTGAATT).

Musca strains and crosses: Strains were reared as described previously (Schmidt *et al.* 1997a; Hediger *et al.* 2004). The strains were as follows: (1) wild-type strain, females *XX; +/+*, males *XY; +/+*; (2) autosomal *M* strain A, females *XX; pw bwb/pw bwb*, males *XX; M pw*$^{+}$ *bwb*$^{+}$*/pw bwb*; (3) autosomal *M* strain B, females *XX; pw bwb w/pw bwb w*, males *XX; M pw*$^{+}$ *bwb*$^{+}$ *w/pw bwb w*; (4) F^{D}/F^{man} strain, females *XX;* F^{D} *Ba/*F^{man} *Ba*$^{+}$, males *XX;* F^{man} *Ba*$^{+}$*/*F^{man} *Ba*$^{+}$; (5) F^{man} strain, females *XX;* F^{man} *Ba*$^{+}$*/*F^{+} *Ba*, males *XX;* F^{man} *Ba*$^{+}$*/*F^{man} *Ba*$^{+}$; (6) multimarked strain, females *XX; ac/ac; ar/ar; bwb/bwb; ye/ye; snp/snp*, males *XY; ac/ac; ar/ar; bwb/bwb; ye/ye; snp/snp*; and (7) *Ag* strain, females and males *XX; Ag/+* or *XX; +/+*.

To obtain a pure female progeny, wild-type females (strain 1) were crossed to no-*M* males of strain 5. Pure male progeny was obtained by crossing wild-type females (strain 1) to males homozygous for the autosomal *M* factor.

To map *Mdtra*, females of strain 5 were crossed to males from the multimarked stock (strain 6). Subsequently, F_1 males were backcrossed to females of strain 6. F_2 flies heterozygous for only one of each marker were collected for further analysis.

To determine the genotype of females of strain 7 (*Ag/+ vs. +/+*), single females were crossed to no-*M* males of the same strain. Those that produced only sons were *Ag/+* females, whereas those that produced only daughters were *+/+* females.

For sequence analysis of the $Mdtra^{D}$ allele, we used F^{D} females collected from natural populations in various parts of the world: Japan (Inoue and Hiroyoshi 1982), Turkey (S. Cakir, unpublished data), Spain, France, Tanzania, South Africa (kindly provided by Leo Beukeboom, University of Groningen), North America, and Australia (kindly provided by Rhonda Hamm, Itaka University). To test for presence of F^{D}, females were crossed to males homozygous for M^{III} (strain 2). We analyzed *Mdtra* sequences of only those females that produced male and female offspring.

Cryosections and *in situ* hybridizations: Ovaries were embedded in tissue-freezing medium (Jung) and frozen in liquid nitrogen. Sections (30 μm) were made with a cryostat at −14° and collected on gelatinized microscope slides. Antisense digoxigenin riboprobes for the *Mdtra* gene were generated using standard methods. The female-specific probe included exons E2a, E4, E5, and E6, while the male-specific probe contained exons E2b and E3. RNA *in situ* hybridization visualized by alkaline phosphatase was performed as described by Vosshall *et al.* (1999), with the modification that hybridization was carried out at 55°.

Injection of dsRNA: dsRNA was generated and injected into early blastoderm embryos as described earlier (Hediger *et al.* 2001, 2004). The fragment used for *Mdtra* silencing has a length of 621 bp and extends from exon 1 to exon E5, lacking the male-specific exons (E2b and E3) and the arginine/serine-rich domain in exon 5.

Transgenic constructs and germ-line transformation: The pBac (3xP3-eGFP; hsp70-Mdtra^{F1}) transgene was cloned by introducing a 1.2-kb *Not*I fragment spanning the complete female-specific Mdtra-ORF (MdtraF1; PCR sequence from primer Mdtra-19 to Mdtra-33) into the *Not*I-digested pBacHsp70 vector (kindly provided by E. Wimmer). This vector contains the eGFP marker under the control of the 3xP3 eye-specific promoter and a "Dmhsp70-polylinker-Dmhsp70poly(A) trailer" cassette. Coprecipitation with the helper plasmid and injection into housefly embryos of strain 3 were performed as described previously (Hediger *et al.* 2004). Transformed flies were identified by the GFP expression in their pigmentless, *white* (*w*) mutant eyes. Expression of the MdtraF1 cDNA was induced by repeated heat-shock pulses given throughout development. Each cycle consisted of 1 hr at 45° followed by 4 hr at 25°.

RESULTS

Isolation of *tra* orthologs in Musca and Glossina: Since TRA belongs to a family of rapidly evolving proteins with low complexity, a direct attempt to isolate a Musca ortholog on the basis of homology at the coding sequence level was expected to be severely hampered (Kulathinal *et al.* 2003; O'Neil and Belote 1992). Therefore we took a different strategy on the basis of the observation that the Mediterranean fruit fly ortholog *Cctra* is a target of *Cctra2* and contains several clusters of well conserved TRA/TRA2 binding sites (Pane *et al.* 2002; Salvemini *et al.* 2009). Given that

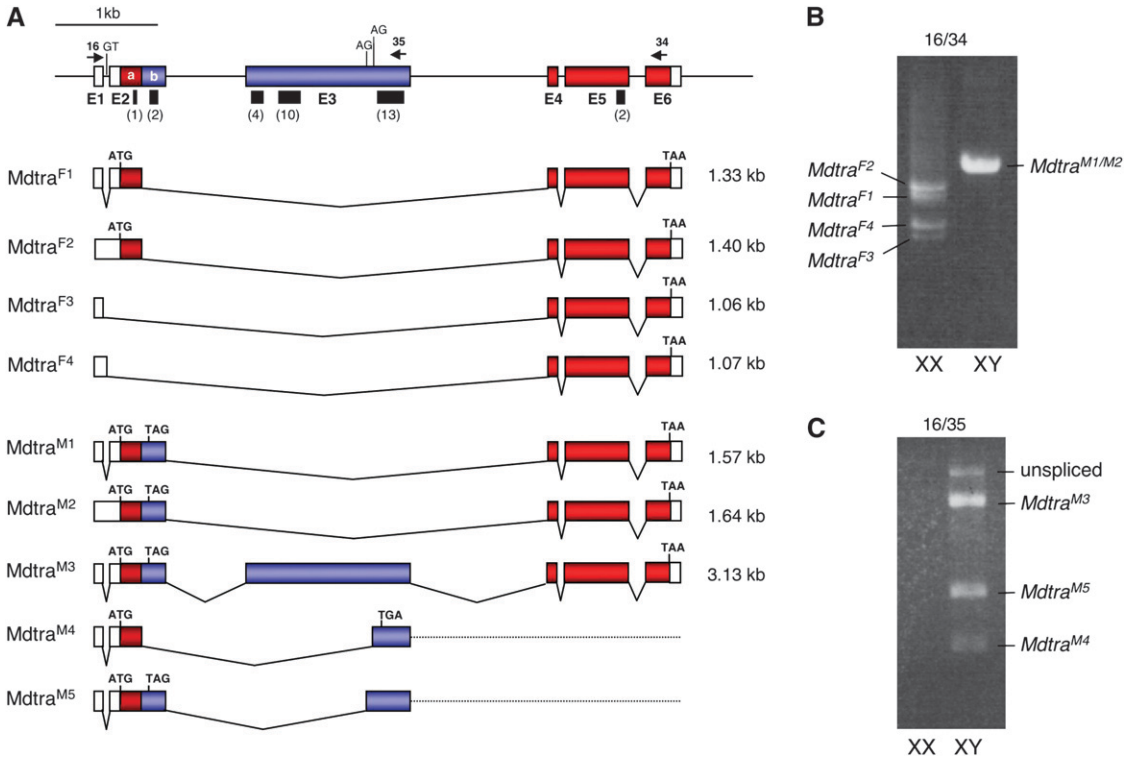

FIGURE 1.—Gene organization and splice variants of *Mdtra*. (A) Exons in red (E2a, E4, E5, and E6) contain the long ORF that is intact in the female-specific transcripts $Mdtra^{F1}$ and $Mdtra^{F2}$. Exons in blue (E2b and E3) are male specific and contain several in-frame translation termination signals. Black bars indicate the location of clusters of putative TRA/TRA2 binding sites with the numbers of sites given in parentheses below (see Figure S1 for sequences). Arrows represent the position of primers used in RT–PCR experiments. (B) RT–PCR analysis of total RNA from male and female adults with primers Mdtra-16 and Mdtra-34. (C) Minor splice variants $Mdtra^{M3–M5}$ in males are detected by amplification with primers Mdtra-16 and Mdtra-35, which is located in the male-specific exon E3.

the gain-of-function allele F^D cannot be silenced by *Mdtra2* RNAi, we presumed that *Mdtra*, the prime candidate for *F*, may also be a direct target of *Mdtra2* (BURGHARDT *et al.* 2005). We conducted a genomewide screen for sequences containing TRA/TRA2 binding sites using degenerate primers, which were designed from the three putative sites previously found in the *Mddsx* gene (HEDIGER *et al.* 2004). With primer pair MdDSX-69/MdDSX-70 we amplified a 360-bp genomic fragment that contains a cluster of nine sites (see MATERIALS AND METHODS). Using the same set of degenerate primers on cDNAs prepared from male embryos, additional sequences flanking this genomic region were isolated. Within this fragment that encompasses exons E2b and E3, a total of 29 putative binding sites were identified (Figure 1 and supporting information, Figure S1).

To retrieve full-length transcript sequences, 5′ and 3′ RACE were performed on cDNAs prepared from male and female adult houseflies. Alignment of the extended cDNA sequences to the corresponding genomic sequences revealed the presence of at least six exons (Figure 1). A genomic region of ~6 kb in length harbors a diverse pattern of alternatively processed transcripts. Of particular interest was the presence of sex-specific splice variants at all developmental stages tested (Figure 1). The only transcripts that contain an intact long ORF, $Mdtra^{F1}$ and $Mdtra^{F2}$ (Figure 1A), were exclusively found in female XX individuals (Figure 1B). We identified two additional transcripts $Mdtra^{F3}$ and $Mdtra^{F4}$ that are present only in females. In contrast to $Mdtra^{F1}$ and $Mdtra^{F2}$, which both encode a full-length protein of 367 aa, these transcripts lack exon 2 sequences and the first translational start signal. The next available start signal is located in exon 6 and, as a result, these transcripts are expected to give rise to a truncated protein of 64 aa. Transcripts that are predominantly detected in males, on the other hand, contain additional sequences that introduce in-frame stop signals causing premature termination of translation (Figure 1A, blue boxes). In RNA preparations from males we generally find a more variable pattern of differently sized transcripts, suggesting that the male splice mode is less robust than that of the female. We identified at least five different male-specific transcripts, $Mdtra^{M1–M5}$, by sequence analysis, but additional low abundance variants may yet exist (Figure 1, B and C). Importantly, none of these transcripts contain a long ORF. Instead, all male-specific ORFs appear prematurely truncated and give rise to small and presumably nonfunctional peptides.

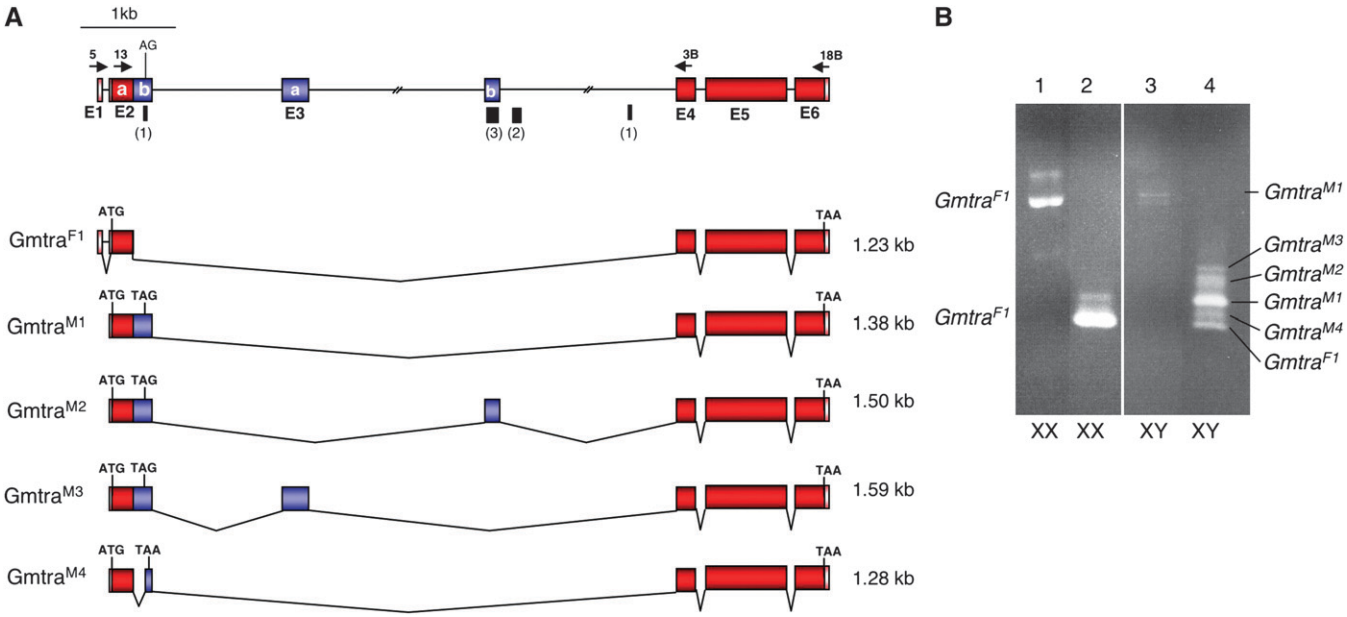

Figure 2.—Gene organization and splice variants of *Gmtra*. (A) Exons in red (E2a, E4, E5, and E6) contain the long ORF that is intact in the female-specific transcripts *Gmtra*^{F1}. Exons in blue (E2b, E3a, and E3b) are male specific and contain several in-frame translation termination signals. Black bars indicate the location of small clusters of putative TRA/TRA2 binding sites with the numbers of sites given in parentheses (see Figure S1 for sequences). Arrows above the top line represent the position of primers used for expression analysis. (B) RT–PCR analysis of total RNA extracted from adult XX and XY flies. Results from female (XX) samples are shown amplified with primers 5 and 18B (lane 1) and with primers Gmtra-13 and Gmtra-3B (lane 2). RT–PCR results from male (XY) samples are shown with primers Gmtra-5 and Gmtra-18B (lane 3) and with primers Gmtra-13 and Gmtra-3B (lane 4).

The exon–intron organization of this gene largely coincides with that of *tra* orthologs found in Tephritidae and Calliphoridae (Pane *et al.* 2002; Lagos *et al.* 2007; Ruiz *et al.* 2007; Concha and Scott 2009). Similarly to these *tra* orthologs, the sex-specific splicing regulation of *Mdtra* is mainly based on exon-skipping mechanisms and 5′ alternative splicing, rather than 3′ alternative splicing as observed for the Drosophila *transformer* gene. In addition, the housefly gene is flanked at the 3′ end by a divergently transcribed homolog of the *l(3)73Ah* gene (data not shown). Close linkage between *tra* and *l(3)73Ah* is also observed in *D. melanogaster*, *C. capitata*, *B. oleae*, and *A. obliqua* (Irminger-Finger and Nothiger 1995; Pane *et al.* 2002; Lagos *et al.* 2007; Ruiz *et al.* 2007).

We extended the analysis of *tra* homologs in the Calyptratae group to the tsetse fly *G. morsitans*. A BLAST search with *Mdtra* sequences led to the identification of a partial cDNA in the Glossina EST database (http://www.sanger.ac.uk/Projects/G_morsitans/). With 3′ and 5′ RACEs this cDNA fragment was extended on both sides and revealed a transcriptional unit that has a genomic organization very similar to that of *Mdtra* (Figure 2A). Two observations make this Glossina gene a likely candidate for representing the *tra* ortholog in tsetse flies. First, this gene generates several splice variants, all of which are sex-specifically enriched (Figure 2B). Of these, only the *Gmtra*^{F1} variant that is predominantly detected in female XX individuals has a long uninterrupted ORF. The four male-specific variants *Gmtra*$^{M1–M4}$ include additional sequences, which introduce translational stop signals shortly after the first AUG and thus encode small peptides. Second, four small clusters of putative TRA/TRA2 binding sites were found in the intron that is differentially spliced in the two sexes (Figure 2A). Given that this Glossina gene shares these critical features with the *tra* orthologs in tephritids and in Musca it is likely that it represents the *tra* ortholog in tsetse flies.

***Mdtra* and *Gmtra* encode SR proteins with homology to TRA and FEM:** Alignment of the long ORF of female-specific MdTRA^{F1} and GmTRA^{F1} with TRA polypeptides of Drosophila, Ceratitis, Glossina, Lucilia, and the *tra*-like FEM protein in the honeybee, *Apis mellifera*, shows a low (but some) degree of similarity at the amino acid level (Figure S2). TRA belongs to a class of rapidly diverging splicing regulatory (SR) proteins, which share domains enriched in arginine/serine dipeptides (RS domain) and a proline-rich domain at the C-terminal end (Kulathinal *et al.* 2003; Hasselmann *et al.* 2008). Both of these structural features are also preserved in MdTRA^{F1} and GmTRA^{F1} (Figure 3). In addition, using the ClustalW alignment program we identified a short highly conserved domain of 21 aa in the amino-terminal regions of all these TRA isoforms. This domain was termed TRA-CAM (C, Ceratitis; A, Apis; and M, Musca). Ruiz and co-workers (Ruiz *et al.* 2007) showed that the N-terminal domain displays the highest level of similarity among the TRA homolog in the tephritids. Another strikingly preserved feature is the position of the sex-

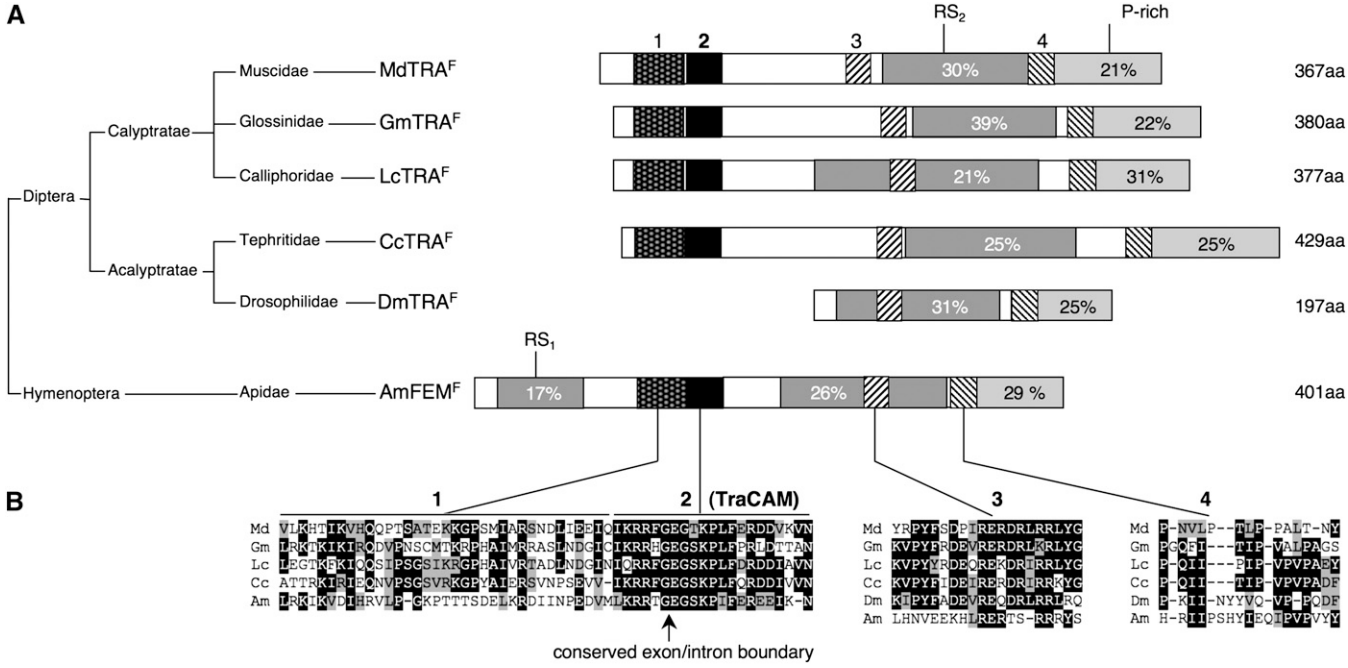

FIGURE 3.—Small conserved protein motifs in TRA homologs. (A) Schematic alignment of proteins encoded by the *tra* ortholog of *Musca domestica* (MdTRA^F), *Glossina morsitans* (GmTRA^F), *Lucilia cuprina* (LcTRA^F), *Ceratitis capitata* (CcTRA^F), *Drosophila melanogaster* (DmTRA^F), and *Apis mellifera* (AmFEM^F). The percentage of arginine/serine residues in the RS domains (dark shading) and the percentage of proline residues in the P-rich domains (light shading) are indicated within the boxes. The relative position and size of the four conserved motifs are indicated by differently shaded boxes (B) Sequence alignment of these small motifs. The conserved exon/intron boundary in motif 2 (TRA-CAM) is indicated by an arrow. In Musca, the glutamine (Q) directly upstream of the conserved TRA-CAM domain is replaced by an arginine (R) in the dominant allele *Mdtra^D*; see also Figure 7A and text.

specifically spliced intron at the same position within this TRA-CAM domain. Apart from the TRA-CAM domain the ClustalW algorithm identified three additional blocks of sequence similarity (domains 1, 3, and 4; Figure 3). Domain 1 is present just upstream of the TRA-CAM domain and both domains are absent in DmTRA^F. In addition, the TRA protein in dipterans reveals a short conserved motif, domain 3, which is located within or flanking the RS domain at the amino-terminal end. Likewise, domain 4, which is juxtaposed to the proline-rich domain in the carboxy-terminal region, occurs preferentially in the dipteran orthologs of *tra*.

***Mdtra* is necessary for female development:** To test for potential function of *Mdtra* in sex determination, we injected dsRNA fragments corresponding to exons E2a and E4 and upstream sequences of exon E5 into early preblastoderm embryos of an autosomal *M* strain (see MATERIALS AND METHODS), in which the male-determining factor *M* is linked to the wild-type alleles of *bwb* (*brown body*) and *pw* (*pointed wings*). This RNAi treatment causes a strong sex reversal phenotype only in flies with a female genotype (*pw bwb/pw bwb*) (Table 1 and Figure 4, A–H). A description of the sexual dimorphic structures is presented in the Figure 4 legend. The vast majority of these individuals (96%) developed into mosaic intersexuals displaying weak to strong degrees of masculinization (Figure 4, C and D). A small fraction of these no-*M* males (4%) were completely transformed into males (Figure 4, E and F). These no-*M* males were fertile and produced exclusively daughters when crossed to wild-type females, confirming the absence of the male determiner *M*. No detectable phenotypes were observed in genotypically male individuals (*M pw$^+$ bwb$^+$/pw bwb*) to which the same treatment was employed and in buffer-injected control flies of both sexes. The masculinization of RNAi-treated no-*M* animals correlates with a shift in the splice pattern of *Mddsx* from the female to the male mode (Figure 4M), placing *Mddsx* downstream of *Mdtra*.

To test whether *Mdtra* is needed for female development in animals carrying the dominant female-determining allele of *F* (*F^D*), we injected the same dsRNA fragments into embryos collected from *F^D* mothers. This time, the vast majority of *F^D* individuals (97%) developed into normal-looking females and only few intersexes or males were recovered (Table 1). However, all of the RNAi-treated *F^D* animals contained gonads that were clearly male-like in size and morphology. Microscopic sections through these testis-like gonads revealed the presence of different spermatogenic stages (Figure S3). The finding that in *F^D* animals only the gonads are masculinized is likely due to a transient silencing effect of *Mdtra* in early development affecting primarily gonad differentiation.

Activation of *Mdtra* is sufficient to instruct female development: The female-to-male transformation caused by loss of *Mdtra* activity reflects the situation in

TABLE 1

Masculinization of flies with a female genotype caused by *Mdtra* RNAi

Strain	Injected substance	No. of injected embryos	Total no. of hatched adults	Female genotype		Male genotype	
				No.	External sexual phenotype	No.	External sexual phenotype
F^{+a}	Ringer	2733	246	107	Female	139	Male
	Mdtra dsRNA	2070	234	0	Female	0	Female
				105	Intersexual[c]	0	Intersexual
				4	Male	134	Male
F^{Db}	Ringer	950	44	24	Female	20	Male
	Mdtra dsRNA	3227	189	89	Female[d]	0	Female
				2	Intersexual[c]	0	Intersexual
				1	Male	97	Male

[a] Autosomal M strain: Female genotype is *XX; bwb pw/bwb pw;* F^+/F^+ (phenotype bwb pw), and male genotype is *XX; M pw*$^+$ *bwb*$^+$*/pw bwb;* F^+/F^+ (phenotype bwb$^+$ pw$^+$).

[b] Strain F^D/F^{man}: Female genotype is *XX;* F^D *Ba/*F^{man} *Ba*$^+$ (phenotype Ba), and male genotype is *XX;* F^{man} *Ba*$^+$*/*F^{man} *Ba*$^+$ (phenotype Ba$^+$).

[c] Flies with mixed external sexually dimorphic structures, *e.g.*, with male genitalia but female eye distance, or flies with mixed external genitalia and male or female eye distances.

[d] All with ovotestes.

individuals that are homozygous for the loss-of-function allele of *F*, the key switch in sex determination of Musca. If *Mdtra* indeed corresponds to *F*, it is expected that forced expression of female *Mdtra* activity in genotypically male flies will impose female development, overriding the repression by *M* and, thus, mimicking the gain-of-function allele F^D. To this end, a construct expressing the full-length protein MdTRA^{F1} under the control of the Drosophila *hsp70* promoter, hs70::*Mdtra*^{F1}, was generated and again introduced into a strain that allows simple phenotypic distinction of individuals with and without M^{III}. In two independent lines, we observed a strong male-to-female transformation in M^{III}-bearing individuals after exposure to repeated heat pulses throughout development. These individuals display typical female traits such as a wider distance between the eyes and several female structures in the genital region such as the ovipositor (Figure 4, K and L). In line 14.2 the feminizing effect of hs70::*Mdtra*^{F1} was the strongest, causing a complete sex reversion of M^{III}-bearing flies to fertile females. In this line, the transgene inserted onto chromosome 3 to a site close to the genetically mapped location of the M^{III} factor. We ruled out the possibility that feminization was a result of insertional inactivation of M^{III} by the transgene, by crossing these females to males homozygous for M^{III}. The offspring carrying the transgene developed into fertile females, indicating that it is the activity of the *Mdtra*^{F1} transgene that overrules the male-promoting activity of M^{III}. In accordance with the expected position of *Mdtra* upstream of *Mddsx*, the splicing pattern of *Mddsx* is shifted from the male to the female mode in sex-reverted animals carrying the *Mdtra*^{F1} transgene (Figure 4M). We can thus conclude that the activity of *Mdtra* is not only required but also sufficient to direct female splicing of *Mddsx* in a genotypically male background.

Flies homozygous for the partial loss-of-function allele of *F*, F^{man}, develop as males (Schmidt *et al.* 1997a). To test whether expression of MdTRA^{F1} can substitute for loss of *F* activity in these animals we crossed line 14.2 into an F^{man} homozygous background. All 165 animals homozygous for F^{man}, carrying the *Mdtra*^{F1} transgene, developed into morphologically normal looking and fertile females. Hence, *Mdtra*^{F1} expression not only overrules repression by *M* but also substitutes for lack of *F* function, suggesting that it acts downstream or at the level of *F*. Taken together our data provide evidence that *Mdtra* is not only essential for female development but also sufficient to instruct female differentiation and thus acts as a *bona fide* genetic on/off switch in the sex-determining pathway of the housefly.

Female-specific splicing of *Mdtra* requires *Mdtra* and *Mdtra2*: In Drosophila sex-specific splicing of *tra* is controlled by *Sxl*, whereas in tephritids splicing regulation of the corresponding *tra* ortholog requires the activity of *tra* itself (Pane *et al.* 2002; Lagos *et al.* 2007). In addition, Salvemini and co-workers (Salvemini *et al.* 2009) recently reported that the *tra2* ortholog in Ceratitis is also required for female-specific splicing of *Cctra*. The presence of multiple clusters of putative TRA/TRA2 sites, ISSs, and RBP1 binding sites in *Cctra* and other tephritid *tra* orthologs gives further support to the notion that *tra* in these species directly controls its activity by directing the female-specific splicing mode.

Likewise, we find multiple clusters of ISSs and RBP1 binding sites located within the transcribed sequences of *Mdtra* and *Gmtra* and, in particular, several clusters of TRA/TRA2 binding sites in the alternatively spliced region (Figure 5, A and B). Hence, splice regulation of *tra* in these Calyptratae species may also involve a direct autoregulatory activity. Consistent with this notion, a shift from the female to the male splice mode of

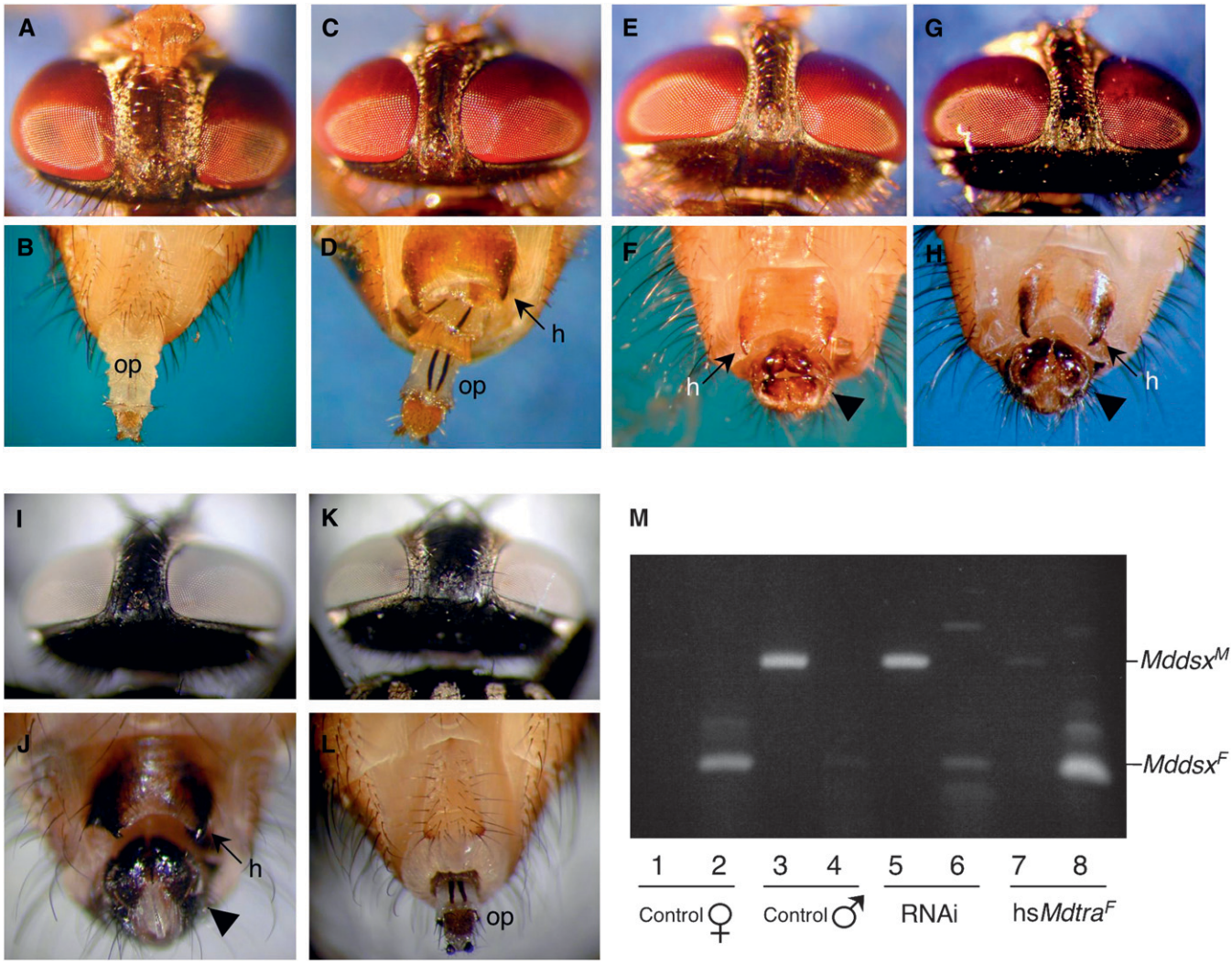

FIGURE 4.—Silencing or misexpression of *Mdtra* causes complete sex transformations in the housefly. Experiments were conducted with the M^{III} bwb^{+} / *bwb* strain to allow rapid and easy phenotypic distinction between *M*-bearing individuals (bwb^{+}) and no-*M* individuals (*bwb*) (see MATERIALS AND METHODS). Phenotypically, males can be recognized by a significantly narrower interocular distance. Also, males display a darkly pigmented copulatory apparatus (arrowhead) and exhibit characteristic horn-like structures (h) at the tip of sternite 5. The external female genitalia are characterized by the presence of an ovipositor (op). (A–H) Female–male sex reversion caused by injections of *Mdtra* dsRNA into syncytial embryos. (A) Head and (B) genital region of a control female (*bwb*) and (G and H) of a control male (bwb^{+}), respectively, are shown. (C and D) Intersexual no-*M* individual (*bwb*) treated with *Mdtra* RNAi displaying a male-like interocular distance and sexually mixed genitalia composed of an almost complete ovipositor and a male-like sternite 5 with hornlike structures. (E and F) Completely sex-reverted no-*M* individual treated with *Mdtra* RNAi displaying normal male morphology in the head and genital region. (I–L) Male–female sex reversion caused by transgenic expression of $Mdtra^{F1}$. (I and J) Head and genital region of a nontransgenic control male (bwb^{+}). (K and L) Complete feminization of an *M*-bearing individual (bwb^{+}) after heat-induced induction of $Mdtra^{F1}$ expression. This fertile individual displays normal female morphology in the head and genital region. (M) *Mddsx* splicing patterns in sex-reverted individuals. Transcripts of *Mddsx* are analyzed by RT–PCR with primer pairs specific for the male splice variants (lanes 1, 3, 5, and 7) and the female splice variants (lanes 2, 4, 6, and 8). Total RNA was extracted from single flies: control female (lanes 1 and 2), control male (lanes 3 and 4), no-*M* individual (*bwb*) treated with *Mdtra* RNAi (lanes 5 and 6; see also E and F), and *M*-bearing individual (bwb^{+}) expressing the $Mdtra^{F1}$ transgene (lanes 7 and 8; see also K and L).

endogenous *Mdtra* RNA was observed in genotypically female flies in which *Mdtra* was silenced by injecting dsRNA fragments into blastoderm embryos (Figure 5C). In the reciprocal experiment splicing of endogenous *Mdtra* nascent transcripts shifts from the male to the female mode in *M*-bearing animals in which $MdTRA^{F1}$ is ectopically expressed (Figure 5C). In accordance with the presumed function of *Mdtra2* as an essential co-factor in *Mdtra*-dependent splice regulation, silencing of *Mdtra2* in genotypically female individuals by injecting dsRNA fragments of *Mdtra2* also causes a shift in the splice pattern of *Mdtra* from the female to the male mode (Figure 5C). We conclude that the female splicing pattern of *Mdtra* requires functional products of *Mdtra* and *Mdtra2*. This is consistent with the findings in *C. capitata* where it has been shown that *tra*, once activated,

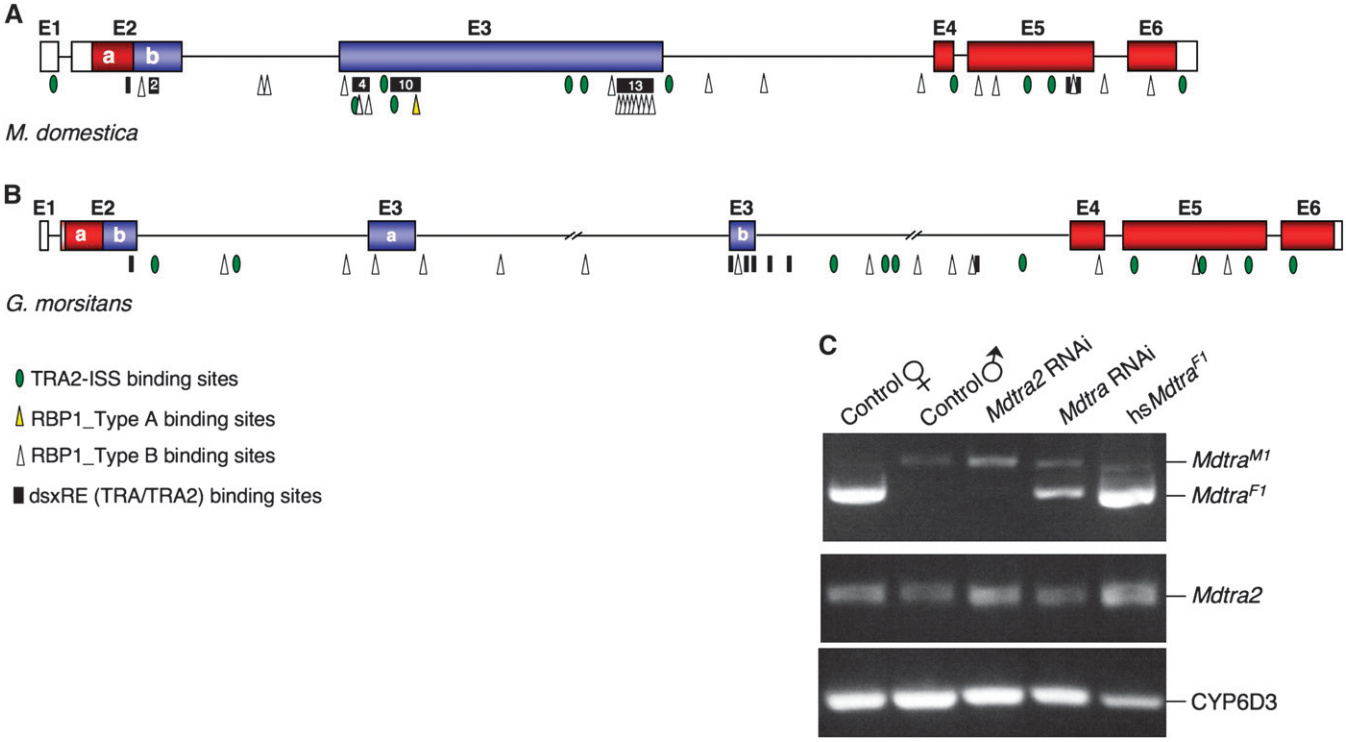

FIGURE 5.—*Mdtra* splicing regulation. Sites of putative *cis*-regulatory regions in the genomic regions of *Mdtra* (A) and *Gmtra* (B) are shown. Boxes represent exons, lines represent introns. The locations of TRA/TRA2 (boxes), RBP1 types A and B (triangles), and TRA2-ISS (ovals) binding sites are shown (for sequences see Figure S1). If not otherwise indicated (numbers within the boxes), each mark represents one binding site. (C) Splicing patterns of *Mdtra* in no-*M* individuals (*bwb*) treated with either *Mdtra* or *Mdtra2* RNAi and in *M*-bearing individuals expressing the *hsMdtra*^{F1}. Total RNA was extracted from single flies: control *bwb* female (line 1), control *bwb*$^{+}$ male (line 2), masculinized no-*M* individual treated either with *Mdtra2* (line 3) or *Mdtra* (line 4) RNAi, and feminized *M*-bearing individual containing the *hsMdtra*^{F1} transgene (line 5). Endogenous *Mdtra* transcripts were specifically amplified with primer Mdtra-24, which is located in 3′-UTR *Mdtra* sequences not present in the transgene. Amplification of *Mdtra2* and *MdCYP6D3* transcripts serves as an internal standard and quality control of extracted RNA.

is needed together with *Cctra2* to maintain a continuous production of active products by a positive feedback mechanism (SALVEMINI *et al.* 2009). Likewise we propose that the activities of *Mdtra* and *Mdtra2* are continuously required to maintain the active female splicing mode.

Maternal requirement for female-specific splicing of *Mdtra*: Regarding *Mdtra*'s autoregulatory function some important questions arise about when and how the productive female splicing mode of *Mdtra* is initiated in individuals with a female genotype. How does the positive feedback loop become established in the female zygote to maintain the selected female fate? One likely scenario is that the components necessary for activating the self-sustaining loop are maternally provided. In a previous report we described that *Mdtra2* transcripts are abundantly present in unfertilized eggs (BURGHARDT *et al.* 2005). To test whether *Mdtra* also provides maternal products, we performed *in situ* hybridizations with DIG-labeled *Mdtra* RNA probes encompassing exons E2a, E4, E5, and E6. Sections through adult ovaries unveiled the presence of large amounts of *Mdtra* transcripts in nurse cells and oocytes (Figure 6A). The predominantly localized *Mdtra* transcripts in the cytoplasm are female processed variants, since no cytoplasmic labeling is observed with probes containing only male-specific sequences (Figure 6B). Instead, these *Mdtra*^{M} specific probes gave a distinct punctated staining pattern that is confined to sites within the large polyploid nuclei of the nurse cells (Figure 6C). These labeled foci most likely represent intranuclear deposits of unprocessed *Mdtra* RNA. Moreover, when examining transcripts in unfertilized eggs, exclusively female-processed *Mdtra*^{F1} transcripts are detected (Figure 6D).

In a mixed population of staged male and female embryos, male-specifically spliced transcripts of *Mdtra* are first detected within 2–3 hr after egg laying before cellularization of the blastoderm (Figure 6E). It seems likely that these male transcripts are generated and processed *de novo*, suggesting that *Mdtra* must already be transcribed at the early blastoderm stage. It is thus conceivable that maternal supplies of *Mdtra* and *Mdtra2* activities present during the initial period of zygotic *Mdtra* transcription can impose the female processing mode in embryos with a female genotype. Likewise, it follows that, in embryos with a male genotype, *M* acts very early to prevent the establishment of the feedback loop by maternal *Mdtra*.

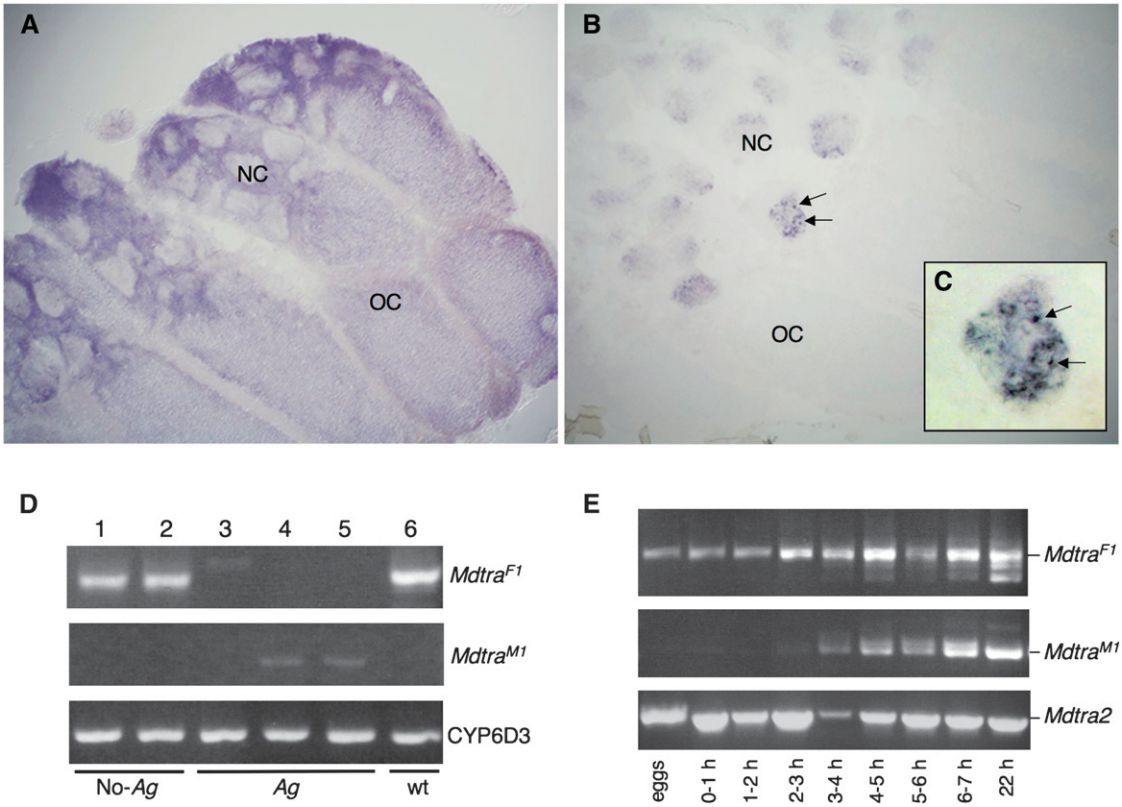

FIGURE 6.—Maternal and early zygotic expression of *Mdtra* in wild-type and *Ag* mutant females. *In situ* hybridization on frozen sections of adult ovaries is shown with (A) a *Mdtra*-specific riboprobe containing female-specific sequences (exons E2a, E4, E5, and E6) and (B) a riboprobe containing exclusively male-specific sequences (exons E2b and E3). Hybridization signals were visualized with an alkaline phosphatase-based detection system. (A) *Mdtra* transcripts predominantly accumulate in the cytoplasm of nurse cells (NC) and in the developing oocyte (OC). (B) Male-specific *Mdtra* sequences are detected only within the polyploid nuclei of nurse cells (arrows). The punctated staining pattern depicts nuclear sites where unprocessed *Mdtra* RNA accumulates. (C) Enlarged view of the punctated staining pattern in nurse cell nuclei. (D) RT–PCR analysis of *Mdtra* transcripts in unfertilized eggs collected from wild-type females (wt, lane 6), from *Ag*/+ mutant females (*Ag*, lanes 3–5) that produced an all male progeny, and from +/+ females of the same strain (No-*Ag*, lanes 1 and 2) that produced only females. Total RNA was isolated from unfertilized eggs laid by a single female over a period of 24 hr. Primers Mdtra-16 and Mdtra-33 were used to amplify $Mdtra^{F1}$ transcripts, and Mdtra-16 and Mdtra-36 were used to amplify $Mdtra^{M1}$ transcripts. Amplification of *MdCYP6D3* transcripts served as an internal control for amount and quality of RNA samples. (E) Profile of *Mdtra* transcripts during embryogenesis. Total RNA was isolated from staged wild-type egg collections and amplified with primers Mdtra-16 and Mdtra-33 for detection of $Mdtra^{F1}$ transcripts and with primers Mdtra-12B and Mdtra-20 to detect $Mdtra^{M1}$ transcripts. Amplification of *Mdtra2* transcripts served as an internal control for amount and quality of RNA samples. Eggs, unfertilized eggs; h, hours after egg deposition.

Further support for the conception that activation of zygotic *Mdtra* depends on maternally supplied *Mdtra* activity comes from studies of *arrhenogenic* mutant females (*Ag*/+). These females produce only male offspring due to a failure in activating zygotic *F* as shown in previous genetic studies (SCHMIDT *et al.* 1997b). We examined whether the dominant mutation *Ag* has any bearings on the maternal inheritance of $Mdtra^{F}$ products. None of the eggs collected from *Ag*/+ females contained detectable levels of $Mdtra^{F}$ (Figure 6D, lanes 3–5) whereas those collected from wild-type sibling females had normal levels of female transcripts (Figure 6D, lanes 1 and 2). Instead, low levels of male-processed $Mdtra^{M1}$ transcripts were found in eggs from mutant *Ag* females. We infer from these data that the *Ag* mutation prevents the production of a sufficient supply of maternal $Mdtra^{F}$ needed to activate zygotic *Mdtra*. Altogether, these results are consistent with the notion that maternal supplies of female *Mdtra* transcripts and *Mdtra2* transcripts are inherited to the zygote to serve as an initial source for directing female splicing of the zygotically active *Mdtra* gene.

Molecular lesions in *Mdtra* sequences derived from F^{man} and F^{D}: Besides the functional correspondence to *F* described above, there are also several structural features of *Mdtra* that make it a strong candidate for being *F*. The *F* gene was previously identified by two mutations: the recessive loss-of-function allele F^{man} (SCHMIDT *et al.* 1997a) and the dominant gain-of-function allele F^{D} (RUBINI *et al.* 1972). We isolated *Mdtra* sequences from these mutant backgrounds and aligned them with a reference sequence, which was assembled from *Mdtra* sequences from seven strains of different origins. While practically no polymorphisms were found in the reference

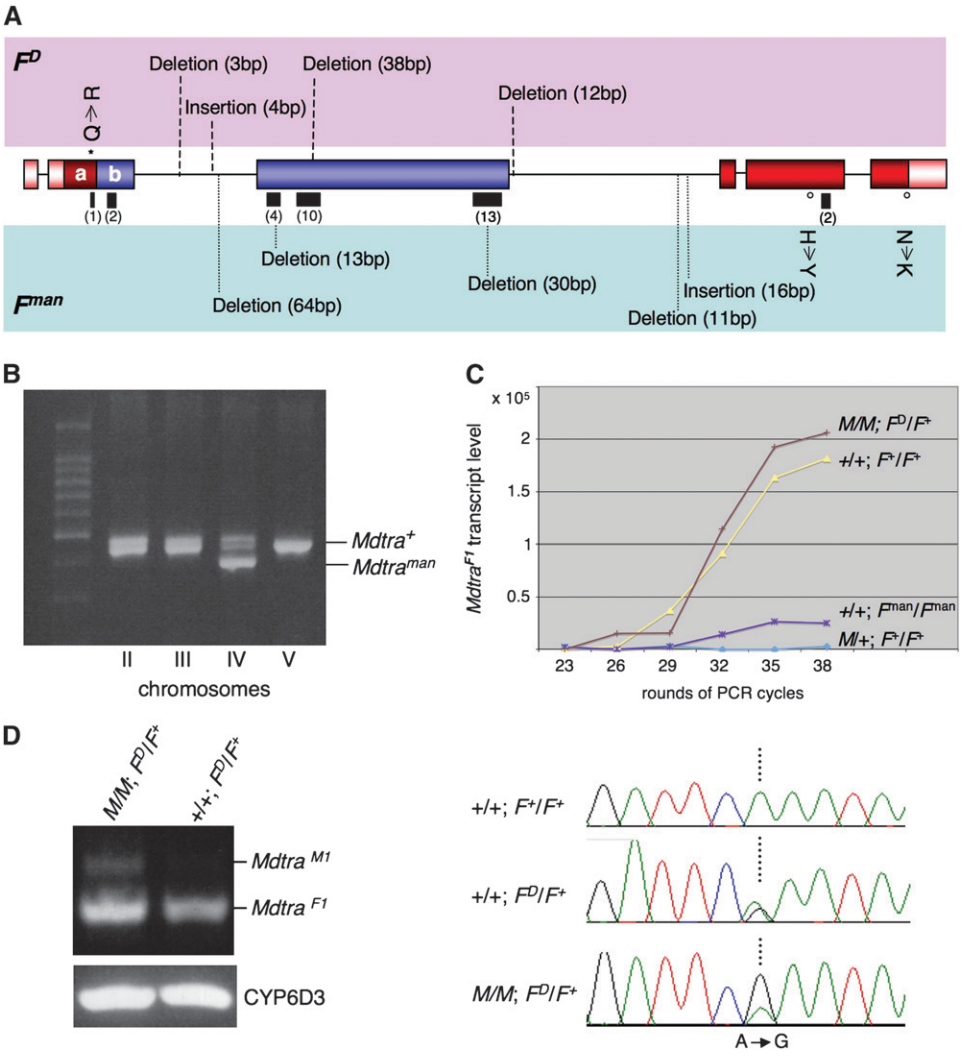

Figure 7.—Analysis and expression of *Mdtra* sequences in F^D and F^{man} mutant backgrounds. (A) Molecular lesions in the *Mdtra* genomic region of F^D and F^{man} animals. The positions of insertions and deletions are indicated by either dashed lines in the F^D allele (top row) or dotted lines in the F^{man} allele (bottom row). Lengths of insertions and deletions are given in parentheses. Amino acid substitutions are marked with an open dot for the F^{man} allele and with a star for the F^D allele. (B) The region encompassing a 64-bp deletion in the F^{man} allele was amplified from flies heterozygous for one of the four marked chromosomes II, III, IV, and V (see MATERIALS AND METHODS). Only flies heterozygous for the marker on chromosome IV also contain both the $Mdtra^+$ and the $Mdtra^{man}$ allele. (C) Semiquantitative analysis of $Mdtra^{F1}$ transcript levels in flies of different genotypes: control males ($M/+$; F^+/F^+), No-*M* males ($+/+$; F^{man}/F^{man}), control females ($+/+$; F^+/F^+), and F^D females homozygous for *M* (M/M; F^D/F^+). Total RNA was isolated from five flies per genotype and RT–PCR was performed with primer pairs Mdtra-9 and Mdtra-20. After each of the indicated cycles, 5 μl of the amplification reaction was analyzed. Intensity of the $Mdtra^{F1}$ bands was normalized using levels of amplified *Mdtra2* products as an internal control. (D) RT–PCR of RNA from M/M; F^D/F^+ females detects male $Mdtra^{M1}$ (primers Mdtra-18 and Mdtra-3) and female $Mdtra^{F1}$ transcripts (primers Mdtra-18 and Mdtra-20), while RT–PCR of RNA from $+/+$; F^D/F^+ females produces only female $Mdtra^{F1}$ transcripts (*MdCYP6D* internal standard). Analysis of the sequence chromatogram of the region encompassing the $Mdtra^D$-specific nucleotide substitution A to G in exon E2a allowed us to compare the relative amounts of F^+ (A) and F^D (G) -derived *Mdtra* transcripts in the absence or the presence of *M* and reveals that in M/M; F^D/F^+ females $Mdtra^{F1}$ transcripts are almost exclusively derived from the F^D allele. In contrast, in $+/+$; F^D/F^+ females, both *F* alleles produce equal amounts of $Mdtra^{F1}$ transcripts.

sequences, the *Mdtra* sequences from F^{man} and F^D animals displayed an unusually high number of small deletions and insertions in the region that is differentially processed (Figure 7A, Figure S4). Consistent with the different origins of the two mutant *F* strains, these indels do not overlap and thus must have arisen independently. We used the lesions in the *Mdtra* sequence derived from F^{man} as a molecular signature to assign the location of this gene to a specific chromosome in a multimarked background. The *Mdtra* variant of the F^{man} allele specifically segregates with markers on chromosome IV (Figure 7B), consistent with the genetic location of *F* (McDonald *et al.* 1978; Schmidt *et al.* 1997a).

We noticed that some of the small deletions in the *Mdtra* sequence of homozygous F^{man} individuals remove a substantial number of TRA/TRA2 binding sites (Figure S4). The loss of these putative binding sites correlates with a marked reduction of levels of female-specific *Mdtra* transcripts in homozygous F^{man} animals (Figure 7C). The strong hypomorphic character of F^{man} can thus be explained by impaired regulation of *Mdtra* to uphold the female-specific splicing mode due to the loss of binding sites. In addition, we detected several single-base-pair substitutions within the *Mdtra* sequence of the F^{man} allele. Two of the five single-base-pair substitutions that reside within the coding region lead to an amino acid exchange in exon 5 (H → Y) and exon 6 (N → K), respectively.

Regarding their effect on *Mdtra* functions the lesions found in the *Mdtra* sequence of the gain-of-function F^D

allele are less well explained. Among the deviations from the reference sequence we also find a small deletion that removes several TRA/TRA2 binding sites and a putative RBP1 type A binding site in one of the clusters (Figure S4). Nevertheless, normal levels of female *Mdtra* transcripts are detected in *M/M; F^D/F^+* females (Figure 7C). A specific nucleotide substitution (A to G) in exon E2a, resulting in a Q to R aa change, allowed us to test whether female *Mdtra* transcripts are derived from the F^D (G) and/or from the F^+ (A) allele. In F^D/F^+ females with no *M* factor present, both alleles produce comparable levels of female transcripts (Figure 7D). However, in F^D/F^+ females, which are homozygous for the *M* factor on chromosome III, female transcripts are almost exclusively derived from the *Mdtra* allele on the F^D chromosome. In this background some low levels of male transcripts are detected that presumably are generated by the *Mdtra* allele on the F^+ chromosome. From these observations we conclude that *M* is not capable of preventing the female splicing of the *Mdtra* allele on the F^D chromosome. On the other hand, this allele seems not to be able to transactivate the female splicing of the *Mdtra* allele on the F^+ chromosome when an *M* factor is present. This is also consistent with the notion that the F^D-derived *Mdtra* allele does not depend on autoregulation to exert its female-promoting activity. For instance, F^D, in contrast to F^+, can restore female differentiation at late stages after recovery from dsRNA-based silencing during embryonic development. In this regard the F^D-derived *Mdtra* allele behaves exactly like F^D in genetic tests where it has been demonstrated that zygotic F^D does not require maternal *F* to be active (Dubendorfer and Hediger 1998).

DISCUSSION

***Mdtra* corresponds to the female determiner *F*:** Since *Mdtra* displays all the relevant features that have been specifically assigned to the female determiner *F* in genetic studies, we propose that these genes are identical. Changes in *Mdtra* activity precisely mimic the reciprocal phenotypic effects of the loss- and gain-of-function alleles of *F*, F^{man}, and F^D. More compelling, female development can be restored in homozygous mutant F^{man} animals by transgenic expression of the female variant, $Mdtra^F$. This expression can impose female development even in the presence of the repressor *M*, reflecting the feminizing and *M*-resistant properties of the gain-of-function allele F^D. Changes in the activity of *F* or *Mdtra* cause corresponding shifts in the sex-specific splicing pattern of *Mdsx*, placing them upstream of *Mddsx* in the pathway. On the other hand, *F* and *Mdtra* are both susceptible to the silencing effects of *Mdtra2* RNAi, positioning them downstream and/or parallel to *Mdtra2* (see below). Besides occupying the same relative position in the pathway, they behave similarly in several other aspects. For instance, it has been previously shown in clonal studies that the activity of *F* is irreversibly set around the blastoderm stage and that its female-promoting activity is continuously required from there on (Hilfiker-Kleiner *et al.* 1994). This is in line with our finding that transcription of *Mdtra* already starts in the early embryo before cellularization when the presence of an *M* factor irreversibly locks it into the male nonproductive mode of splicing. In partially transformed individuals we often observed a graded response to *Mdtra* RNAi with strongest effects close to the injection site and lesser effects farther away from the injection site. It thus seems that the RNAi response in the syncytial embryo is not even but depends on the local concentration of dsRNA, which at the single-nucleus level may or may not be sufficient to cause an irreversible collapse of the feedback loop. This early commitment to the female or male fate based on the stable setting of a key switch is reminiscent of the situation in Drosophila, where activity of *Sxl* is irreversibly set during the early cleavage stages prior to cellularization (Keyes *et al.* 1992). Another important feature that *F* and *Mdtra* have in common is their dependence on their own maternal contribution to activate the female-promoting function in the zygote (Dubendorfer and Hediger 1998). Our findings based on studies of the maternal effect of the *Ag* mutation suggest a requirement of maternal *Mdtra* activity for female processing of *Mdtra* in the zygote. Finally, we identified a specific set of lesions in *Mdtra* sequences derived from the two *F* mutant backgrounds, F^D and F^{man}, which are likely to impinge on proper regulation of *Mdtra* as is discussed below.

Regulation of *Mdtra*: In Drosophila regulation of *tra* is achieved at the level of splicing to generate functional products only in individuals committed to the female fate. Likewise, alternative splicing of *tra* seems to be a common regulatory strategy in other dipterans to restrict expression of functional proteins to females. However, unlike in Drosophila, this splicing regulation appears not to be controlled by *Sxl* (Meise *et al.* 1998; Saccone *et al.* 1998). It was first demonstrated in the Medfly, *C. capitata*, that regulation of the *tra* ortholog, *Cctra*, is fundamentally different from that in Drosophila in that it involves an autoregulatory activity to maintain its female mode of splicing (Pane *et al.* 2002). The participation of the auxiliary factor *Cctra2* in *Cctra* splicing and the presence of TRA/TRA2 binding sites in *Cctra* pre-mRNA suggested that this regulation is direct (Salvemini *et al.* 2009). Here we report that *Mdtra* and *Mdtra2* are both required to uphold female splicing of *Mdtra*. The presence of several clusters of putative TRA/TRA2 binding sites in the *Mdtra* pre-mRNA suggests that *Mdtra* and *Mdtra2* are also directly involved in splicing regulation. In our initial survey for TRA/TRA2 binding sites in the genome we recovered only three fragments, of which one contained the *Mdtra* sequences. Clustering of these repeats in the Musca genome thus seems to be scarce and otherwise found only in

Mddsx and the recently discovered *fruitless* ortholog, both of which are targets of *Mdtra* (this article and N. Meier and D. Bopp, unpublished results). Likewise, the clustering of putative ISS and RBP1 binding sites in *Mdtra* and *Gmtra* is an unusual structural feature that is shared with *tra* orthologs of tephritids (Ruiz *et al.* 2007). Whether these sequences are involved in splicing regulation, however, remains to be investigated. Support for the relevance of putative TRA/TRA2 binding sites in *Mdtra* splicing comes from sequence analysis of the hypomorphic allele $Mdtra^{man}$. The only lesions found in this allele are several small deletions that remove a substantial number of putative TRA/TRA2 binding sites. It seems reasonable to assume that these deletions will lower the overall binding affinity of MdTRA/MdTRA2 complexes to *Mdtra* pre-mRNA and, thereby, decrease the efficacy of female splicing. The $Mdtra^{D}$-specific lesions, on the other hand, cause *Mdtra* to be locked into the female mode of splicing irrespective of whether *M* is present or not. In addition, we showed that female-specific processing of $Mdtra^{D}$ is reestablished after transient silencing of *Mdtra* or *Mdtra2*, indicating that activation of this allele does not depend on an autoregulatory function. This is in accordance with genetic results, which demonstrated that the $Mdtra^{D}$ allele is active in zygotes that lack maternal activity of *Mdtra* (Dubendorfer and Hediger 1998).

To reconcile the phenotypic effects with the lesions found in $Mdtra^{D}$ and $Mdtra^{man}$ we propose a model in which splicing of *Mdtra* consists of three superimposed levels of control (Figure S5). An important inference of this model is that the most subordinate level, the male mode of splicing, does not simply represent a deregulated state but requires the interaction of *Mdtra* pre-mRNA with a specific set of yet unidentified **m**ale-splicing **p**romoting **f**actors (MPFs). This interaction is required to activate the male-specific splice sites. The association of these MPFs with *Mdtra* pre-mRNA is antagonized by maternally supplied products of *Mdtra* and *Mdtra2*. Binding of these maternal factors to the *Mdtra* pre-mRNA prevents MPFs from binding and instead the female-specific splice sites are preferentially utilized. This second level of control initiates the production of functional MdTRA, which will continually prevent MPFs from activating the male splice sites. However, when an *M* factor is introduced into the system, it will antagonize female processing of *Mdtra*, either by hindering MdTRA/MdTRA2 to repress the function of the MPFs or by stabilizing the association of MPFs with *Mdtra* pre-mRNA. According to this model, we postulate that the $Mdtra^{D}$ lesions primarily affect *cis*-regulatory regions required for interaction with MPFs. As a result, the inclusion of the male-specific exons is no longer supported and instead female splice sites are preferentially used by default.

Putative TRA/TRA2 binding sites have also been found in the alternatively spliced regions of *tra* orthologs in other tephritids (Lagos *et al.* 2007; Ruiz *et al.* 2007), in *L. cuprina* (Concha and Scott 2009), and in *G. morsitans* (this article). From this it can be inferred that the dependence of female splicing of *tra* on its own activity is a common feature of *tra* regulation not only in members of the tephritids but also in the Calyptratae. In addition, this study provides evidence that expression of $Mdtra^{F}$ alone has the capacity to shift the splicing mode from male to female. Hence, *Mdtra* acts as a *bona fide* switch in the pathway in that it is not only required but also sufficient to activate and maintain the female mode of development. This feedback mechanism may serve as a cellular memory that ensures the proper implementation of the female program throughout the life cycle. A similar strategy has been described in Drosophila, where it is the gene *Sxl* that uses a feedback mechanism to sustain its female-promoting activity throughout development (Cline and Meyer 1996).

Evolutionary diversification of the sex determination pathway in the housefly: Although the process of sex determination is fundamental to all sexually reproducing organisms, the genetic logic underlying this process appears to be highly variable. Already in the insect world, an astounding variety of sex-determining strategies seem to exist. Nevertheless, it has been postulated that these differences may simply reflect variations on a common theme (Nöthiger and Steinmann-Zwicky 1985). These authors proposed that, in insects, a common principle underlies sex determination consisting of three basic components, a primary signal, a binary switch gene, which responds to the signal in an on/off manner, and a downstream bifunctional switch, which, depending on the setting of the binary switch, directs either overt male or female differentiation. These elements have been proposed to form the variables for rapid evolutionary diversification as observed for instance in the housefly (Dubendorfer *et al.* 2002). The molecular identification and characterization of the master switch *F* in Musca in this study gives us now a suitable framework to explain the mechanistic basis of this diversification. The findings presented here led us to propose the following model for sex determination in the housefly.

Mdtra acts as the central on/off switch in the pathway. Its continuous activity is required to properly implement female development. This perpetuation of *Mdtra* activity is normally ensured by a positive feedback loop that is established already in the very early zygote during the cleavage stages when zygotic *Mdtra* becomes first transcribed. Maternal deposits of functional *Mdtra* and *Mdtra2* products will initiate and activate the loop in the zygote (Figure 8A). In principle, the loop is continually active in the female lineage, as the female-promoting *Mdtra* activity is incessantly passed from mothers to daughters. Hence, presence of these maternal *Mdtra* activators predisposes the eggs for female development and would lead to a continuous production of females

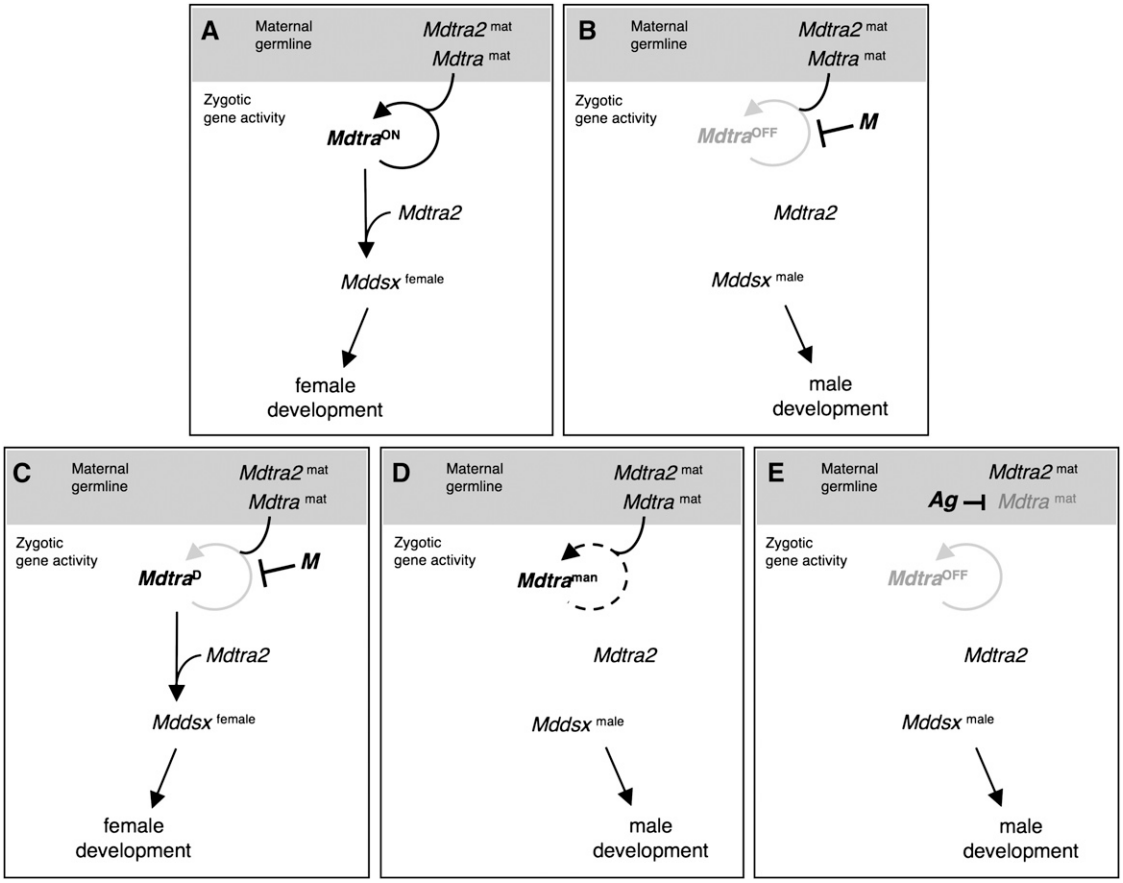

Figure 8.—Model to account for the different sex-determining mechanisms in the housefly. (A) In a "standard" XX zygote, maternal *Mdtra2*^{mat} and *Mdtra*^{mat} products activate zygotic *Mdtra*, which, once activated, maintains its productive (ON) mode of expression throughout development by a positive feedback loop. Together with *Mdtra2*, *Mdtra* sets its direct downstream target *Mddsx* into the female mode of expression, which leads to overt female differentiation. (B) In a standard *M*-containing zygote, activation or maintenance of the zygotic loop of *Mdtra* is prevented by the paternally transmitted *M*. As a result, *Mdtra* is locked into the nonproductive (OFF) mode of expression and *Mddsx* is set by default into the male mode of expression and male development ensues. (C) Dominant female determiner I: Presence of an *Mdtra*^{D} allele in the zygote uncouples the productive mode of *Mdtra* expression from the need of maternal factors for activation and from repression by *M*. This allele is by default locked into the productive mode of expression and sets *Mddsx* into the female mode of expression. (D) Dominant female determiner II: In *Mdtra*^{man} mutant zygotes, female *Mdtra* expression is severely reduced to levels below the threshold required to set *Mddsx* into the female mode of expression. In this background, presence of a wild-type *Mdtra* allele will behave as a dominant female determiner by providing sufficiently high levels of zygotic *Mdtra* activity required to impose female development. (E) Maternal sex determination: Eggs laid by an *Ag*/+ mutant mother are devoid of maternal *Mdtra* products due to a dominant *Mdtra* repressing activity of *Ag* in the germ line. As a result, zygotic *Mdtra* cannot be activated and male development follows. On the other hand, +/+ females in this strain produce eggs with normal levels of maternal *Mdtra* products. When fertilized by male *Ag*/+ siblings, all zygotes will develop into females since *Ag* does not prevent somatic activation of *Mdtra*.

from one generation to the other. To disrupt this "female" continuity the activation of the loop in the early zygote needs to be prevented, a compulsory measure for male development, which is usually achieved by a specific repressor such as the paternally transmitted *M* factor. Our model proposes that the sole function of *M* is to prevent the establishment of the female-promoting self-regulatory loop of *Mdtra*. Once the loop collapses, cells will not be able to resume a female identity and instead follow the alternative male fate (Figure 8B). The straightforwardness by which a male determiner can interfere with female development may explain the manifold occurrence of *M* factors in natural Musca populations (Hiroyoshi 1964; Rubini and Palenzona 1967; Wagoner 1969). Interference at any regulatory level of *Mdtra*, *e.g.*, transcriptional, post-transcriptional, or post-translational, would be sufficient to prevent the establishment of the loop. However, the observed resistance of *Mdtra*^{D} to *M* repression suggests that *M* does not compromise overall functions of *Mdtra*, at least not those involved in *Mddsx* regulation. Rather it seems to specifically impair the functions that are involved in *Mdtra* splicing. *M* factors have been found at different genomic sites in natural housefly populations. It has not yet been resolved whether they are transposed versions of the same gene or whether these are different genes that have adopted the function of a dominant loop breaker. It is conceivable that *M* factors evolved from

different genes by acquiring a dominant antimorphic or neomorphic mutation that specifically impinges on the autoregulatory loop of *Mdtra*.

Musca strains that differ from the standard type described above can be explained on the basis of discrete mutational changes that primarily affect regulation of *Mdtra*. For instance, we propose that lesions in the dominant gain-of-function allele $Mdtra^{D}$ uncoupled it from the requirement for *Mdtra* and *Mdtra2* to initiate and maintain the female mode of expression. Consequently, its disengagement from the feedback loop made this allele resistant to repression by *M* and converted it into a dominant female determiner (Figure 8C). On the other hand, lesions in the $Mdtra^{man}$ allele have the opposite effect such that female splicing of this allele is severely reduced even when maternal *Mdtra* and *Mdtra2* activities are supplied. Homozygosity for this allele leads to male development, since levels of female *Mdtra* products remain below a level that is required to promote female development (Figure 8D). In heterozygous animals, on the other hand, the presence of a wild-type *Mdtra* allele provides sufficient activity to implement female development. In this strain, hence, the wild-type allele of *Mdtra* behaves as a dominant female determiner. Finally, the maternal type of sex determination found in Musca can be derived from the dependence of zygotic *Mdtra* on maternally provided factors. We propose that the dominant *Ag* mutation in this maternal strain specifically represses expression of *Mdtra* in the female germ line without affecting the somatic functions of *Mdtra*. As a result, eggs of these arrhenogenic mothers are devoid of a sufficiently large supply of maternal *Mdtra* products to activate the self-sustaining loop in the zygote (Figure 8E). This is also in line with a previous postulate based on genetic data that *Ag* acts as an *M* derivative that specifically represses *F* in the female germ line without affecting *F* function in the soma (Schmidt *et al.* 1997b).

Taken together, these examples in Musca convincingly illustrate how subtle changes at the level of *Mdtra* regulation can lead to profound differences regarding the genetic logic of how the sexual fate is specified. This plasticity in the Musca system gives support to the proposition of Nothiger and Steinmann-Zwicky (1985) that seemingly different strategies can arise from small variations in an otherwise well conserved pathway. It seems from a number of studies in Acalyptratae and Calyptratae that a system that is primarily based on *tra* autoregulation represents the most common and possibly the ancestral mode of sex determination in higher Diptera. The most notable exceptions are found in the Drosophila lineage. In *D. melanogaster*, for instance, the sex determination cascade has been extended by incorporating *Sxl* as an upstream regulator of *tra*. The Drosophila system seems to be a derivative of the ancestral *tra*-based system in which the key functions, selection and maintenance of the sexual fate, are both delegated to *Sxl*. It has been proposed that *Sxl* was initially recruited as an additional coregulator of *tra* premRNA splicing, thereby establishing a functionally redundant circuit to *tra* autoregulation (Siera and Cline 2008). In this context, *Sxl* may have gradually relieved *tra* from upholding the female mode of its splicing to become eventually the exclusive regulator of *tra*. As a result, the mechanism based on autoregulation of *tra* and its repression by *M* became obsolete and eventually completely disappeared in Drosophila.

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Molecular Characterization of the Key Switch *F* Provides a Basis for Understanding the Rapid Divergence of the Sex-Determining Pathway in the Housefly

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	transformer* locus of *M. domestica	***transformer* locus of *G. morsitans***
putative TRA/TRA2 binding sites	Ex2a : TCAGCAACCAACA Ex2b : TCTTCAATCAACA TCATCAATCAACA Ex3,cluster 1: TCATCAATCAACA (4 sites) GCACCATTCATCA ACAAATATCAACA GCAGCATTCAACG Ex3,cluster 2: TCATCAATCAACA (10 sites) CCAAGAATCAACA TCAGCAATCAACA ACATCAAACAACA ACAACAATCAACA (3x) ACAACTATCAACA ACAACAATCAACT ACAACAATCAAAA Ex3,cluster 3: TCTACAATCAACA (2x) (13 sites) TCTTCAATCAACA (7x) TCTTCCATCAACA TCTGCAATCAACA (2x) ACAACATTCAACA Ex5 : ACTTCTTCCAACG TGTTCTTCCAACA	Ex2b : ACAACAATCAACA Ex3b : TCAACAATCAACG ACATCAATCAACT TCTACAATCAACA Intron 4: TCAACAATCAACA TCTTCAATCAATA TCTGCAATCAACA
putative ISS sequences	CAAGA (8x) CAAGG (3x)	CAAGA (10x)
putative RBP1 Type A binding sites	Ex3,cluster 2: TCAACTTTA	NONE
putative RBP1 Type B binding sites	Ex2b: ATCCTAA Intron 2: ATCCATA ATCCCTA Ex3,cluster 1: ATCTACA ATCTATA ATCCACA Ex3,cluster 2: ATCCATA ATCTTCA (6x) ATCTACA ATCTGCA Intron 3: ATCTAGA ATCTAAA ATCTTAA Ex5: ATCCGGA ATCTGGA ATCCAAA Intron 5: ATCCTTA Ex6: ATCCCCA	Intron 2: ATCTAGA ATCTTTA Ex3a: ATCTTAA Intron 3: ATCTCTA ATCTTGA ATCTTCA Ex3b: ATCTTGA INTRON 4: ATCCGAA ATCTGAA ATCTGCA (2x) Ex4: ATCTAGA Ex5 ATCTCCA ATCTAAA

FIGURE S1.—Sequences of putative TRA/TRA2, RBP1 and TRA2-ISS binding sites in the genomic region of *Mdtra* and *Gmtra*. The number of identical sequences are indicated in parentheses. In total, 32 putative TRA/TRA2 binding sites, one putative RBP1 Type A binding site, 23 putative RBP1 Type B binding sites and 11 putative TRA2-ISS binding sites are found in *Mdtra* sequences. In *Gmtra*, we detected 7 putative TRA/TRA2 binding sites, site, 14 putative RBP1 Type B binding sites and 10 putative TRA2-ISS binding sites, but no putative RBP1 Type A binding site. For the location of these sites in the genomic region see Figure 5.

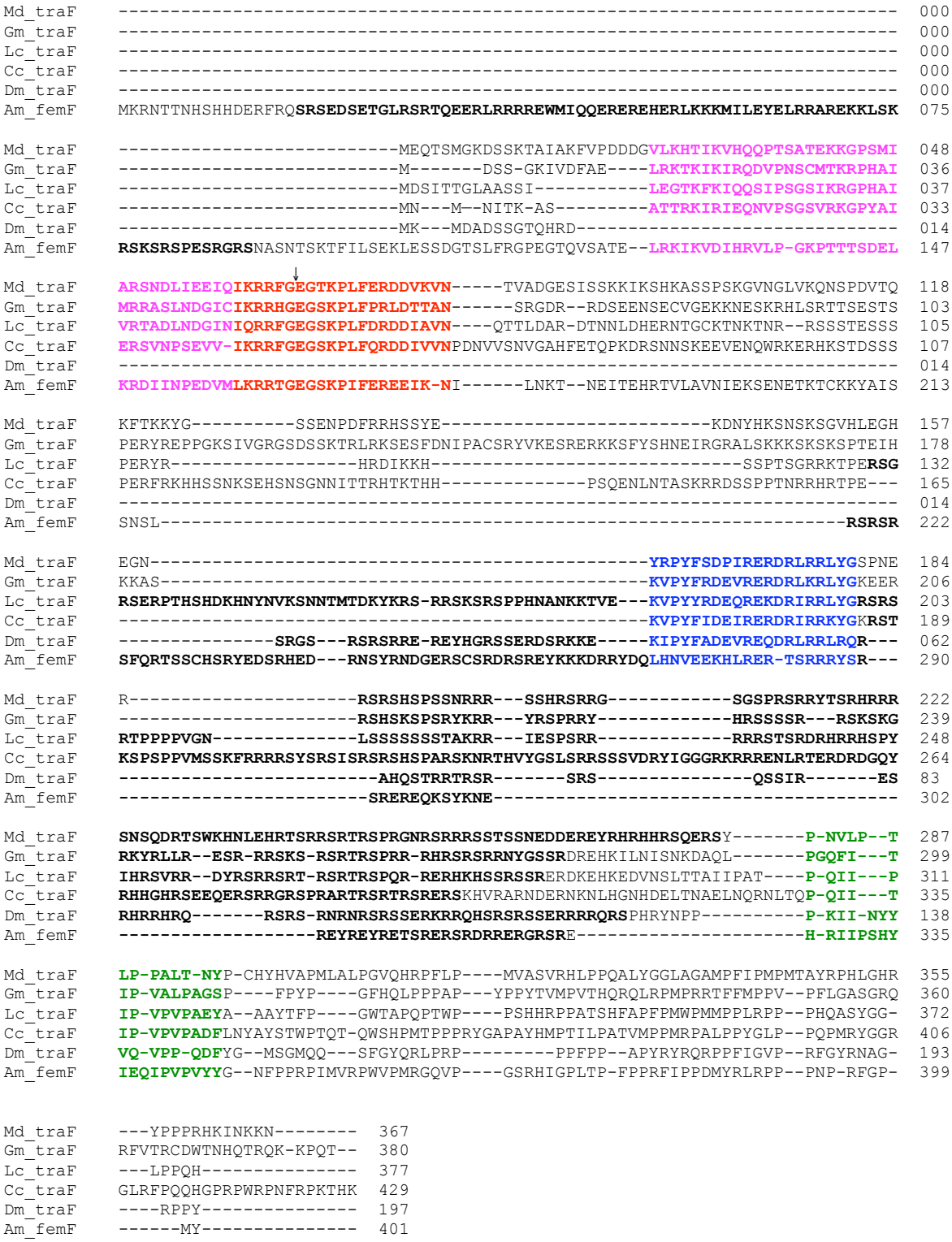

FIGURE S2.—Multiple sequence alignment of putative TRA proteins. Amino acid sequences of full-length TRA protein encoded by the orthologs in different species were aligned using the ClustalW program and manual adjustments. TRA protein of *Musca domestica* (Md, this paper, GenBank accession no. GU070694), of *Glossina morsitans* (Gm, this paper, GenBank accession no. GU070695), of *Lucilia cuprina* (Lc, Concha and Scott 2009, GenBank accession no. FJ461619), of *Ceratitis capitata* (Cc, Pane et al. 2002, GenBank accession no. AF434936), of *Apis mellifera* (Ap, Hasselmann et al. 2008; GenBank accession no. EU101388), of *Drosophila melanogaster* (Dm, GenBank accession no. AAF49441).The arginine /serine-rich domains are indicated in bold (black), the conserved motifs 1- 4 are indicated in color (pink: domain 1, red: domain 2, TRACAM, blue: domain 3, green: domain 4; see also Figure 3). The position of a conserved exon/intron boundary in the TRACAM domain is indicated with an arrow.

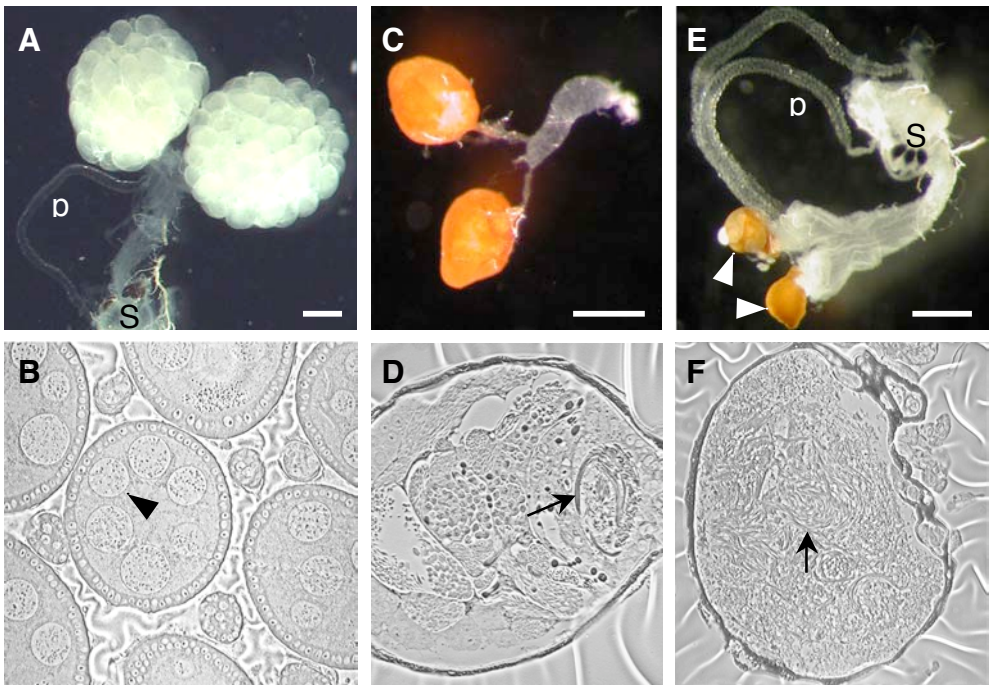

FIGURE S3.—Silencing of *Mdtra* by RNAi affects gonadal differentiation in *M/M*; F^{D}/F^{+} females.. (A) Adult ovaries dissected from a control *M/M;* F^{D}/F^{+} female with fully differentiated eggs (p = parovaria, s = spermathecae). (B) Section through the control ovary displays several cysts containing polyploid nuclei of nurse cells (arrowhead). (C) Adult testes dissected from a control *M/M;* F^{+}/F^{+} male of the same strain. Testes are pear-shaped and covered by a darkly pigmented epithelial sheath. (D) Section through the control testis displays bundles of elongating spermatids (arrow). (E) Gonads of an adult *M/M;* F^{D}/F^{+} female injected with *Mdtra* dsRNA at the blastoderm stage. Some female structures (parovaria and spermathecae) are still present, but gonads are substantially reduced in size and covered by a darkly pigmented male-like epithelial sheath (arrowhead). (F) Section through these gonads reveals the presence of elongated bundles of spermatids (arrow) and absence of polyploid nurse cells.

Aberrations in the *Mdtra* sequence of F^D

Intron 2	Deletion (3bp):	TTTGGCGTACGAAATATA...TTATGTTAATTAAAAAATAGGG
	wt sequence	TTTGGCGTACGAAATATATTGTTATGTTAATTAAAAAATAGGG
Intron 2	Insertion (4bp):	CCAAATTTACATTACAAT**T**ACTGTCTCTTT (bold = bp substitution)
	wt sequence	CCAAATTTACATTA....**G**ACTGTCTCTTT
Exon 3	Deletion (38bp):	C.....TTTA
	wt sequence	CACCTTTA**ACAACTATCAACA**TCATCA**ACAACAA**<u>**TCAACT**</u>TTA
		(bold = two possible dsxRE, underlined = possible RBP1_type A)
Intron 3	Deletion (12bp):	CTATATGTGGACCAATAAACCAAA.....TATTATTTTTATTA
	wt sequence	CTATATGTGGACCAATAAACCAAAAATACGGAACAATATTATTTTTATTA

Aberrations in the *Mdtra* sequence of F^{man}

Intron 2	Deletion (65bp):	C.T**GCCA**TCTT**C**TG.....G
	wt sequence	CTT**TGTC**TCTT**T**TGTAGTCTCATTTTTTATTGAAACGCGTTTATTCTCTTGGCAAAATATTCCTACAACATTTAATTG
		(bold = bp substitutions)
Intron 2	bp substitution:	ATCCTA**G**
	wt sequence	ATCCTA**A** (bold = nucleotide substitution in first RBP1 type B site)
Intron 2	bp substitution:	AT**T**CATA
	wt sequence	AT**C**CATA (bold = nucleotide substitution in second RBP1 type B site)
Exon 3	Deletion (13bp):	A.....TCATCA
	wt sequence	AGTAATT**GCACCATTCATCA** (bold = suboptimal dsxRE)
Exon 3	Deletion (30bp):	A.....T
	wt sequence	A**TCTTCAATCAACA**AA**TCTACAATCAACA**AAT (bold = possible dsxRE)
Intron 3	Deletion (11bp):	T.....A
	wt sequence	TGAGAAAAAAAAA
Intron 3	Insertion (16bp):	AAAAATACAATTGTTTAT
	wt sequence	A.....T

FIGURE S4.—*Mdtra* sequence alterations in F^D and F^{man}. Nucleotide sequences of indels identified in the *Mdtra* sequence of F^D and F^{man}, respectively. *Mdtra* sequences from the standard XX-XY strain was used as the wildtype reference.

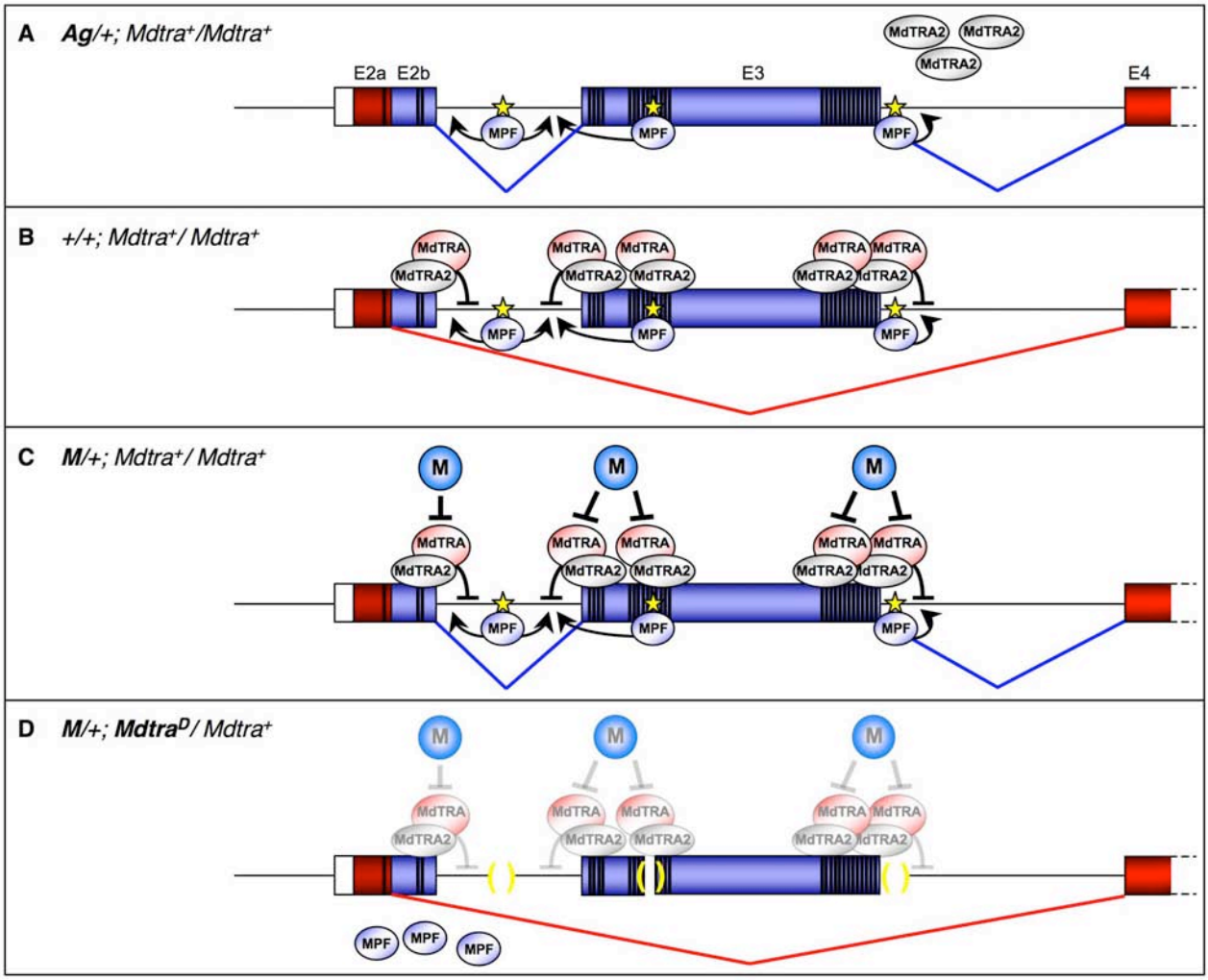

FIGURE S5.—Model for *Mdtra* splicing regulation. Based on expression studies and sequence analysis of the two *Mdtra* alleles we propose that (A) the first level of *Mdtra* splicing regulation involves a set of splice regulators referred to as MPFs (male-splice promoting factors) which, by interacting with specific sites on the *Mdtra* pre-mRNA (indicated with stars), entails the male splicing mode. When maternal *Mdtra2* or *Mdtra* products are absent (e.g. in eggs of *Ag/+* mothers), MPFs will activate the male-specific splice sites and/or prevent the utilization of the female splice sites. (B) Presence of maternal *Mdtra2* or *Mdtra* products in the early zygote prevents the MPFs from interacting with *Mdtra* pre-mRNA. As a result, the female splice sites are preferentially used (e.g. in *+/+; Mdtra*$^{+}$*/Mdtra*$^{+}$). This interference requires MdTRA/MdTRA2 to interact with specific binding sites in the pre-mRNA. When these sites are missing (e.g. in the *Mdtra*^{man} mutant), MdTRA/MdTRA2 can no longer effectively prevent the MPFs from imposing the male mode of splicing. (C) The male-determining factor *M* acts as an antagonist of MdTRA/MdTRA2 action. This inhibition allows MPFs to interact with *Mdtra* pre-mRNA and activate the male splice sites. (D) Lesions in *Mdtra*^{D} sequences do not allow MPFs to effectively interact with the pre-mRNA. As a result, male splice sites cannot be recognized and instead the female mode of splicing takes place. Hence, the *Mdtra*^{D} allele is active by default. There is no need for an autoregulatory function to activate and maintain the female mode of processing. (Shown is only the regulated splice region between exons E2 and E4. Red boxes = exons encoding a full length ORF; blue boxes = male-specific exons; yellow star = binding sites for MPFs; yellow parentheses = missing MPF binding sites; black bars within the exons = single TRA/TRA2 binding sites)

TABLE S1

Primer information for the *Mdtra* locus

Name	Sequence (5' → 3')	Location
Mdtra-3as	CTCTTTGGCAGCTTGGATTGGA	Exon 2b (male-specific)
Mdtra-9s	CTGCTACAGAAAAGAAAGGCC	Exon 2a
Mdtra-12Bs	GTGATCCTAACAATCAGCTAG	Exon 2b
Mdtra-16s	GTATCGGGTACTCTGTTCGA	Exon 1 (untranslated)
Mdtra-18s	CTTCCCGTCCATGCGCAAGTA	Exon 2a (untranslated region)
Mdtra-20as	TTGCTGCTGGGGGAATGTG	Exon 5
Mdtra-24as	GATGCATTTTGTGCATCGCAA	Exon 6 (untranslated region)
Mdtra-33as	GAATATTCGACTTGGATAGTG	Exon 6 (untranslated region)
Mdtra-34as	CTGGGAGGTGGATATCTATG	Exon 6
Mdtra-35as	TTGTTCCTTTGTAGGTTTGTTG	Exon 3 (male-specific)
Mdtra-36as	ATCACAATTGTTGCCTCCGC	Exon 3 (male-specific)

s = sense
as = antisense