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The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate

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SUMMARY

The medfly *Ceratitis capitata* contains a gene (*Cctra*) with structural and functional homology to the *Drosophila melanogaster* sex-determining gene *transformer* (*tra*). Similar to *tra* in *Drosophila*, *Cctra* is regulated by alternative splicing such that only females can encode a full-length protein. In contrast to *Drosophila*, however, where *tra* is a subordinate target of *Sex-lethal* (*Sxl*), *Cctra* seems to initiate an autoregulatory mechanism in XX embryos that provides continuous *tra* female-specific function and act as a cellular memory maintaining the female pathway. Indeed, a transient interference with *Cctra* expression in XX embryos by RNAi treatment can cause complete sexual transformation of both germline and soma in adult flies, resulting in a fertile male XX phenotype. The male pathway seems to result when *Cctra* autoregulation is prevented and instead splice variants with truncated open reading frames are produced. We propose that this repression is achieved by the Y-linked male-determining factor (*M*).

Key words: *Ceratitis capitata*, Sex determination, *transformer*, Autoregulation

INTRODUCTION

A broad variety of genetic cues that determine the sexual fate of a developing individual are known. Even within a minor taxonomic group, for example, dipteran insects (Marin and Baker, 1998; Schutt and Nöthiger, 2000), we find male heterogamety with a male-determining Y chromosome (*Musca*, *Ceratitis*) or with a single autosomal factor (*Megaselia*, *Culex*), female heterogamety (*Musca*, *Chironomus*), chromosomal balance systems (*Drosophila*, *Sciara*), and maternal effects (*Chrysomya*). This variety raises the issues of how these different mechanisms have evolved and how much they differ at the genetic and molecular level. Comparative analyses of different species can be used to address these. We have chosen the economically important medfly (Mediterranean fruitfly), *Ceratitis capitata* (*Tephritidae*). In this species a Y-linked factor, *M*, determines maleness, and absence of *M* leads to female development (Willhoeft and Franz, 1996). However, how this signal is relayed to genes responsible for expressing dimorphic traits is completely unknown.

The genetic cascade regulating sexual development in *Drosophila* is well known down to molecular details (Cline, 1993; Cline and Meyer, 1996). In contrast to *Ceratitis*, the primary signal in *Drosophila* is polygenic and is formed by the ratio of X chromosomes to sets of autosomes, the so-called X:A ratio. When this ratio is 1.0 (XX:AA), the gene *Sex-lethal* (*Sxl*) is activated; with a ratio of 0.5 (X:AA), *Sxl* remains inactive. *Sxl* now acts as the key ON/OFF switch that controls all aspects of somatic sexual dimorphism via a short cascade of subordinate regulatory genes (Nagoshi et al., 1988). When the gene is active, it dictates female development; when it is inactive, male development follows. Once the gene is activated in females, its products initiate a positive autoregulatory mechanism that guarantees the continuous production of SXL, thus forming a cell memory of the sex and maintaining the cells on the female pathway throughout development (Bell et al., 1991). In males, however, where *Sxl* is not activated, the gene will remain functionally OFF. *Sxl* produces sex-specific mRNAs by alternative splicing: the female-specific mRNAs encode full-length functional Sxl protein, while the male-specific ones have an additional stop-containing exon and encode a truncated non-functional Sxl peptide. The ON/OFF state of *Sxl* activity is set early during embryogenesis by a complex combination of transcriptional and post-transcriptional gene regulation (Bell et al., 1991; Keyes et al., 1992). The initial activation of *Sxl* in XX embryos relies on the use of an alternative XX-embryo-specific promoter that responds to the genes signaling the *X:A* ratio (Parkhurst et al., 1990). *Sxl* pre-mRNAs produced from this promoter have such a structure that they are spliced in a female-specific mode by the spliceosome independently of additional trans-acting factors, such as the Sxl protein itself (Horabin and Schedl, 1996; Zhu et al., 1997). The RNA-binding Sxl proteins translated from these early mRNAs then initiate the autoregulatory loop by directing the female-specific processing of the pre-mRNAs produced from the late *Sxl* promoter. The late pre-mRNAs, in contrast to the early *Sxl* pre-mRNAs, can

be spliced in the female-specific mode only in the presence of Sxl protein.

To execute the correct developmental program, *Sxl* transmits the determined state to *transformer* (*tra*) (Boggs et al., 1997), the next gene in the cascade. At this level, *Sxl* regulates the choice between two alternative 3′ splice sites in the pre-mRNA of *tra* (Inoue et al., 1990; Valcárcel et al., 1993). In absence of SXL, the more proximal site is used resulting in a *tra* mRNA that encodes a truncated inactive protein. When SXL is present, it will bind to the *tra* pre-mRNA and enforce the use of the distal 3′ splice site to produce an mRNA with a full-length ORF (Sosnowski et al., 1989). The state of activity of *tra* is then transmitted to *doublesex* (*dsx*) (Burtis and Baker, 1989), the last component of the pathway. In females, TRA, together with the constitutively expressed TRA-2, binds to *dsx* pre-mRNA directing its female-specific splicing, such that a mature mRNA encoding the DSX^F protein is generated (Hoshijima et al., 1991; Tian and Maniatis, 1993). In males, absence of TRA causes male-specific splicing and the production of a DSX^M protein. The two proteins, DSX^F and DSX^M, are transcription factors that regulate the activity of sex-specific differentiation genes (Burtis and Baker, 1989).

Previous studies have indicated that control of sexual development in the medfly follows a different route. In particular, the *Ceratitis* homolog of *Sxl* does not appear to have a switch function: the gene is expressed in both sexes, irrespective of whether the male-determining Y is present or absent (Saccone et al., 1998), which is inconsistent with a main sex-determining function. However, preliminary data suggest that the bottom-most component of the pathway, *dsx*, is not only present in *Ceratitis* (*Ccdsx*), but has conserved a role in sexual differentiation (Saccone et al., 2000). The pre-mRNA of this gene is also alternatively spliced giving rise to sex-specific products that show a remarkable structural conservation when compared with the corresponding male and female products in *Drosophila*. Sequence analysis of *Ccdsx* revealed the presence of putative TRA/TRA-2-binding sites close to the regulated splice site, suggesting that the underlying mechanism of sex-specific splicing is conserved and under the control of proteins homologous to TRA and TRA-2 (Saccone et al., 2000; Saccone and Polito, 2002). To extend our comparative analysis, we isolated the *Ceratitis* homolog of the *Drosophila transformer* gene.

In this report, we demonstrate that a homolog of this gene (*Cctra*), although highly diverged in sequence, is indeed present in the genome of Ceratitis and that, as in *Drosophila*, *Cctra* has a female-determining master function. However, in contrast to the *Drosophila tra*, *Cctra* plays an essential role in *Ceratitis* sex determination by maintaining the female sexual cell state through a positive feedback loop and by forming an epigenetic memory of the sex of the organism (analogous to *Sxl* in *Drosophila*) (Jablonka and Lamb, 1995).

MATERIALS AND METHODS

PCR and RT-PCR

Total RNA was extracted, as described elsewhere (Andres and Thummel, 1994), from adult individuals and from unfertilized eggs. Oligo-dT-primed cDNA was made from DNaseI-treated total RNA of unfertilized eggs, male and female flies using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Gibco BRL). RT-PCR reported in Fig. 1B was performed with the following primers:

164+ (5′-CAGTGGTTCGGTTCGGAAG-3′) located in *Cctra* exon 1

900– (5′-TCCATGATGTCGATATTGTCC-3′) located in *Cctra* exon 4

Cctra male specific cDNA M1 and M2 were amplified by RT-PCR using the following oligonucleotides:

F+ (5′-CATGAACATGAATATTACAAAGGC-3′)

E– (5′- TCGCGTTCTCTAATCTCGTC-3′)

These primers were derived from female-specific *Cctra*F1 cDNA. RT-PCR was performed on RNA from unfertlized eggs, using 164+/900- primers. Cycling conditions were denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minutes, annealing at 60°C for 1 minute and extension at 72°C for 2.5 minutes, with a final 5 minute extension at 72°C. The PCR products were gel-purified, cloned using the Sure Clone Ligation Kit (Amersham Pharmacia Biotech) and sequenced by T7 Sequencing Kit (Amersham Pharmacia Biotech). Y-specific repetitive elements were amplified from genomic DNA by PCR using the following oligonucleotides:

Y-spec1 (5′-TACGCTACGAATAACGAATTGG-3′)

Y-spec2 (5′-GCGTTTAAATATACAAATGTGTG-3′)

To perform positive control experiment *Cctra*-specific primers 164+ (described above) and 481– (5′-CTGGAATGGCACTGGTATTG-3′) were used.

RT-PCR experiments to analyze *Ccdsx* expression pattern were performed using a mix of the following *Ccdsx*-specific primers:

Non-sex-specific 1400+ (5′-GGCATCAAGGCGTATAGAAGA-3′)

Male-specific 1130– (5′-CTGGTGGTGACATCGTATCG-3′)

Female-specific 2000– (5′-ACGACGGCATGACCTTTAAC-3′)

For the negative RT-PCR controls reverse transcriptase was not included in the first strand cDNA synthesis reaction.

Northern blot analysis

We separated 2 μg polyA(+) RNA by formaldehyde gel electrophoresis and transferred RNA onto a Hybond NX membrane filter (Amersham Pharmacia Biotech). For hybridization, we incubated filters at 42°C overnight in a buffer of 50% formamide, 5×SSPE buffer, 5×Denhardt solution and 1% SDS. A *Cctra* probe was prepared by nick-translation labeling of full-length *Cctra*F1 cDNA in the presence of [α^{32}P]dCTP (NEN; 3,000 Ci/mmol).

RNAi

Cctra dsRNA was obtained and injected as described for *Drosophila* (Kennerdel and Carthew, 1998). A *Cctra*F1 fragment from positions 164 to 900 was amplified with primers that introduced a T7 promoter sequence at each of the product ends. In vitro RNA transcriptions were performed with the Megascript Kit (Ambion). Sense and antisense RNAs were separately obtained and equal amounts of the two ssRNA were mixed together, ethanol precipitated and resuspended in the injection buffer (Rubin and Spradling, 1982). Embryos were collected 1 hour AEL (after egg laying), hand dechorionated and microinjected with either 5 μM or 15 μM dsRNA solutions. We set up 27 cages, each containing single apparently normal males chosen from the injected flies and three Benakeion females. Twenty cages produced bisexual progenies, each consisting of a number of flies ranging from two to 51 individuals. Seven cages gave female-only progenies, each consisting of a number of flies ranging from seven to 66 individuals (7, 17, 20, 28, 33, 52 and 66).

Genomic and cDNA library screening

To identify *Ceratitis l(3)73Ah* genomic clones, we screened a *Ceratitis* genomic library in the EMBL3 vector using standard methods. A probe was obtained from a 500 bp RT-PCR product (oligonucleotides: l(3)981+, 5′-TAACGTGCAAGATCTGCGGC-3′ and l(3)1581–, 5′-TTGGCCACCAGCTTCTTGAG-3′) corresponding to a conserved region of *Drosophila melanogaster l(3)73Ah* gene

(GenBank Accession Number, X84372). Genomic inserts were subcloned in pBluescript (Stratagene) and sequenced using the T7 Sequencing Kit (Amersham Pharmacia Biotech). To clone the female-specific *Cctra* F1 cDNA we screened an adult female cDNA library in Lambda-Zap vector (Stratagene), using a probe obtained from a *Cctra* 400bp *Hin*cII genomic fragment corresponding to a region of the common exon 2.

Sequence analysis

Protein alignment was performed by MACAW (http://www.ebi.ac.uk/clustalw/) with default settings (NCBI, NIH, Bethesda, USA). The TRA/TRA-2 binding sites were identified in *Cctra*, by MACAW and by DNA Fasta sequence comparison between *Ceratitis* and *Drosophila* sequences.

GenBank Accession Numbers

Ccl(*3*)*73Ah* cDNA, AF436077; *Cctra* F1 cDNA, AF434936; *Cctra* M1 cDNA, AF434937; *Cctra* M2 cDNA, AF4349378; *Ccdsx* F cDNA, AF 435087; and *Ccdsx* M, AF434935.

RESULTS

Isolation of *tra* in *Ceratitis* by synteny

Given the unusually high degree of sequence divergence among *tra* homologs in *Drosophila* (O'Neil and Belote, 1992), we decided to attempt the isolation of the *tra* gene in the medfly by exploiting its close linkage in *Drosophila* to a well-conserved gene, *l*(*3*)*73Ah* (Irminger-Finger and Nöthiger, 1995). Hence, as a first step towards the isolation of *tra*, we isolated cDNA and genomic *Ceratitis* sequences that cross-hybridized to a 500 bp *Drosophila* cDNA fragment of *l*(*3*)*73Ah* at reduced stringency. These isolates indeed contained a structurally well conserved homolog of *l*(*3*)*73Ah* as confirmed by sequencing and comparison (*Ccl*(*3*)*73Ah*). We then continued to sequence a 4 kb long genomic region downstream of the *l*(*3*)*73Ah* homolog and identified a putative ORF that showed by Blast search significant sequence similarity at the amino acid level to *tra* in *Drosophila* (ranging from 32% to 40% identity scattered over 120 amino acids) and contained an arginine-serine-rich domain (SR-rich region) commonly found in splicing regulators (Manley and Tacke, 1996). As in *Drosophila*, the two genes are transcribed in opposite orientation and sequence analysis of corresponding cDNA clones revealed that they overlap by about 200 bp (data not shown). We conclude that this gene arrangement must have already existed in the common ancestor of these fly species. Though the significance of this synteny is unknown, it provided an ideal entry point to the molecular identification of the *tra* homolog in *Ceratitis* (*Cctra*).

Cctra produces sex-specific transcripts

If this *tra* homologous gene indeed corresponds to the *tra* switch gene in *Drosophila*, we expect it to be regulated sex specifically. A Northern blot containing poly(A)+ RNA from different developmental stages of the medfly was probed with a genomic fragment derived from the *Cctra* locus. We find that *Cctra* transcripts are continuously present from embryonic stages until adulthood (Fig. 1A). Furthermore, this probe detects sex-specific transcripts in samples from adult flies (Fig. 1A). The Ceratitis *tra* locus expresses four different mRNA variants: two products, of 1.6 kb and 3 kb in size, are found only in female adults, while two mRNAs, of 1.9 kb and 2 kb in size, appear only in male adults. In embryos two mRNAs are detected having sizes similar to those of the adult female-specific transcripts. In RNA sample extracted by larvae of mixed sexes all four transcripts can be detected suggesting that, as in *Drosophila*, *Cctra* sex-specific processing may already operate early in *Ceratitis* development.

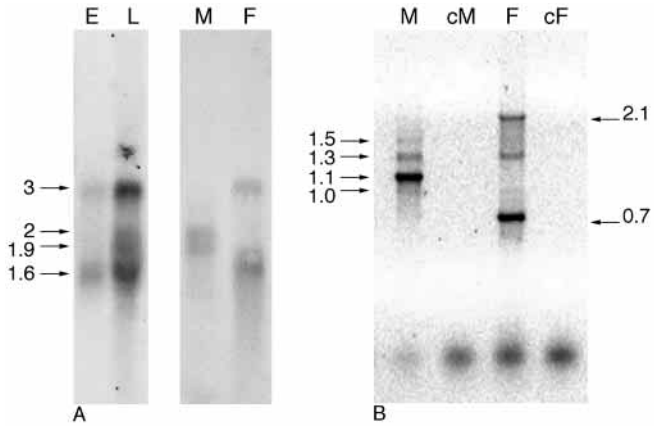

Fig. 1. Analyses of *Cctra* transcripts. (A) Northern blot analysis on poly A+ RNA from embryos (E), larvae (L), and adult males (M) and females (F), using as probe the F1 cDNA clone. In males, two predominant transcripts 1.9 kb and 2 kb long are detected, while two different transcripts 1.6 kb and 3 kb long are present in females. In embryos, two transcripts are detected and in larvae all four transcripts are detected. (B) RT-PCR amplification of *Cctra* on adult males (M) and females (F) total mRNA samples. Three main products are present in the female lane (F), which are 0.7 kb, 1.3 kb and 2.1 kb long. In the male lane (M) four bands are detectable which are 1 kb, 1.1 kb, 1.3 kb and 2.1 kb long. Male (cM) and female (cF) RT-PCR negative controls (reactions without reverse transcriptase) are shown.

A female-specific cDNA corresponding in size to the 1.6 kb transcript was isolated and entirely sequenced; a comparison with partial genomic sequences revealed that it is composed of three exons (Fig. 2). Using RT-PCR with various pairs of *Cctra*-specific primers, sex-specific amplification products were recovered from RNA samples of adult flies (Fig. 1B). The 164+/900– pair amplified (only in males) an abundant fragment of 1.1 kb and three minor bands of 1 (faint), 1.3 and 1.5 kb, whereas in females, they amplified a prominent 0.7 kb long fragment, and three minor bands of 1 (faint), 1.3 and 2.1 kb (Fig. 1B). The size of the female-specific 0.7 kb cDNA product corresponds to the one expected on the basis of the F1 cDNA structure. The non-sex-specific fragments of 1 and 1.3 kb, in other RT-PCR experiments, were sometimes undetectable. They most probably represent partially spliced and/or unstable *Cctra* RNAs. The 2.1 kb female-specific cDNA was isolated and entirely sequenced; a comparison with genomic sequences revealed that it is an unspliced product (data not shown). The size of this cDNA product suggests that it is derived from the 3 kb female-specific transcript.

The F+/Z1– pair of primers (Fig. 2A) amplified a male-specific 1.7 kb cDNA product, named *Cctra*M1 (data not shown). The nucleotide sequence alignment of *Cctra*M1 and *Cctra*F1 revealed that they are colinear with the exception of two additional exons present in the male-specific cDNA. The

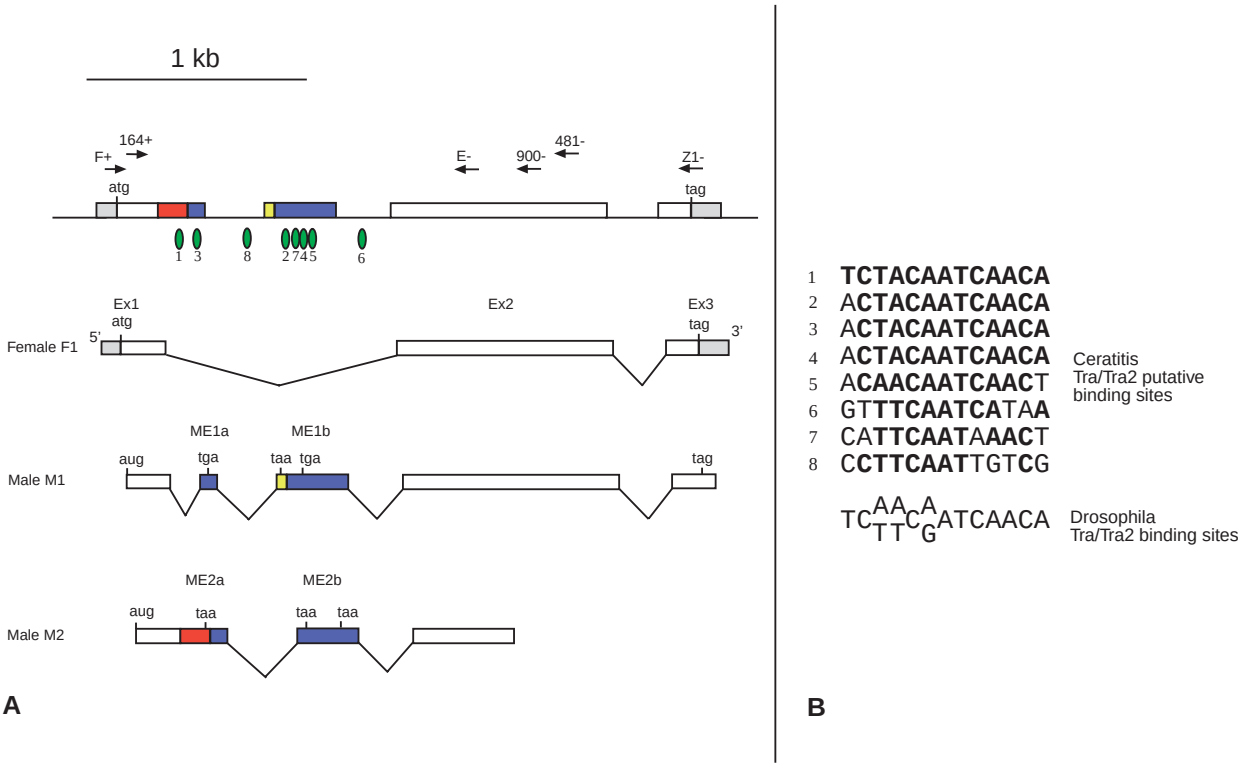

Fig. 2. Genomic organization of the *Ceratitis capitata tra* gene. (A) The top line represents the genomic DNA encompassing the *Cctra* locus. The positions of exons in the *Cctra* mRNAs are shown above the line, with Ex1, Ex2 and Ex3 representing exons in common between the male and the female mRNAs, the blue boxes representing male-specific exons, the yellow box indicating a male-specific exon in the M1 mRNA, and the red box representing a male-specific exon included in the M2 mRNA. Numbered green ovals indicate TRA/TRA-2-binding sites (see B). Introns are represented by solid lines. Open boxes represent the ORF of the female-specific 1.6 kb long mRNA (Female F1) encoding the putative 429 amino acid TRA protein (see Fig. 3). Gray boxes indicate 5′ and 3′ untranslated regions. Arrows above the first line represent the positions of the oligonucleotides used in the RT-PCR experiments. The bar indicates the scale of the figure. (B) Sequence alignment of eight putative TRA/TRA-2 binding sites found in the *Cctra* genomic sequence (see A). Conserved positions between *Ceratitis* and *Drosophila* are indicated in bold.

male-specific exons are located between the first and the second exon of *Cctra*F1 and they are 40 bp (ME1a) and 203 bp (ME1b) in length (Fig. 2A). Another pair of primers, F+/E– (Fig. 2A), amplified a male-specific fragment of 0.9 kb (data not shown), named *Cctra*M2, that was cloned and sequenced, showing with respect of *Cctra*F1 two additional exon sequences of 210 bp (ME2a) and 176 bp (ME2b). ME2a is an alternative exon including the previously described exons 1 and ME1a, plus the intervening intronic region (Fig. 2A). This ‘composed’ new exon is produced by skipping the first 5′ splice donor site. ME2b has an identical sequence to ME1b but it lacks the first 27 bp because of the usage of a downstream 3′ alternative splice site (Fig. 2A).

Cctra female-specific transcript encodes a SR-rich protein

An alignment of *Cctra*F1, *Cctra*M1 and *Cctra*M2 cDNA sequences with the genomic sequence exposes the organization of *tra* in *Ceratitis* (Fig. 2A). The gene is composed of five exons. The first, fourth and fifth exons are included in the mature transcripts of both sexes, while the second and the third exons are male specific. The most important finding is that the female-specific transcript has a long open reading frame, while the male-specific mRNAs contain stop codons that abort prematurely the protein translation. Indeed partially different intronic sequences are retained in the M1 and M2 cDNA clones, adding stop codons in different positions (Fig. 2A). This finding suggests that a functional full-length TRA is only encoded by the female-specific transcripts. This mode of sex-specific regulation at the level of splicing is well documented for the *tra* gene in *Drosophila* (Boggs et al., 1997). Different from *Drosophila*, however, where sex-specific regulation is based on the alternative use of two 3′ splice acceptor sites, sex-specific regulation in *Ceratitis* appears more complex and is achieved by a combination of exon skipping and differential use of 5′ donor and 3′ acceptor sites.

The long ORF in the female-specific *Cctra*F1 encodes a putative protein of 429 amino acids. The CcTRA protein exhibits a low degree of similarity to TRA proteins in *Drosophila* species and it is significantly larger in size in both N and C termini. Sequence processing tools of MACAW led to the identification of five small blocks of sequence similarity dispersed throughout the longest ORF of the female-specific transcripts (Fig. 3). The regions with highest similarity (identified also by FastA analysis) are located between CcTRA positions 150-230, 286-292 and 332-342 (Fig. 3). The SR-rich region in *Ceratitis* TRA and possibly the other conserved domains may confer specific RNA binding and protein-protein

interactions consistent with a proposed role in splicing regulation (Manley and Tacke, 1996). The male-specific truncated protein isoforms lack the conserved boxes, the SR-rich region and do not show significant similarity with other known proteins.

tra is essential for female development in *C. capitata*

The confinement of transcripts with a long ORF to females suggested to us that this gene had an essential role in female development of the medfly. To test its function, we employed the RNAi technique that permits functional studies of genes in genetically less amenable organisms (Kennerdel and Carthew, 1998; Hunter, 1999). A 900 bp fragment of *Cctra*F1 was used as a template to produce dsRNA that was then injected as a 15 μM solution into either the anterior or the posterior poles of embryos of two different laboratory strains (*Benakeion* and

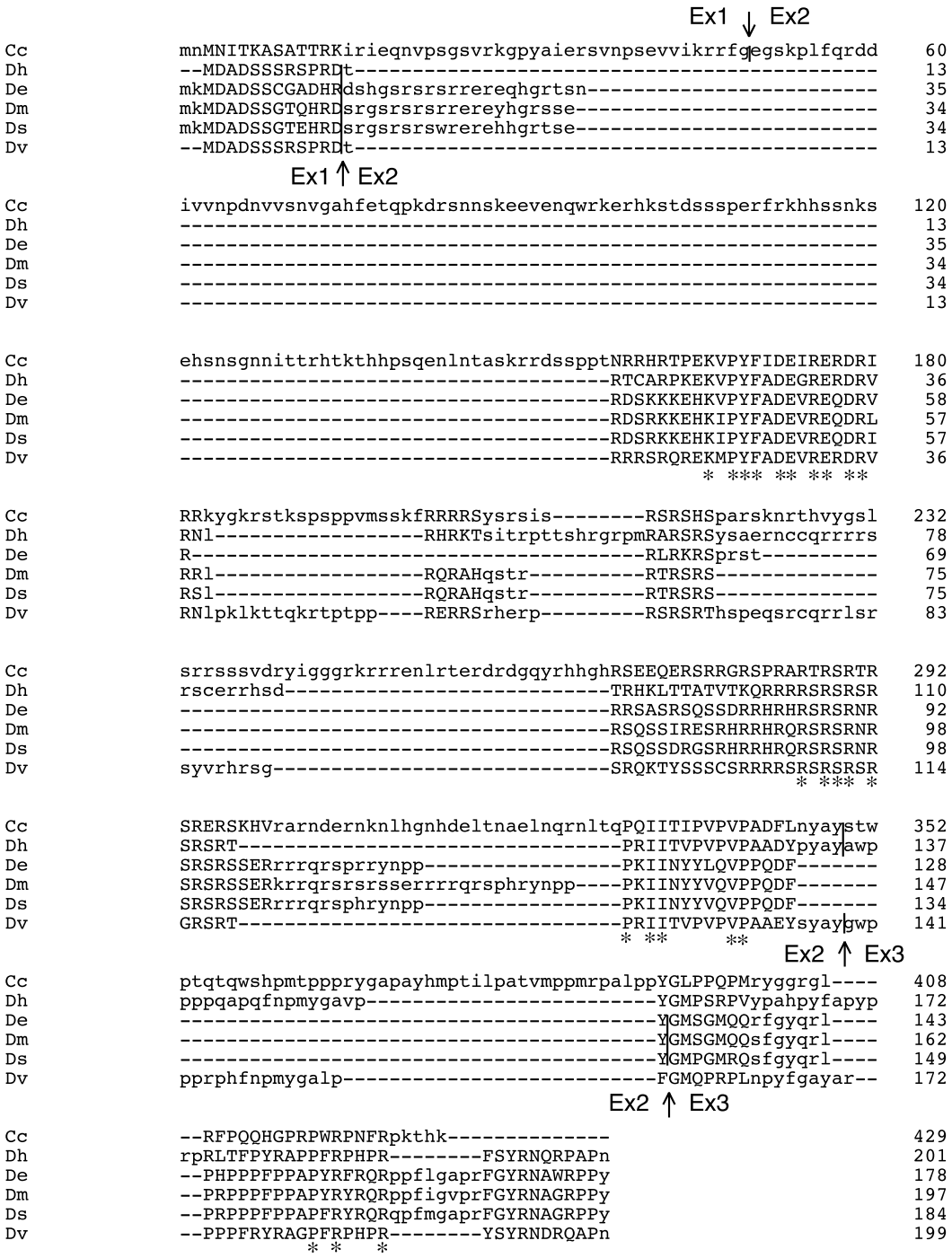

Fig. 3. Multiple sequence alignment of TRA proteins. *Ceratitis capitata* (Cc), *D. melanogaster* (Dm), *D. erecta* (De), *D. simulans* (Ds), *D. virilis* (Dv) and *D. hydei* (Dh). Asterisks indicate amino acid identity in all species. Intron/exon boundaries are indicated by vertical arrows. Amino acid residues occurring in the conserved regions are indicated by capital letters.

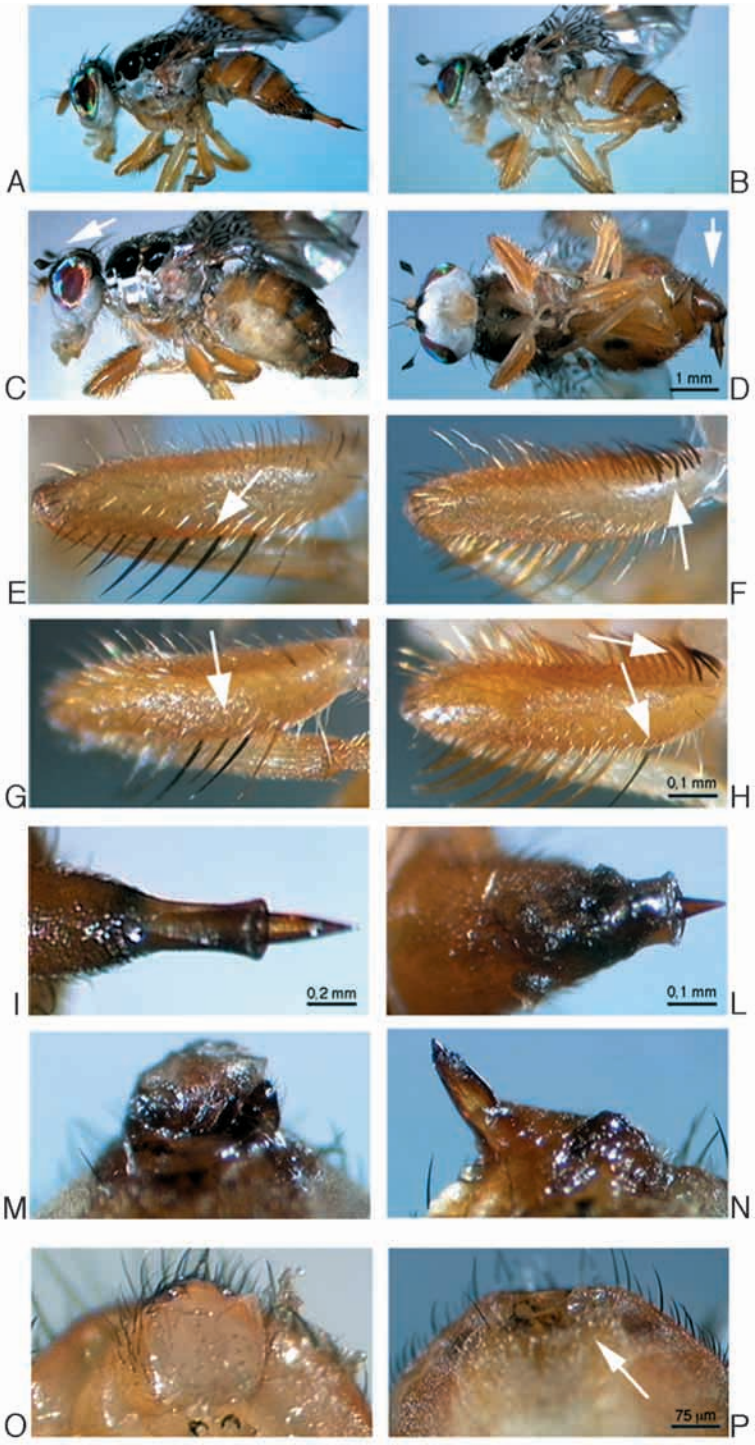

Fig. 4. Phenotypic analysis of RNAi intersexes. (A) Wild-type female has long pigmented bristles on the femur pointing towards the coxa of the foreleg (arrow in E) and the ovopositor (A,I). (B) Wild-type male exhibits two spatulated bristles on the head (B), a row of non-pigmented bristles on the ventral part of the femur towards the coxa of the foreleg, short pigmented bristles grouped on the dorsal part of the femur (arrow in F) close to the coxa of the foreleg (F) and male genitalia (B,O). (C,D) Intersexes obtained by dsRNA injection into the anterior pole of the embryos exhibit male-specific spatulated bristles on the head (arrow in C), male-specific bristles (upper arrow in H) and female-specific bristles (arrow in G; lower arrow in H) mixed together on the femur of the foreleg (G,H) and female genitalia (C,D). Some intersexes show various degrees of abnormal gonadal development exhibiting bent (arrow in D), deformed (L-N) or completely absent (arrow in P) genitalia. Scale bar in D applies at A-D; scale bar in H applies to E-H; scale bar in I applies to I; scale bar in L applies to L; and scale bar in P applies to M-P.

white-eye). From a total of 900 injected embryos, 272 adult flies were recovered and grouped by their sexual phenotype. A strong sex ratio bias was observed in favor of males. Out of 272, 231 flies (84.9%) showed a normal male morphology, 37 flies (13.6%) exhibited various degrees of intersexuality (Fig. 4) and the remaining four (1.4%) were the only flies recovered with a normal female phenotype. All of the 37 intersexes exhibited an anteroposterior pattern of intersexuality. More tellingly, the position of male tissues correlated exactly with the initial injection site in the embryo: injection into the anterior pole resulted in the formation of male-specific spatulated bristles on the head of intersexes (Fig. 4C,D), male-specific blue eye reflections (data not shown), male-like bristles mixed with female-like bristles on the femur toward the coxa of the foreleg (Fig. 4G,H), but the genitalia at the posterior remained female-like (Fig. 4C). Conversely, injection into the posterior pole gave rise to mosaic adults with male genitalia but with female bristles on the head and female-specific green eye reflections (data not shown). The intersexes showed also various degrees of abnormal gonadal development, with abnormally bent (Fig. 4D) or deformed ovopositor (Fig. 4L) and with mixed male-like and female-like tissues (Fig. 4M,N). A few intersexes apparently lacked genitalia (Fig. 4P).

Karyotypic analyses of RNAi-treated adults

To assess the sexual karyotype of affected flies, we performed a PCR amplification of genomic DNA using *Ceratitis* Y-specific primers (Anleitner and Haymer, 1992). No products were detected in single preparations of 10 randomly chosen intersexes (data not shown) and six out of 10 phenotypic males did not reveal the presence of a Y chromosome by this test, indicating that all these animals have a female XX karyotype (Fig. 5). These results are in agreement with the expected loss of female-promoting activity when *tra* function is impaired by RNAi. On the contrary, male development of XY flies seems not to be affected by RNAi of *tra*, suggesting that the gene, as in *Drosophila*, is dispensable in this sex. The occurrence of intersexes and of few females is most likely due to incomplete penetrance of the RNAi effect. Indeed, when a lower concentration of dsRNA (5 μM versus 15 μM) was injected into the anterior embryonic region, we obtained 64 intersexes, 76 males and four females out of 144 adult flies. Therefore the percentage of intersexes increased from 14% to 44%, while the percentage of males decreased from 84% to 52%, suggesting that XX individuals were only partially masculinized. From these results, we conclude that *tra* is required for female

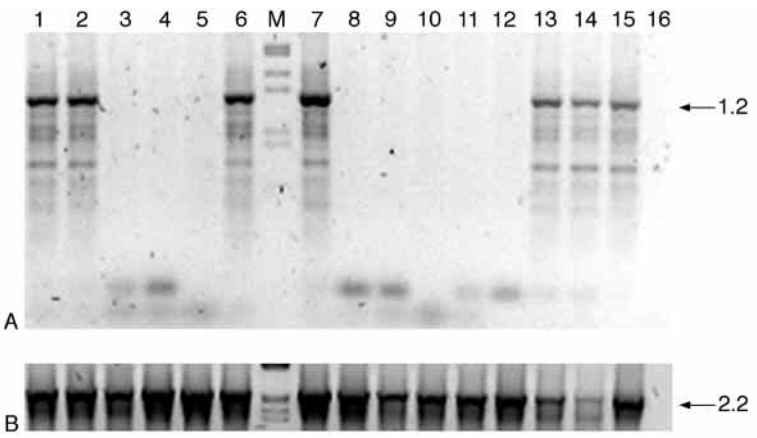

Fig. 5. Karyotypic analysis of RNAi treated individuals. (A) PCR with Y-specific oligonucleotides carried on medfly genomic DNA. From lane 1 to 10, PCR on single males developed from dsRNA-injected embryos; lanes 11 and 12, PCR on single wild-type females; lanes 13 and 14, PCR on single wild-type males. PCR on flies of mixed sexes and a negative control are shown, respectively, in lanes 15 and 16. The PCR amplification patterns in lanes 1,2,6 and 7 correspond to those of wild-type males, indicating that the analysed adults have an XY karyotype. By contrast, no bands are detected in lanes 3-5,8-10 indicating that these males lack a Y chromosome and therefore are XX sexually transformed males. (B) Positive PCR control with *Cctra* specific primers (*Cctra*164+ and *Cctra*481–) showing that medfly genomic DNA is present in all samples. Lane M (A,B) presents the molecular weight marker.

development in *Ceratitis*. Moreover, it is conceivable that absence of *tra* activity constitutes a signal that triggers the male fate. Thus, as in *Drosophila*, *Ceratitis tra* may act as a genetic switch between female (when functionally ON) and male (when functionally OFF) development. The male-specific short peptides encoded by the alternatively spliced male-specific transcripts seem to be non-functional, at least at early embryonic stages, because the RNAi has no evident effects on the development of XY males. We cannot determine, however, whether they play a function at later stages, when the RNAi starts to lose its efficiency.

Adult XX males developed from RNAi-injected embryos are fertile

To investigate the fertility of the RNAi-treated adults, 27 males obtained from embryos injected with 15 μM dsRNA solution were individually crossed with wild-type females. We predicted that if XX males are fertile than they should give a female-only progeny when crossed with wild-type virgin females. Indeed out of 27, seven crosses gave a unisexual female-only progeny. The karyotype of these seven males was then analyzed by PCR, as previously described, confirming that they were XX fertile males. As expected, PCR karyotypic analyses of those males giving a bisexual progeny revealed that they were XY males (data not shown). Our data demonstrate that the Y-chromosome does not carry genes necessary for male fertility, as previously suggested by others (Willhoeft and Franz, 1996).

Activity of *tra* is maintained by autoregulation

Next, we wanted to investigate the mechanisms which control the activity of *tra* in *Ceratitis*. In *Drosophila*, regulation of *tra* activity is achieved at the post-transcriptional level based on 3′ splice site selection (Boggs et al., 1997). When SXL protein is present, it prevents the use of a distal acceptor site, thereby promoting the use of the next downstream available 3′ splice site, and it shifts about 50% of the pre-mRNA molecules from a non-sex-specific splicing to a productive female-specific mRNA (Sosnowski et al., 1989). It has been shown that this regulation requires the direct binding of SXL to a poly $(U)_8$ stretch upstream of the regulated splice site (Kanaar et al., 1995). Several findings argue against a similar mechanism for conferring sex-specific splicing of *tra* in *Ceratitis* (Saccone et al., 1998). First, *Cctra* splicing is based on a combination of exon skipping and 5′ and 3′ splice site regulation, rather than on 3′ splice site selection. Second, CcSXL protein is present in both sexes of *Ceratitis*. However, upon close inspection of the *Cctra* sequence, we made an important discovery: within the two male-specific exons and the male-specifically retained intron, eight repeats were found by DNA sequence comparison that are structurally related to the TRA/TRA-2 binding sites (13 nucleotides long) in the *dsx* gene of *Drosophila* (Tian and

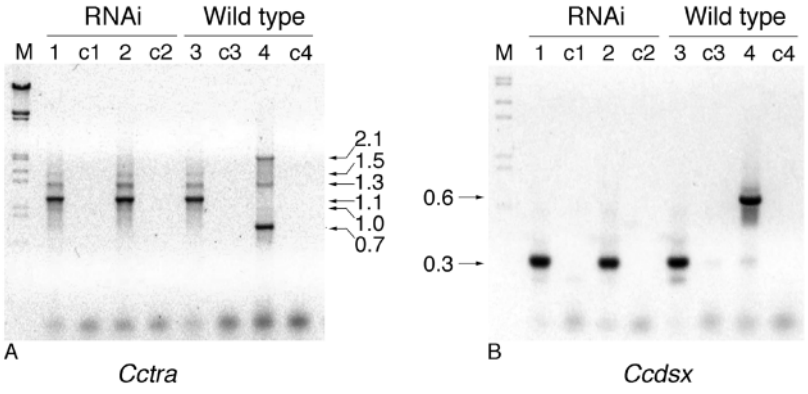

Fig. 6. Analysis of *Cctra* and *Ccdsx* splicing patterns in adult individuals. (A) RT-PCR with *Cctra* specific primers *Cctra*164+ and *Cctra*900– on XY and XX males from dsRNA-injected embryos (lanes 1 and 2) and on wild-type males (lane 3) and females (lane 4). Lanes c1-c4 show RT-PCR negative controls. The dsRNA injection in XX embryos induces a permanent shift in the splicing pattern of *Cctra* that turns from a female to a male mode. (B) RT-PCR with *Ccdsx*-specific primers (*Ccdsx*1400+, *Ccdsx*1130– and *Ccdsx*2000–) on the same cDNA samples used in A. The 0.6 kb fragment corresponds to a region of *Ccdsx* female-specific transcript, while the 0.3 kb fragment represents a region of *Ccdsx* male-specific transcript. A consequence of the *Cctra*-specific RNAi is a persistent change in *Ccdsx* regulation that turns from a female-specific to a male-specific splicing mode. A molecular weight marker is also shown in lane M (A,B).

Maniatis, 1993) (Fig. 2A,B). Similar repeats are also detected in the female-specific exon of the *dsx* homolog in *Ceratitis* (Saccone et al., 2000). Their high sequence similarity to *Drosophila* TRA/TRA-2 binding sites (Fig. 2B) and peculiar localization within the *Cctra* gene led us to believe that these sequences are involved in the sex-specific splicing regulation of *Cctra* itself. In *Drosophila*, *dsx* and *fru* genes these cis-elements act as, respectively, 3′ and 5′ splice enhancers by recruiting the TRA/TRA-2 complex to promote the use of the regulated splice site (Tian and Maniatis, 1993; Heinrichs et al., 1998). The presence of potential TRA/TRA-2-binding sites in and around the male-specific exons suggests that the female-specific CcTRA could inhibit their usage and led us to investigate whether an autoregulatory function of *Cctra* is involved in the process of sex-specific splicing.

If female-specific splicing of *tra* pre-mRNA indeed depends on *tra* activity, we reasoned that a transient depletion of *tra* activity should no longer be able to sustain the female mode of splicing. To test this supposition, we analysed sex-reversed XX males recovered from *Cctra* dsRNA injections. By RT-PCR analysis, only male-specific *tra* products were detected in adult tissues of injected XX and XY individuals, but no female-specific products (Fig. 6A). In addition, the same males contained predominantly male-specific splice variants of *dsx*, a probable downstream target of *tra* also in *Ceratitis* (Fig. 6B). We infer from these results that early application of RNAi transiently eliminates *Cctra* mRNAs and, thus, prevents continued production of TRA protein. Once *tra* pre-mRNA production is resumed at a later stage in development, the unproductive male mode of *tra* splicing is launched because of the absence of functional TRA. Likewise, absence of TRA causes its direct target *dsx* to be spliced in the male mode. These results are compatible with our postulate that *Cctra* sustains the productive mode of its splicing by an autoregulatory feedback loop and mediates female differentiation, at least in part, by the control of its target gene *dsx*. The initiation of the autoregulatory loop in XX embryos could be based on maternal *Cctra* mRNAs that have been detected in unfertilized eggs by RT-PCR experiments (data not shown). These mRNAs are spliced in the female mode and hence could provide a source of CcTRA activity that allows female-specific splicing of zygotic *Cctra* pre-mRNA.

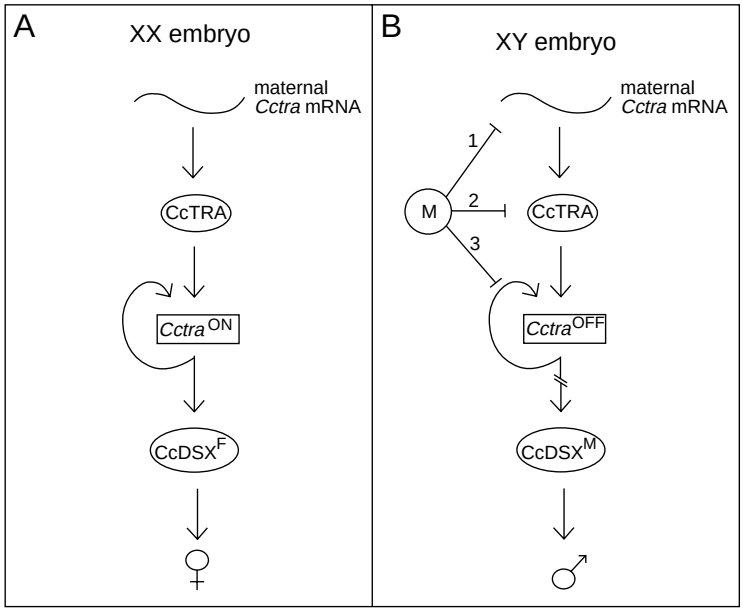

Fig. 7. Model for sex determination in *Ceratitis capitata.* (A) In XX embryos, a maternal *Cctra* mRNA provides full-length CcTRA protein that initiates a positive feedback regulation. This protein drives a female-specific splicing of the zygotically transcribed *Cctra* pre-mRNA so that new CcTRA protein can be produced. The newly synthesized protein controls the maintenance of *Cctra* autoregulation and the female-specific splicing of *Ccdsx* pre-mRNA. Therefore a $CcDSX^F$ protein is produced that induces, at least in part, female development. (B) In XY embryos, *Cctra* autoregulation is impaired by the male determining *M* factor. The *M* factor could prevent the translation of the maternal *Cctra* transcript (1) or inhibit the function of the protein that is produced by this mRNA (2). It is also conceivable that the *M* could interact with the spliceosome or repress *Cctra* transcription initiation in the zygote (3). In any case, the result is always that a full-length CcTRA protein is not produced in XY embryos and, thus, the autoregulatory loop can not initiate. In absence of CcTRA protein, *Ccdsx* is expressed by default to produce the $CcDSX^M$ isoform, which induces, in turn, male development.

DISCUSSION

We have isolated a gene, *Cctra*, which is an ortholog of *Drosophila tra* and acts as key regulator in sex determination of the medfly *Ceratitis capitata*. *Cctra* is regulated, as in *Drosophila*, by sex-specific splicing and encodes a protein showing, as expected, low sequence conservation, when compared with TRA proteins of *Drosophila* species (O'Neil, and Belote, 1992). We present evidence that female development depends on an active *Cctra* that, in XX individuals, seems to promote the productive mode of processing its own mRNA, thus initiating an autoregulatory mechanism to continuously produce a full-length protein. Interference at this level, for example, by injection of *Cctra* dsRNA, leads to a breakdown of the regulatory loop and to the production of male-specific mRNAs encoding truncated peptides. Thus, *Cctra* can be regarded as (1) an early binary switch in the sex-determining pathway of *Ceratitis*: when ON, female development ensues, when OFF, male development follows; (2) a key gene controlling an epigenetic cell 'memory' system of *Ceratitis* sex determination with evident analogies with the *Drosophila Sxl* gene.

A comparison between *Ceratitis tra* gene and its homolog in *Drosophila*: parallels and differences

Our results show that *Ceratitis* and *Drosophila* sex-determining cascades share a conserved *tra>dsx* genetic module to control sex determination and sexual differentiation as well as that *tra* sex-specific splicing regulation differs in the two species. In *Drosophila*, TRA protein, together with TRA-2, binds to the TRA/TRA-2 recognition sequences on the *Drosophila dsx* pre-mRNA and promotes the use of a nearby

female-specific acceptor site. We show that *Cctra* is needed to impose the female-specific splicing of *Ccdsx*, most probably by a similar mechanism as in *Drosophila*, invoking the existence of a *Cctra2* homolog (Saccone et al., 2000; Saccone and Polito, 2002). This hypothesis is also supported by the finding of TRA/TRA-2 recognition sequences located in close vicinity to the female-specific acceptor site in *Ccdsx* pre-mRNA (Saccone et al., 2000).

In *Drosophila*, *tra* female-specific splicing is promoted by SXL, which blocks the use of the non-sex-specific splice site present in the *tra* pre-mRNA. In *Ceratitis*, the presence of multiple TRA/TRA-2-binding elements within the *Cctra* male-specific exonic sequences strongly suggests that CcTRA and a hypothetical CcTRA-2 proteins could bind to them mediating a direct autoregulation. The unusually strong phenotypic effects of the RNAi against this gene also support this model of *Cctra* regulation. The localization of the putative regulatory elements within the *Cctra* gene indicates a repression mode by which CcTRA in females prevents the recognition of male-specific splice sites. The mechanism by which *Cctra* seems to promote the female mode of processing of its own pre-mRNA by TRA/TRA-2-binding elements appears to be different also from the female-specific splicing of *dsx*. Rather than activating a splice site nearby the regulated exon, as in the case of *dsx*, inclusion of male-specific *Cctra* sequences is suppressed when CcTRA is present. Although this would be a novelty with the respect to known *Drosophila* TRA/TRA-2 activities, it has been previously shown that the 'behavior' of these cis elements is context dependent and that changing the location of splicing enhancers can transform them into negative regulatory elements (Kanopka et al., 1996; Lopez, 1998).

A model for sex determination in *Ceratitis capitata*

In *Drosophila*, the presence of the Y chromosome is necessary for male fertility but not for male development (Hardy et al., 1981). By contrast, RNAi-treated *Ceratitis* embryos with a female XX karyotype can develop into fertile males, which indicates that transient repression of *Cctra* by RNAi is sufficient to implement fully normal male development. The cases of complete sexual transformation of genetic *Ceratitis* females (XX) into fertile males by RNAi demonstrate that the Y chromosome, except for the dominant male determiner *M*, does not supply any other contribution to both somatic and germline male development, as suggested by previous Y-chromosome deletion analysis (Willhoeft and Franz, 1996). Other dipteran species, such as *Musca domestica* (Hilfiker-Kleiner et al., 1994) and *Chrysomya rufifacies* (Ullerich, 1984) show a female and male germline sex determination that is completely dependent on the sexual fate of the soma. However, in *Drosophila*, the XX and XY germ cells seem to respond differently to sex determining somatic cues (Waterbury et al., 2000; Steinmann-Zwicky et al., 1989). Indeed the XY germ cells have also an autonomous stage-specific sex determination mechanism that probably integrates the somatic signal (Janzer and Steinmann-Zwicky, 2001). In *Ceratitis*, *Cctra* could be required in XX somatic cells to let them induce the XX germ cells to differentiate as oogenic cells. Alternatively, *Cctra* could be required in XX germ cells to 'feminize' them. This case would be a novelty with the respect of the known *Drosophila transformer* gene functions.

As zygotes that carry a Y chromosome do not activate *Cctra* female-specific splicing and autoregulation, we propose that the Y-linked male-determining *M* factor prevents this activation (Fig. 7). It is conceivable that *Cctra* is a direct target of the *M* factor. Presence of this *M* factor in the zygote may prevent the production of CcTRA protein. The *Cctra* positive feedback loop is a probable target for regulation, because of its sensitivity (already shown by RNAi). An important question to be addressed is how autoregulation of *Cctra* is initiated in XX embryos of *C. capitata* and how this is prevented in XY embryos. A possible explanation is suggested by the *Cctra* female-specific mRNAs encoding the full-length protein, which have been detected in unfertilized eggs. Depositing these *Cctra* transcripts in eggs may provide a source of activity that can be used later for 'female-specific' processing when *Cctra* is zygotically transcribed (Fig. 7). Once zygotically activated in XX embryos, *Cctra* promotes its own female-specific splicing maintaining the female sex determination and the female-specific splicing of the downstream *Ccdsx* gene. Taken together, these events induce the female differentiation (Fig. 7A). In our model for sex determination of medfly, the *M* factor is directly involved in the *Cctra* sex-specific regulation (Fig. 7B). Thus, in the presence of *M Cctra*, autoregulation is blocked and the gene produces male-specific transcripts encoding short and possibly non-functional CcTRA peptides. The absence of CcTRA leads *Ccdsx* to produce male-specific transcripts by default, promoting male differentiation (Fig. 7B). The control of the *M* factor upon *Cctra* expression could be exerted at different levels. The male determiner *M* could, for example, act at the pre-translational level blocking the production of CcTRA protein from the maternal transcripts. *M* could act at the post-translational level antagonising the formation of protein complexes necessary for the female splicing mode. Or *M* could act as a transient transcriptional repressor of *Cctra* to reduce the amount of active CcTRA below a threshold needed to maintain the feedback loop. The proposed autoregulatory model of *Cctra* may also explain the remarkable efficiency of sex reversal by *Cctra* RNAi: a transient silencing of *Cctra* by injecting dsRNA is sufficient to let the loop collapse. Furthermore, the sensitivity of this positive autoregulation could be an evolutionary widely conserved pre-requisite to permit a 'faster' recruitment/replacement of different upstream regulators and to easily evolve different sex determining primary signals, as observed in dipteran species.

Sex can even be determined by a maternal effect in dipteran species such as *Sciara coprophila* (Crouse, 1960) and *Chrysomya rufifacies* (Ullerich, 1984). Our hypothesis of a *Cctra* maternal contribution to the activation of the zygotic *Cctra* gene has similarities to the model of sex determination proposed for *Musca domestica* (Dübendorfer and Hediger, 1998). In the common housefly, the maternal product of the key switch gene *F* is needed to activate the zygotic function of *F* in females. Musca male development results whenever *F* cannot become active in the zygote. This happens when the male-determining *M* is present in the zygotic genome, or when maternal *F* is not functional because of either the presence of *M* or the mutational loss of function of *F* (F^{man}) in the germline (Dübendorfer et al., 2002). More interestingly, embryonic RNAi against the *Musca tra-2* homolog caused sex reversion of *Musca* XX adults into

intersexes and fertile males, although this gene is not sex-specifically expressed (Dübendorfer et al., 2002). These recent data in *Musca* and our results in *Ceratitis* support the idea that *F* of *Musca* functionally corresponds to the *Ceratitis tra* gene, that seems to autoregulate and maternally contribute to its own activation, rather than to the *Drosophila tra* gene.

Evolution of sex determining cascades

Our data show that a basic structure of sex determination is conserved in the two dipteran species, namely the flow of 'instructions' from *tra* to *dsx*. This confirms the model of 'bottom-up' evolution (Wilkins, 1995), suggesting that during evolution developmental cascades are built from bottom up and that the genes at the bottom are widely conserved, while further upstream new regulatory elements may be recruited. Our results show that *Ceratitis* and *Drosophila* sex-determining cascade differ at the level of *transformer* as well as upstream of it. Indeed the gene has conserved its function during evolution, but it has female-specific positive autoregulation in *Ceratitis*, while in *Drosophila* it needs *Sxl* as upstream regulator to express its female determining function. More likely the sex-determining function of *Sxl* was co-opted after *Drosophila* and *Ceratitis* had separated more than 100 Myr ago (Saccone et al., 1998; Beverley and Wilson, 1984). Furthermore, it is conceivable that the autoregulatory mechanism of *Sxl* could have been selected to overcome a mutation impairing the *tra* autoregulation. Hence, in both species the female pathway is maintained by a single gene positive-feedback mechanism through sex-specific alternative splicing. Single gene autoregulation by alternative splicing seems not to be infrequent in nature, especially in those genes encoding splicing regulators. Indeed, other genes encoding RNA-binding proteins are thought to autoregulate their expression by controlling the processing of their own pre-mRNAs (Mattox and Baker, 1991; Boelens et al., 1993; Chabot et al., 1997; Jumaa and Nielsen, 1997). Such a single-gene network with positive regulation is capable of bistability (Hasty et al., 2001). This suggests that the emergence of analogous positive autoregulation in different genes such as *Drosophila Sxl* and *Ceratitis tra* genes would have been selected, during evolution, to guarantee a similar ON/OFF-female/male bistable cell state.

As *Ceratitis capitata* is a major agricultural pest in many areas of the world, the isolation of a key sex-determining gene such as *Cctra* will substantially aid the development of new strategies to optimize the efficacy of currently used male sterile techniques for pest control (Saccone et al., 2000; Robinson et al., 1999). We expect that *tra* is also a key sex-determining gene in many other insect species. Hence, the isolation of corresponding *tra* genes will open new means to control not only agricultural pests but also medically relevant vectors of diseases such as *Glossina palpalis* and *Anopheles gambiae*.

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