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A *cis*-regulatory Sequence Within the *yellow* Locus of *Drosophila melanogaster* Required for Normal Male Mating Success

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ABSTRACT

Drosophila melanogaster males perform a courtship ritual consisting of a series of dependent fixed-action patterns. The *yellow* (*y*) gene is required for normal male courtship behavior and subsequent mating success. To better characterize the requirement for *y* in the manifestation of innate male sexual behavior, we measured the male mating success (MMS) of 12 hypomorphic *y* mutants and matched-outbred-background controls using a y^+ rescue element on a freely segregating minichromosome. We found that 4 hypomorphs significantly reduced MMS to varying degrees. Reduced MMS was largely independent of adult pigmentation patterns. These mutations defined a 300-bp regulatory region upstream of the transcription start, the mating-success regulatory sequence (MRS), whose function is required for normal MMS. Visualization of gene action via GFP and a Yellow antibody suggests that the MRS directs *y* transcription in a small number of cells in the third instar CNS, the developmental stage previously implicated in the role of *y* with regard to male courtship behavior. The presence of Yellow protein in these cells positively correlates with MMS in a subset of mutants. The MRS contains a regulatory sequence controlling larval pigmentation and a 35-bp sequence that is highly conserved within the genus Drosophila and is predicted to bind known transcription factors.

REPRODUCTION usually involves heterosexual courtship behavior that is central to the divergence and diversity of animal species and is of obvious adaptive significance. In many species the basic program of courtship behavior is innate. These inborn, instinctual behaviors are likely to be a result of gene action during development that establishes the potential for behavior and motivates the animal to perform the behavior, given the appropriate external stimulation (Baker *et al.* 2001). It can be hypothesized that there are genes required for building into the central nervous system (CNS) the ability to process information specific to a behavior and the specific neural output pathway for signaling the performance of that behavior (Baker *et al.* 2001).

Individual fruit flies of *Drosophila melanogaster* routinely perform innate behaviors. One such behavior that is well-characterized is the courtship ritual performed by males for females prior to heterosexual copulation. The male ritual is in essence a series of dependent fixed-action patterns: tapping, following, orienting, horizontal wing extending, wing vibrating ("singing"), genital licking, and attempted copulation (Bastock and Manning 1955; Bastock 1967; Hall *et al.* 1982; Hall 1994a; Yamamoto *et al.* 1997; Greenspan and Ferveur 2000). Typically, these behaviors must be performed in the correct sequence with some repetition over a modest period of time (2–10 min) to significantly stimulate a female to be receptive to copulation. Such a stereotypic courtship sequence is common to many animals (*e.g.*, Darwin 1874; Bastock 1956; Morris 1970; Walters 1988; Bradbury and Vehrencamp 1998; Carew 2000; Judson 2002).

Many genes whose functions are required for normal *D. melanogaster* reproductive behavior have been described (Sturtevant 1915; Hall *et al.* 1982; Hall 1994a; Greenspan 1997; Yamamoto *et al.* 1997, 1998; Goodwin 1999; Yamamoto and Nakano 1998, 1999; Gaines *et al.* 2000; Orgad *et al.* 2000; Romanova *et al.* 2000; Greenspan and Ferveur 2000; Sokolowski 2001; Boll and Noll 2002; Dauwalder *et al.* 2002; Subocheva *et al.* 2003; Grosjean *et al.* 2004). However, there is a paucity of information about how these genes function in the development of innate courtship behavior. At present, studies of the *fruitless* (*fru*) gene provide most of our information about how genes specify *D. melanogaster* male courtship behavior. Wild-type *fru* function is required for most aspects of the male courtship ritual, and therefore of male mating success (MMS)

This article is dedicated to Melvin M. Green for the question he asked that led to this work.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under the accession nos. DQ099804–DQ099893.

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(Ryner et al. 1996; Villella et al. 1997). The *fru* gene encodes a predicted BTB/POZ family zinc-finger transcription factor, which has been shown to be a member of the *D. melanogaster* somatic sex determination hierarchy (Ito et al. 1996; Ryner et al. 1996; Heinrichs et al. 1998). The use of an antibody specific to male-limited FRU proteins (FRU^M) (Lee et al. 2000; Lee and Hall 2001) has demonstrated that the spatial and temporal distribution of FRU^M proteins in the CNS is consistent with the timing of the critical period for programming a male fate (Belote and Baker 1987; Arthur et al. 1998) and the regions of the CNS whose maleness is necessary for normal development of male courtship behaviors (Hall 1979; von Schlicher and Hall 1979). Most recently, it has been shown that sex-specific splicing of *fru* specifies the male courtship behavior fate (Demir and Dickson 2005; Manoli et al. 2005; Stockinger et al. 2005). The available evidence suggests that male-specific FRU transcription factors form the apex of a developmental genetic hierarchy responsible for many aspects of the male courtship ritual and MMS in *D. melanogaster* (reviewed by Baker et al. 2001; Drapeau 2001a; Greenspan and Ferveur 2000; Sokolowski 2001; Hall 2002).

The direct and indirect targets of FRU^M are largely unknown. We recently demonstrated that the *yellow* (*y*) gene is in a pathway downstream of *fru* in a small number of cells in the third instar CNS (Drapeau et al. 2003). Yellow protein accumulation in the CNS is disrupted in at least two *fru* mutants, and ectopic FRU^M is sufficient to increase levels of Yellow protein throughout the CNS, even in females (Drapeau et al. 2003). On the basis of this evidence we predict that *y* is either a direct target of FRU^M or a downstream gene in the pathway.

The *y* gene is an excellent candidate to study further in relation to the development of male sexual behavior. It has long been known that *y* is required for normal amounts of wing extension (WE) behavior during male courtship and consequent MMS (*e.g.*, Sturtevant 1915; Bastock 1956; Burnet et al. 1973). Male-limited WE is important because it is performed in *D. melanogaster* for the sole purpose of singing the courtship song, which stimulates females to be receptive to the male's mating advances (reviewed in Hall 1994a; Greenspan and Ferveur 2000). Males carrying null alleles of *y* have a reduction of ~50% normal WE levels, which is likely to contribute to the well-characterized decrease in *y* mutant MMS (Bastock 1956; Burnet et al. 1973; Drapeau et al. 2003). We found that y^+ expression at the third instar developmental stage was sufficient to rescue male WE and competitive MMS to normal levels (Drapeau et al. 2003), suggesting that *y* is crucial for laying the foundations of the elaborate courtship repertoire.

Understanding the relationship between a gene and a behavior requires analysis of behavioral phenotypes of numerous mutants, followed by characterization of the wild-type gene and its product(s) in the manifestation of behavior. Some of the most powerful evidence that the function of a specific gene is required for a behavior is the identification of different mutant alleles that have varying effects on the behavior (*e.g.*, *period*, Konopka and Benzer 1971; *fruitless*, Villella et al. 1997; *foraging*, de Belle et al. 1989). In this report, we characterize 3 null and 12 hypomorphic alleles of *yellow* and find a spectrum of allelic effects on MMS. Knowing the molecular basis of the hypomorphic lesions allowed us to define a 300-bp regulatory region of *y* whose function is necessary for normal MMS. We name this region the mating-success regulatory sequence (MRS). On the basis of additional genetic and biochemical experiments, we conclude that the MRS is important for the regulation of *y* expression in the developing CNS. A 35-bp region of the MRS is highly conserved within the genus Drosophila, and this region is predicted to bind at least one transcription factor involved in somatic sexual differentiation. These data suggest that *y* plays a critical role in the development and evolution of a complex adult behavior with relevance to organismal fitness.

MATERIALS AND METHODS

Fly culture conditions: *D. melanogaster* flies were raised in half-pint glass milk bottles on standard banana/corn syrup/yeast/agar medium at room temperature in constant light. Bottles for experiments were always cleared of parents before 1 week after egg laying, and usually within 3–4 days. Parents were sometimes fed a baker's yeast/distilled water paste while laying eggs, which became progeny for experiments. We note that Dow (1977c) has demonstrated that neither light regime (24:0 L:D constant light *vs.* a 12:12 L:D regime) nor temperature (15° *vs.* 20° *vs.* 25°) has a strong influence on the relatively poor mating success of y^1 *vs.* wild-type males, assayed with wild-type females.

Mutant *yellow* strains: Descriptions of the *y* mutants used in these experiments, including their full names, abbreviations, origin, molecular genetic phenotypes, and relevant references and sources are summarized in Table 1. The mutants include spontaneous mutants with lesions in the endogenous *y* locus and mutants constructed *in vitro* and then inserted into a *y* null background using standard germline transformation protocols (*e.g.*, Rubin and Spradling 1982). All the mutant stocks are highly inbred, but experiments were carried out in completely outbred genetic backgrounds; see below.

The hypomorphic *y* mutants that we measured for MMS were chosen because each in some manner eliminated the sequence and/or function of regulatory regions, or potential regulatory regions, of the *y* locus. For a given mutant, this was accomplished in one of four ways: insertion of foreign sequences, an inversion breakpoint within the *y* locus, deletion of *y* sequence, and "*gypsy* insulation" of regulatory elements. The last method requires a brief explanation. Transposable elements of the *gypsy* class in *D. melanogaster* (Lindsley and Zimm 1992; FlyBase 2003) contain binding sequences for the Suppressor-of-Hairy-wing [Su(Hw)] protein. Bound Su(Hw), in combination with other proteins, blocks the interactions of distal enhancers with target promoters and constitutes the *gypsy* insulator. Except for the spontaneous y^2 mutant, which contains a full-length *gypsy* element insertion (Geyer et al. 1986; Parkhurst and Corces 1986), and $y^{-300gin}$ (see below),

TABLE 1

Descriptions of the alleles used in this study

yellow allele	Full allele name	Molecular genetic basis	Endogenous or transgene	Pigmentation allele class	Source	Representative references
y^{1}	*yellow 1*	Point mutation (A-to-C transversion) in translation initiation codon	Endogenous	Null	Bloomington[a]	Geyer *et al.* (1990)
$Df(1)y\text{-}ac^{22}$	*Deficiency(1) yellow achaete 22*	Deletion covering entire *y* locus	Endogenous	Null	Bloomington	Biessmann and Mason (1988)
y^{59b}	*yellow 59b*	Deletion of 1.2 kb from −700 to +519 bp and insertion of 3.2 kb of *gypsy* element at deletion site	Endogenous	Null	Bloomington	Geyer *et al.* (1990)
y^{-893}	*yellow -893*	Insertion of insulating vector at −893 bp	Transgene	Hypomorph	Victor Corces	Geyer and Corces (1992)
y^{2}	*yellow 2*	Insertion of full-length *gypsy* element at −700 bp	Endogenous	Hypomorph	Bloomington	Campuzano *et al.* (1985); Chia *et al.* (1986); Parkhurst and Corces (1986)
y^{3P}	*Inversion(1) yellow 3 of Patterson*	Inversion with left inversion breakpoint at ∼−700 bp	Endogenous	Hypomorph	Bloomington	Campuzano *et al.* (1985); Biessman (1985)
$y^{-300gin}$	*yellow -300 gypsy in*	Gene replacement: insertion of insulating vector at −300 bp	Transgene	Hypomorph	Pamela Geyer	Parnell *et al.* (2003)
y^{bl}	*yellow bristle*	Three small aberrations 5′ of −700 bp	Endogenous	Hypomorph	Bloomington	Biessman (1985)
y^{-91}	*yellow -91*	Deletion from −2873 to −91 bp	Transgene	Hypomorph	Pamela Geyer	Geyer and Corces (1987)
y^{3d}	*yellow 3 dark*	Insertion of ∼7 kb between −300 and 0 + 1 bp	Endogenous	Hypomorph	Bloomington	Chia *et al.* (1986)
y^{2S}	*yellow 2 from Swedish*	Insertion of ∼4.5 kb between −300 and +1 bp	Endogenous	Hypomorph	Bloomington	Chia *et al.* (1986); Parkhurst and Corces (1986)
y^{a77}	*yellow a77*	Insertion of *P* element in reverse orientation at −4 bp	Endogenous	Hypomorph	Pamela Geyer	Morris *et al.* (1999)
$y^{intronless}$	*yellow intronless*	Deletion of entire *y* intron	Transgene	Hypomorph	Pavel Georgiev	Geyer and Corces (1987)
y^{+660R}	*yellow +660R*	Insertion of insulating vector at +600 bp	Transgene	Hypomorph	Victor Corces	Geyer and Corces (1992)
y^{+1310}	*yellow +1310*	Insertion of insulating vector at +1310 bp	Transgene	Hypomorph	Victor Corces	Geyer and Corces (1992)

[a] Bloomington (Indiana) Drosophila Stock Center.

all insulator mutants used were constructed by Geyer and Corces (1992). These authors constructed *P*-element vectors containing 12 Su(Hw)-binding sites inserted at different sites within a *y* transgene. These Su(Hw)-binding sites are sufficient to function as an insulator. Transgenic stocks were produced by germline transformation of a parental *y* deficiency line. The $y^{-300gin}$ allele contains the *gypsy* insulator positioned 300 bp upstream from the transcription start and was generated at the endogenous *y* locus using targeted gene replacement (Parnell *et al.* 2003).

Other Drosophila strains: To further examine the function of *y* regulatory sequences, we searched the Gal4 Enhancer Trap Database (GETDB, http://flymap.lab.nig.ac.jp/∼dclust/getdb.html) for a strain carrying a *P*{GawB} element (Brand and Perrimon 1993) inserted within *X* chromosomal region 1A5, where *y* is located. GETDB strain 6014 drives Gal4 expression in tissues that have *y*-dependent pigmentation (*e.g.*, larval mouth parts, adult dorsal abdominal stripe). This mutant, which we name y^{6014}, contains a *P*{GawB} element inserted at +23 bp relative to the start of transcription. This ∼11.3-kb *P*-element insertion causes a severe reduction in larval and adult pigmentation and the absence of anti-Yellow staining in the third instar CNS, suggesting that y^{6014} is a loss-of-function allele. We used the GAL4 feature within the element to "read out" the surrounding regulatory sequence that may be relevant to the development of behavior. In this experiment, y^{6014}

females were crossed with *w; UAS-nu.GFP* males, which carry a gene encoding nuclear-localized green fluorescent protein that is responsive to a Gal4 element driving *in trans*. We observed GFP staining in the third instar larval CNS of y^{6014}; *UAS-nu.GFP/*+ progeny.

Behavior genetic experimental design: Because of the sensitivity of behavioral phenotypes to environmental influence, the genetic background of the mutant and of the matched-background control males tested in our mating assays was well controlled. This was a consequence of our experimental design, which incorporated a one-generation crossing scheme. For assays of male sexual behavior, we generated outbred and nearly isogenic experimental and control males using a strain that contains a freely segregating *X* chromosome-originating duplication, *Dp(1;f)8-23* [*Dp(1;f)1187,* $P\{ry^{+t7.2} = PZ\}0801$ $P\{ry^{+t7.2} = PZ\}8\text{-}23$ y^+; $In(1)sc^8$, $Df(1)sc^8$ (FlyBase 2003)]. *Dp(1;f)8-23* is a segregating hemizygous 1.3-Mb "minichromosome" (one of the smallest known to segregate in eukaryotes) that contains ~1000 kb of *X* chromosome centromeric heterochromatin and 300 kb of *X* chromosome distal DNA (*e.g.*, Tower *et al.* 1993). This distal euchromatic region includes the endogenous *y* locus and its naturally occurring surrounding regulatory DNA, the *achaete* (*ac*) locus, and the *scute* (*sc*) locus up to the breakpoint named sc^8 (Lindsley and Zimm 1992; FlyBase 2003). Distal to *y* are 10 annotated genes, including 3 genes that have been studied previously, *Or1a*, *cin*, and *ewg* (information from the FlyBase Genome Browser at http://www.fruitfly.org/cgi-bin/annot/gbrowse, analyzing release 3 of the *D. melanogaster* genome). Finally, *Dp(1;f)8-23* has two $rosy^+$ (ry^+) *P*-element insertions (Figure 1A; Lindsley and Zimm 1992; Tower *et al.* 1993; FlyBase 2003; Kumar Hari, personal communication).

To generate mutant and control flies, y^- (representing any *y* mutant in Table 1), virgin females were crossed to y^1 *sc* w^a; ry^{506}; *Dp(1;f)8-23*, y^+ males. Flies segregating from this cross are y^- ; ry^{506}/+ and y^-; ry^{506}/+; *Dp(1;f)8-23*, y^+ (Figure 1B). The flies lacking the minichromosome have the y^- mutant phenotype, and the flies carrying the minichromosome constitute the y^+ control phenotype in an otherwise identical, outbred background. We will occasionally abbreviate these experimental and control genotypes as y^- and y^+, respectively. A minority of mutant *y* alleles studied (y^{-893}, y^{+660R}, y^{+1310}, and $y^{intronless}$) carried transgenes that were transformed into a *Df(1)y* background and were not located at the endogenous *y* locus. These lines were homozygous for the dominantly acting inserts, and so progeny were heterozygous for the inserts and their genotypes were formally *Df(1)y/Y*; $P[y^-]$/+; ry^{506}/+ (*i.e.*, y^-) and *Df(1)y/Y;* $P[y^-]$/+; ry^{506}/+; *Dp(1;f)8-23*, y^+ (*i.e.*, y^+).

Germline segregation of the y^+ minichromosome in our crossing scheme was investigated. We found that the y^+, *Dp(1;f)8-23* minichromosome was transmitted to offspring ~75% of the time, with no significant sex bias. In crosses of y^+, *Dp(1;f)8-23* males to y^1 females, counts of 922 male and 955 female offspring showed that there were 235 (25.5%) y^1 males and 687 (74.5%) y^+ males and 243 (25.4%) y^1 females and 712 (74.6%) y^+ females.

With this protocol, we achieved excellent control over what can collectively be called "background effects," which have the potential to be misleading in behavioral genetics studies (*e.g.*, de Belle and Heisenberg 1996; Crabbe *et al.* 1999). Use of the minichromosome crossing scheme allowed us to examine the behavior of outbred "experimental" and "control" male progeny, which were full sibs, developed under the same environmental and developmental conditions, with identical genetic backgrounds except for the presence or absence of the y^+ minichromosome. Outbred flies with well-controlled background genetic and environmental effects provided excellent material for testing hypotheses associated with the relationship between specific *y* alleles and male mating success.

Noncompetitive mating behavior observations: All noncompetitive mating experiments were carried out in a set of behavior observation chambers collectively called the "Copulatron," the construction of which is described in detail elsewhere (Drapeau and Long 2000). Briefly, the Copulatron is a set of 49 1-inch-diameter Plexiglas circular mating chambers arranged in a 7 × 7 configuration. Each chamber is ~2 cm deep and can be divided into two 1-cm-deep sections. The floor of the chamber is white porous polyethylene, and the ceiling is glass. Fly media was pipetted into side chambers connected to each of the mating chambers (see Drapeau and Long 2000) so that flies would not starve while recovering from anesthesia and general handling for ~24 hr.

Male noncompetitive mating success was assayed in the Copulatron by pairing single virgin males with single virgin females. We always assayed experimental and control males simultaneously in the same Copulatron unit. All flies were collected as 6- to 8-hr-old virgins and stored in groups of four to nine in plastic 8-dram food vials for ~3–4 days. Flies were then briefly (~30 sec) anesthetized with CO_2 and mouth pipetted, or gently picked up with a paintbrush and placed, into the Copulatron's mating chambers. A single male and female were put in each chamber. Fresh standard fly media was always present in the chambers. Males and females were separated by a lightly lubricated (Pam cooking oil wiped on with paper towels) piece of overdeveloped (*i.e.*, darkened) film, which was removed at the start of each assay, simultaneously introducing males and females in all 49 chambers. Flies were allowed to recover from CO_2 anesthesia for ~24 hr before the start of each experiment. The flies were exposed to constant light at room temperature.

Because it is inherently difficult to understand the behavior of mutant flies with very poor courtship behavior by using a measurement such as time to copulation, male noncompetitive mating success was defined as follows. On a given day, during which experimental and control males were without exception assayed simultaneously, the rank order of copulations was determined (*i.e.*, the first male to copulate was given the rank 1, the second was assigned rank 2, etc.). Occasionally, two males who effectively began copulation simultaneously were given a rank that was the average of the next two ranks. Within a given assay on a given day, males who mated before the median rank were defined as successful ("S"). Males who mated at or after the median rank were failures ("F"). Numbers of successes and failures for a genotype were denoted as #S and #F. This ranking scheme circumvents the problem of losing data from *y* males who did not copulate within a reasonable amount of time, which we defined as roughly 1 hr. Normal, healthy, virgin males and females of this species typically achieve copulation within 2–5 min of introduction to each other. We required copulations to last for at least 2 min to be recorded as valid; *D. melanogaster* copulation duration is typically 10–20 min. (Occasionally, a male will attempt to copulate and will appear to succeed, but the female will not allow the male to fully engage. In this situation the male will climb off the female and nearly always resume courtship of her. This series of events typically takes <2 min.)

We performed independent measurements (trials) of male mating success for each *y* mutant. Each trial took place on a separate day. To determine whether there was significant variation in mating success estimates across trials within mutants, we carried out chi-square tests on *y* mutant male #S and #F in an $n \times 2$ format, where n is the number of trials for that mutant. For each of the 15 mutants, we found $P > 0.01$, with 14 of 15 *P*-values $\gg 0.05$ [The $Df(1)y\text{-}ac^{22}$ flies had $P = 0.02$, but in all trials such males had extremely reduced mating success; see

results.] Because data were quantitatively consistent across trials for all mutants, mating success data for each *y* mutant and its matched-background control were combined across all trials, and statistical tests of homogeneity were performed on the overall data to test whether mutants had a different distribution of mating success and failure with wild-type females than did controls. For each mutant, three tests were performed on #S and #F for experimental and control males, with data organized into 2 × 2 contingency tables: a Fisher's exact test, a Pearson's chi-square test, and a Mantel-Heanszel chi-square test.

Competitive mating behavior observations: All competitive mating experiments were performed in 8-dram plastic vials with cotton plugs. The vials contained standard banana/corn syrup/yeast/agar fly media on the bottom. Flies were isolated as 6- to 8-hr-old virgins, and the two sexes were separated and stored in groups of four to seven in food vials at room temperature. At 3–4 days of adult age, control and experimental males were placed in food vials, one of each genotype, using CO_2 anesthesia. We found no evidence for environmental "bottle" effects in pilot experiments in which competing males were placed in a vial together only if they were collected from the same bottle in which they developed (data not shown). Wild-type females were placed singly in food vials. At 4–5 days of adult age, and 24 hr after CO_2 anesthesia (to allow adequate recovery) males were transferred into female vials without anesthesia by "tapping." This is a simple, commonly used assay to ascertain relative mating success of two genotypes (*e.g.*, Sturtevant 1915; Threlkeld *et al.* 1974; Markow 1981; Markow and Manning 1982). For each individual female, the male genotype that achieved copulation first was recorded. Male genotypes were differentiated by pigmentation of the body cuticle or eyes. The fraction of trials in which a given genotype achieves the first copulation can be used as a measure of female preference for one genotype relative to the other. Competitive mating experiment data were analyzed using binomial tests to determine whether the distribution of "first copulations" among the two male genotypes deviated from a 50:50 null hypothesis.

Molecular genetic analysis of *y* insertion mutations: Three *y* mutants found to disrupt male mating success, y^{3d}, y^{a77}, and y^{2S}, were partially characterized at the molecular genetic level before this study (Table 1). These three alleles were known to have inserts of unknown origin within the ∼700 bp 5′ of the endogenous *y* start of transcription: y^{3d} was defined by an ∼7.0-kb insert, y^{a77} was characterized by an insertion of unknown size, and the y^{2S} mutant was defined by an ∼4.5-kb insert (reviewed in Morris *et al.* 1999). To confirm the presence of the inserts in this general region, we performed PCR with forward primer 5′-AATATTTGACCCTCAGTGAATTGTG-3′ and reverse primer 5′-TATATGTTCAGCTATAGGGGGTTC TT-3′. In a wild-type fly the resulting amplicon is 888 bp, covering the entire ∼700-bp region of interest, plus flanking sequence on each side. We carried out 10-μl PCR reactions with the following protocol: 4 min at 94°, 35 × [45 sec at 94°, 45 sec at 56°, 4 min at 72°], 1 min 72°, followed by a 4° hold. Using these primers we were able to confirm the presence of the inserted DNA in the y^{3d}, y^{a77}, and y^{2S} mutants. Then we used forward primer 5′-TTCTGGATTTTTGTCTGCATGT-3′ and reverse primers 5′-ATGATTTTTGGCCTTCATCG-3′ and 5′-GCCTTCGGCTGTGTGATATT-3′ to subdivide the −700- to 0-bp region into two sections from ∼−700 to −300 bp and from −300 to +1 bp to further map the locations of the three insertions.

We partially sequenced the male mating success regulatory region of y^{a77} to further characterize its insert. The following primers were used both for PCR amplification of fragments and for sequencing: forward primer 5′-GCTCAAAATCACC TGCCAAT-3′ and reverse primer 5′-AGGTGATCAGGGTCA CAAGG-3′. The resulting sequence was deposited in GenBank with accession no. DQ099804.

Larval dissection and immunocytochemistry: Third instar larvae were dissected in 1× phosphate-buffered saline (PBS) using fine forceps, incubated for 15 min at room temperature (RT) in 1× collagenase (Sigma, St. Louis) in PBS, incubated 30 min at RT in 4% paraformaldehyde solution, washed two times for 10 min at RT in PBS/1% Triton, blocked in PBS/0.5% Triton/10% horse serum for 45 min at RT, incubated overnight at 4° with primary antibody, washed three times for 10 min at RT in PBS/0.5% Triton, incubated with secondary antibody for 60 min at RT, washed three times as above, and placed in 50% glycerol solution. The CNS of each larva was then dissected using needles on a glass slide. Polyclonal rabbit anti-Yellow antibody has been previously described (Radovic *et al.* 2002; Wittkopp *et al.* 2002a; Drapeau *et al.* 2003) and was used at a dilution of 1:150. For secondary antibody, a dilution of 1:1000 anti-rabbit 594 (red, Molecular Probes, Eugene, OR) was used. Fluorescent images were taken using a Nikon Microphot-SA fluorescent microscope with a Nikon super-high pressure mercury lamp. Confocal images were taken using a Leica TCS SP2 confocal microscope. Images were processed using Adobe Photoshop on a Macintosh.

Sequencing natural *y* alleles of *D. melanogaster*: To identify naturally occurring genetic variation that might contribute to the evolution of male sexual behavior, we sequenced the MRS (see results) and its flanking sequence in 85 strains of *D. melanogaster.* These strains were: Oregon-R and Canton-S (both from the Bloomington Drosophila Stock Center), an isofemale line derived from the outbred Ives strain (from Michael Rose, University of California, Irvine, CA), 71 iso-*X* chromosome strains derived from a recent collection in Napa Valley, California (A. D. Long, unpublished data), 7 iso-*X* chromosome strains derived from a collection from Zimbabwe (from Chuck Langley, University of California, Davis, CA), and four iso-*X* chromosome strains derived from a collection from North Carolina (also from Chuck Langley). We used forward primer 5′-GCTCAAAATCACCTGCCAAT-3′ and reverse primer 5′-AGGTGATCAGGGTCACAAGG-3′ to sequence part of the *y* locus from these strains. These sequences were deposited in GenBank with accession nos. DQ099805–DQ099807 and DQ099812–DQ099893.

Evolutionary analysis of *y* alleles from diverse Drosophila species: To study patterns of change and constraint within the MRS over evolutionary time, we sequenced the appropriate *y* region in a number of Drosophila species. As with the *D. melanogaster* strains, we used forward primer 5′-GCTCAAAAT-CACCTGCCAAT-3′ and reverse primer 5′-AGGTGATCAGG GTCACAAGG-3′ to sequence strains of each of its three sibling species, *D. simulans*, *D. sechellia*, and *D. mauritiana*. The strains that we sequenced were: *D. simulans* sim6 and s132 (from Dave Begun, University of California, Davis, CA), *D. sechellia* S-9 (from the Arizona Drosophila Species Center, Tucson, AZ), and *D. mauritiana* iso-197 (Arizona Drosophila Species Center). These sequences were deposited in GenBank with accession nos. DQ099808–DQ099811.

In addition, we used an evolutionarily diverse set of previously sequenced *y* alleles in our analysis. Species, strain (if available), NCBI sequence identification numbers, and relevant references are as follows: *D. simulans*, Sim-5 G20, AB026336, Takano-Shimizu (1999); *D. orena*, AB026342, Takano-Shimizu (1999); *D. teissieri*, AB026339, Takano-Shimizu (1999); *D. erecta*, AB017574, Takano-Shimizu (1999); *D. yakuba*, AB017573, Takano-Shimizu (1999); *D. subobscura*, from Figure 2 of Munté *et al.* (1997); *D. pseudoobscura* from the Baylor School of Medicine *D. pseudoobscura* Genome Project; *D. virilis*, AY128944, Wittkopp *et al.* (2002b); and *D. novamexicana*, 15010-1031.0, AY165561, Wittkopp *et al.* (2003b).

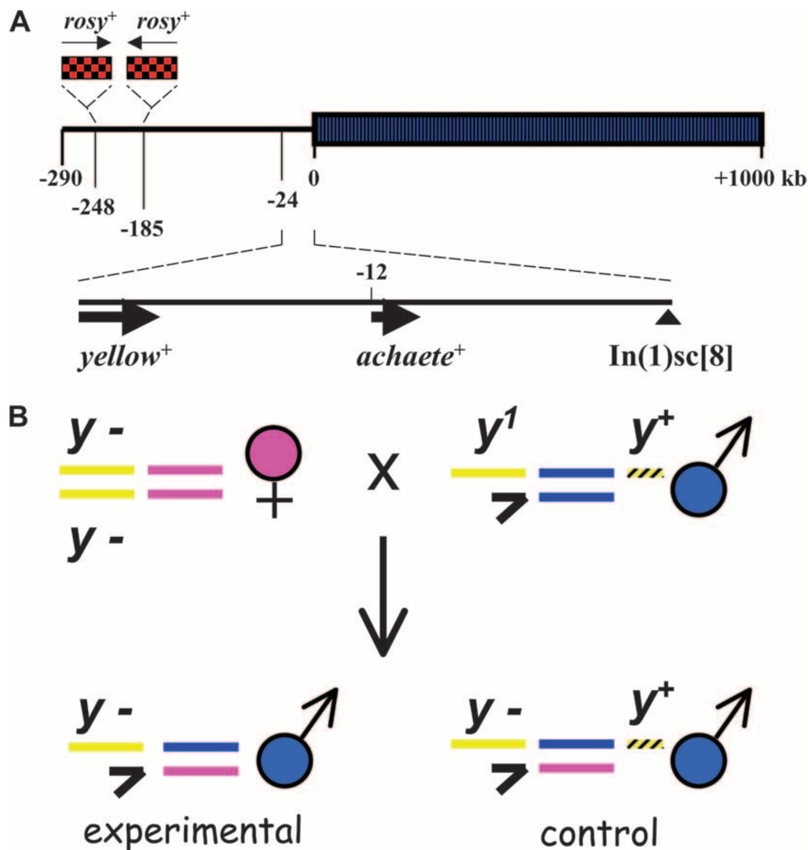

Figure 1.—(A) Using the *yellow*$^{+}$ minichromosome *Dp(1;f)8-23* to rescue *yellow* mutants. This minichromosome was created by a duplication of part of the tip of the *X* chromosome where the *y* locus resides. Minichromosome *8-23* and related minichromosomes are the smallest chromosomes known to be transmitted in eukaryotes. *8-23* carries the entire endogenous *y* locus with all of its normal flanking DNA, which includes previously characterized regulatory regions that act as enhancers. Approximately 12 wild-type genes are carried on the minichromosome, as well as two *rosy*$^{+}$ *P*-element insertions. The *P* elements and the *yellow* and *achaete* loci are shown. The striped rectangle is ∼1000 kb of centromeric heterochromatin. (B) The crossing scheme used to generate experimental and control males. This one-generation crossing scheme takes advantage of the y^{+} locus located in minichromosome *8-23*, and it was used for all of the experiments reported in this article. To generate completely outbred and isogenomic males, we crossed females (with one genetic background, in pink) with the *y* mutant allele of interest (y^{-}) to males (with another genetic background, in blue) that were y^{1}; ry^{506}; *8-23*, ry^{+} y^{+}. (The y^{+} minichromosome is shown by a black/yellow striped line.). All sons receive the same *X* chromosome carrying a *y* mutant from their mother, and ∼75% of the males receive the y^{+} minichromosome from their father (see materials and methods). In this manner, y^{-} ("experimental") and y^{-} ; y^{+} ("control") males are generated for analysis. Experimental and control males have the same set of parents and develop in the same set of bottles, and the behavior of both groups is measured simultaneously in the Copulatron.

Bioinformatic predictions of transcription factor binding sites: Using the 300-bp MRS (see results) as our query, we searched the TRANSFAC database of transcription factor binding sites (Heinemeyer *et al.* 1998) using the TFSEARCH tool (http://www.cbrc.jp/research/db/TFSEARCH.html) (Akiyama 1998). We also utilized the FLY GRID database of genetic and physical interactions (http://biodata.mshri.on.ca/fly_grid/servlet/SearchPage). Finally, empirically determined transcription factor binding sites from the primary literature were used in our analysis.

RESULTS

Null *y* mutants have severely reduced male mating success: The *D. melanogaster* y^{1} allele has been previously characterized as an A-to-C transversion in the first translated codon of the *yellow* gene, which results in an altered mRNA product (Geyer *et al.* 1990). Since Sturtevant's pioneering genetic study of Drosophila sexual behavior (Sturtevant 1915) researchers have recurrently shown that y^{1} males have severely lowered MMS (Spett 1931; Diederich 1941; Merrell 1949; Mayr 1950; Meyers 1953; Bastock 1956; Barker 1962; Schroeck 1971; Burnet *et al.* 1973; Threlkeld *et al.* 1974; Dow 1975, 1977a, b, c, d; Tracey and Espinet 1976, 1977; Wilson *et al.* 1976; Oakeshott and Hayman 1979; Bradman *et al.* 1981; Mizuguchi and de Almeida 1983; Heisler 1984; Hamerlynck 1994; Drapeau *et al.* 2003). The y^{1} allele is a null with regard to its pigmentation phenotypes (FlyBase 2003), but it has never been explicitly demonstrated that the canonical "poor male mating success" *y* mutant, y^{1}, meets the definition of a null/amorphic allele. A more specific question is whether the y^{1} mRNA has a neomorphic function in y^{1} males that alters their courtship behavior and MMS. An alternative hypothesis is that the y^{1} mRNA is nonfunctional.

Because *y* is on the *X* chromosome and males normally have only a single copy, y^{1} will be defined as a null if the y^{1} MMS phenotype is equivalent to that of a male carrying a single *X*-linked deletion of the *y* locus (Muller 1932). Using the y^{+} minichromosome rescue technique (Figure 1), we measured the noncompetitive MMS of y^{1} males as well as of males carrying either of two null mutants of *y*, which have been characterized as deletions: *Df(1)y-ac*^{22}, a deletion of the entire *y* locus, and y^{59b}, which is a deletion of genomic sequence −700 to +519 bp relative to the start of transcription, with an insertion of 3.2 kb of *gypsy* retrotransposon sequence (Table 1).

In noncompetitive mating assays, males carrying any of these three mutants had significantly worse mating success than their matched-background control males

TABLE 2

Statistical tests of homogeneity of *yellow* mutant and matched-control noncompetitive male mating success

yellow allele	Total N^a	No. of trials	% success in experimental flies	% success control flies	Fisher's exact test P-value[b,c]	Pearson's $\chi^{2\ d}$	Pearson's P-value[c]	Mantel-Heanszel chi-square[d,e]	Mantel-Heanszel P-value[c]	Overall male-mating success
y^{1}	120	5	32.9	74.0	8×10^{-6}	19.75	≤ 0.0001	19.58	≤ 0.0001	–
$Df(1)y\text{-}ac^{22}$	83	4	18.2	76.9	6×10^{-8}	28.74	≤ 0.0001	28.39	≤ 0.0001	–
y^{59b}	82	3	13.2	79.5	9×10^{-9}	35.97	≤ 0.0001	35.53	≤ 0.0001	–
y^{-893}	123	4	45.3	58.6	*0.10*	2.14	0.14	2.12	0.15	+
y^{2}	139	5	51.3	47.6	0.73	0.19	0.66	0.19	0.67	+
y^{3P}	70	2	46.7	50.0	0.49	0.08	0.78	0.08	0.78	+
$y^{-300gin}$	126	4	46.8	48.4	0.50	0.04	0.85	0.04	0.85	+
y^{bl}	193	6	51.5	49.0	0.69	0.13	0.72	0.13	0.72	+
y^{-91}	64	2	17.6	90.0	7×10^{-6}	20.13	≤ 0.0001	19.81	≤ 0.0001	–
y^{3d}	174	6	23.5	73.0	4×10^{-11}	42.64	≤ 0.0001	42.32	≤ 0.0001	–
y^{2S}	335	9	44.9	54.8	0.045	3.25	*0.07*	3.24	*0.07*	–
y^{a77}	121	4	29.4	64.3	1×10^{-4}	14.35	≤ 0.0001	14.33	0.0001	–
$y^{intronless}$	166	5	51.4	52.2	0.52	0.01	0.92	0.01	0.92	+
y^{+660R}	88	2	45.8	55.0	0.26	0.73	0.39	0.73	0.39	+
y^{+1310}	109	5	55.2	47.1	0.85	0.72	0.39	0.71	0.40	+

[a] Total N is the sum of the number of experimental and matched-control flies analyzed across all trials.

[b] One-sided Fisher's exact tests were performed because we expected *a priori* that the mating success of mutant males would either not change or be reduced. The P-value is the probability of the exact contingency table or a more extreme one in the same direction.

[c] The P-values that are <0.05 are underlined, and P-values <0.10 but >0.05 are in italics.

[d] All chi-square tests were performed with 2×2 contingency tables and 1 d.f. with a critical chi-square value of 3.841 for a 0.05 significance level.

[e] The Mantel-Heanszel chi-square test is frequently used to analyze data tables in which one or more cells have low values. We utilized this as an additional test because low mating success of some *y* mutants occasionally resulted in low values for "success," and/or very high mating success of some control males resulted in low values for "failure."

carrying the y^+ minichromosome, with statistical significance in each case determined by 2×2 tests of homogeneity within mutants (Table 2; Figure 2A). We additionally evaluated the behavior of males carrying these three mutants in a distinct competitive MMS paradigm. Consistent with the noncompetitive mating test results from the preceding experiment, we found that all three mutants had severely and significantly reduced mating success in a one-mutant male, one-control male competitive situation in a modest-sized arena, with statistical significance in each case determined by a binomial test for a deviation from a 50:50 distribution of y^1:y^+ competitive MMS (Figure 2B).

Statistical significance was extremely high ($P < 0.0001$) in each of the six experiments presented above, suggesting that y^1 acts as a null allele with respect to MMS. To more rigorously determine whether the effects of y^1 on MMS are equivalent to those caused by deletions of the gene [$Df(1)y\text{-}ac^{22}$ and y^{59b}], we carried out additional statistical analyses on the two overall data sets produced from the noncompetitive and competitive mating experiments above. First, we performed two-way ANOVA analysis of arcsine-square root-transformed noncompetitive MMS data with factors "allele" and "minichromosome" (Sokal and Rohlf 1995). Presence or absence of the minichromosome had a highly significant effect on male mating success, as expected since the y^+ minichromosome rescued poor mutant male mating success ($F_{1,18} = 52.7$, $P < 0.0001$). Within alleles, discounting the presence/absence of the y^+ rescue chromosome, there were both successes and failures, and therefore the mutant alleles did not by themselves significantly contribute to the observed variation in overall male mating success ($F_{2,18} = 0.96$, $P = 0.40$). However, the important part of the ANOVA is the interaction between alleles and the minichromosome, which answers the following question: Did the difference between the presence or absence of the minichromosome fluctuate with varying alleles? In this case, as expected, if all three mutant alleles are null and hence equivalent to each other, the interaction was not significant ($F_{2,18} = 1.20$, $P = 0.55$). ANOVA analysis of nontransformed data yielded statistically equivalent results (not shown). Second, we performed a 3×2 chi-square test on the distribution of y^1 *vs.* y^+ first copulations among the three alleles in competitive mating assays and found no evidence for meaningful variation among alleles ($\chi^2 = 0.736$, d.f. $= 2$, $P = 0.692$). Overall, we conclude that y^1 is a null allele with regard to MMS and that lesions in the *y* locus that reduce MMS in a simple noncompetitive assay are likely to have effects in more ecologically relevant, competitive situations.

Hypomorphic *y* alleles have a spectrum of effects on male mating success: The experiments with null alleles presented above provide a foundation for further

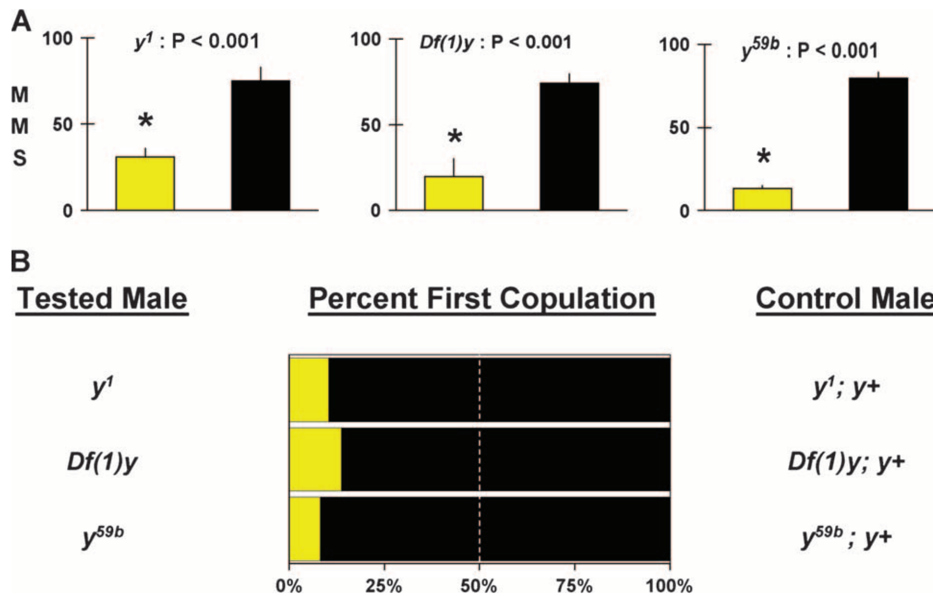

Figure 2.—(A) Noncompetitive male mating success of three *y* null mutants, y^1, $Df(1)y\text{-}ac^{22}$ [here abbreviated *Df(1)y*], and y^{59b}. Yellow bars represent behavioral data collected from experimental (mutant) males, and black bars represent data from control males (carrying the minichromosome). Bars represent means + SEM, calculated across behavioral trials. Data were analyzed using Fisher's exact tests, and the *P*-values from this analysis are shown. Sample sizes and additional statistical analyses are shown in Table 2. (B) Competitive male mating success of the same *y* null mutants. Raw data converted to percentages of total competitions are shown. Here, mating success is measured as the percentage of time that a genotype achieves first copulation with a female when in direct mate competition with another genotype. Data were analyzed with binomial tests to determine for each mutant-and-control combination whether a significant difference from a 50:50 distribution among males existed. In all cases, we found a highly significant difference (see results).

dissection of the *y* locus and analysis of modest molecular perturbations on male sexual behavior. We quantified the noncompetitive mating success of males carrying each of 12 previously characterized hypomorphic *y* alleles (Table 1) to identify alleles that significantly reduced MMS relative to control males carrying the same mutation plus the rescuing y^+ gene on the minichromosome (Figure 1).

Results from these behavior assays are presented in Table 2 and Figure 3. We found that 8 of 12 hypomorphs had MMS that was statistically equivalent to controls (Table 2). In our behavioral assay, this was manifested as ~50% mating success for both the mutant and control genotype (Figure 3, gray bars). To determine whether significant variation in MMS exists among this set of eight alleles, we performed two-way ANOVA analysis with factors "allele" and "minichromosome" on arcsine-square root-transformed data, as for the null alleles above. In this case, as expected, we found no significant main effects of alleles or presence of the minichromosome on overall variation in male mating success, and the interaction term was also not significant (all $P > 0.05$). This statistical outcome is consistent with the following hypotheses: These alleles as a group do not significantly reduce MMS, and the mean mating successes of the different mutant alleles do not significantly differ from each other (they are all ~50%; Figure 3).

In contrast to the eight mutants discussed above, four *y* hypomorphs, y^{-91}, y^{3d}, y^{a77}, and y^{2S}, caused significant reductions in MMS (Table 2, Figure 3, yellow bars). The degree to which each mutation significantly lowered MMS varied from (mean ± SEM of percentage success) 20.6 ± 2.9 for y^{-91} to 41.8 ± 4.9 for y^{2S}. To further investigate this variation in MMS, we performed two-way ANOVA analysis (with factors "mutant allele" and "minichromosome") of arcsine-square root-transformed

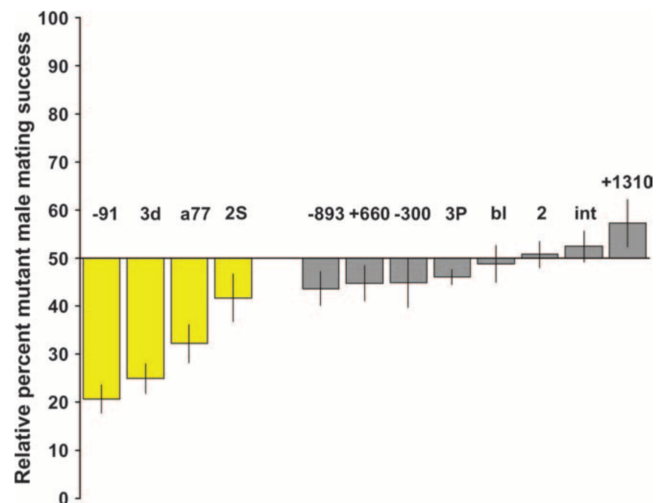

Figure 3.—Noncompetitive mating success of males carrying *y* hypomorphic regulatory mutations. The *y* allele name is centered over the appropriate bar depicting the mean ± SEM of that genotype's mating success, calculated across behavioral trials. Mutant male data are represented by yellow (gray) bars if they had significantly lowered (unchanged) mating success, as determined using Fisher's exact tests. Sample sizes and additional statistical analyses are shown in Table 2. Control male data are also shown in Table 2.

MMS data from these four hypomorphs. We tested the hypothesis that although each individual allele lowered MMS, there was significant variation among alleles in their effects on this behavior. As expected, the presence or absence of the minichromosome had a highly significant effect on MMS, because without exception the y^+ minichromosome rescued poor MMS of the mutants ($F_{1,34} = 66.4$, $P < 0.0001$). Again, the *y* mutant alleles by themselves did not significantly contribute to the observed variation in overall MMS ($F_{3,34} = 0.20$, $P = 0.89$), because within a given allele across the presence/absence of the y^+ rescue chromosome, there were both successes and failures. Differing from the set of null alleles, we found a highly significant interaction between allele and minichromosome ($F_{3,34} = 6.7$, $P = 0.001$), as expected if there is significant variation among alleles for the difference in MMS between males with and without the y^+ rescue chromosome. Identical ANOVA analysis of nontransformed data yielded statistically equivalent results (not shown). We conclude that different molecular perturbations of *y* can lead to a spectrum of effects on noncompetitive MMS.

Additional statistical analyses were carried out to test whether each of these four alleles should be classified as null or hypomorphic with regard to MMS. We determined whether the number of successes and failures differed between the two deletions $Df(1)y\text{-}ac^{22}$ and y^{59b} and the four pigmentation hypomorphs: Nonsignificance suggests that the allele acts as a null for MMS, and a significant difference indicates that the allele is a hypomorph. Using Fisher's exact tests, we found statistical support for the following order of allelic effects on reduction in MMS: $Df(1)y\text{-}ac^{22} = y^{59b} = y^{-91} \geq y^{3d} > y^{a77} \geq y^{2S}$. Specifically, we found that $Df(1)y\text{-}ac^{22}$ and y^{59b} MMS were statistically equivalent and that the MMS of y^{-91} and y^{3d} were comparable to each of the nulls ($P \gg 0.05$ in all comparisons). Analyses of y^{a77} [$P < 0.00001$ ($Df(1)y$) and $P < 0.10$ (y^{59b})] and y^{2S} ($P < 0.001$ in both comparisons) show that while mutant MMS is significantly lower than that of their respective controls, their relative MMS is greater than that caused by null mutants of the *y* gene. We conclude that y^{-91} and y^{3d} are null with regard to MMS and that y^{a77} and y^{2S} are hypomorphs for MMS. On the basis of the marginally significant differences in mating success of y^{2S} males in the original analysis (Table 2), this allele has the weakest effect on MMS. Although in a number of individual trials y^{2S} males had very low MMS, in some trials they were equivalent to control males (not shown). Indeed, detection of an overall statistically significant difference in MMS between y^{2S} mutants and controls was possible only by assaying hundreds of males (Table 2).

Genetic dissection of *y* regulatory sequences reveals 300 bp required for male mating success: The *y* locus is relatively simple, containing two exons, one intron, and a single transcript and protein product (Geyer *et al.* 1986; FlyBase 2003). Six general regions of the *y* locus might contain regulatory sequences affecting MMS: (1) the ~4-kb noncoding region upstream of the start of transcription, (2) the 5′ untranslated region (UTR), (3) the first exon, (4) the large single intron, (5) the second exon, and (6) the 3′-UTR and sequences downstream of the end of transcription. Because sequences regulating the development of cuticular melanization have previously been identified both in the upstream sequence and in the intron, the regions that we inspected were already known to contain sequences with regulatory function (Beissmann 1985; Geyer and Corces 1987, 1992; Martin *et al.* 1989). Therefore, these are excellent candidate regions within which to search for sequences regulating the function of *y* with respect to male-specific behavior.

The behavioral phenotypes of the 12 hypomorphic mutants combined with information about their molecular lesions allowed us to map a 300-bp region of *y* whose function is required for normal MMS. We named this region the MRS (Figure 4). This conclusion is based on the following observations.

First, males carrying three hypomorphic mutants with disruptions in the *y* intron and the sequence downstream of this intron, $y^{intronless}$, y^{+660R}, and y^{+1310}, had normal MMS (Table 2, Figure 3). All three mutants carry transgenes not located at the site of the endogenous *y* locus. The $y^{intronless}$ flies contain a *y* transgene deleted for the ~2.7-kb intron (Geyer and Corces 1987). Mutant y^{+660R} insulates regulatory sequence function downstream of +660 bp, and in an identical manner y^{+1310} insulates regulatory sequence function in the intron sequence downstream of +1310 bp. Both lines contain the *gypsy* insulator within the intron, which begins at nucleotide +409 and ends at nucleotide +3129. Since the y^{+660R} and y^{+1310} insulators disrupt the function of any regulatory sequence downstream of their respective insertion sites, these data suggest that, within the limits of the *y* transgene construct, there is no MMS regulatory sequence located in the *y* coding sequence downstream of the intron (see Geyer and Corces 1992). This includes the second *y* exon, the 3′-UTR, and any downstream regulatory sequences.

Second, males carrying five hypomorphic mutants with disruptions upstream of the *y* transcription start, y^2, y^{bl}, y^{3P}, y^{-893}, and $y^{-300gin}$, had normal MMS (Table 2, Figure 3). Four of these mutants were generated within the endogenous *y* locus: a full-length insertion of *gypsy* in y^2, a mutant characterized by a number of small aberrations named y^{bl}, an inversion with a single breakpoint within *y* named y^{3P}, and a gene-replacement mutant named $y^{-300gin}$, which utilizes the *gypsy* insulator at −300 bp relative to the *y* transcription start (Parnell *et al.* 2003). The remaining mutant, y^{-893}, carries a genetically engineered transgene of the *y* locus utilizing the *gypsy* insulator (Geyer and Corces 1992). The molecular lesions associated with these five mutations are upstream of −300 bp relative to the *y* start of transcription. Therefore, these data suggest that there is no regulatory region required for normal MMS located in the ~7 kb of the *y* locus upstream of −300 bp of the *y* transcription start

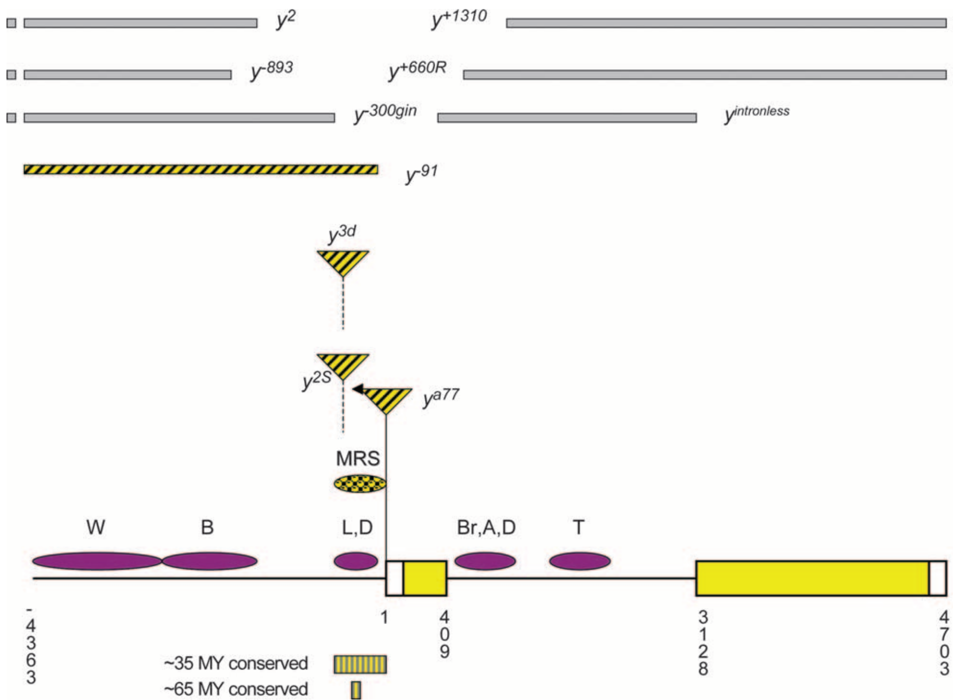

Figure 4.—Summary of behavior genetic data relevant to the mapping of an ∼300-bp regulatory region whose function is necessary for normal male mating success. Numbers labeling the *y* locus are base pairs relative to the start of transcription (at +1 bp). Locations of pigmentation enhancers (purple ovals) are taken from Geyer and Corces (1987, 1992) and Martin *et al.* (1989). W, wing; B, body; L, larval mouth parts; Br, bristles; A, aristae; D, larval denticle belts; T, tarsal claws. Boxes indicating conserved noncoding sequence within the MRS are based on Munte *et al.* (1997) and on analysis in the results and in Figure 7. Most of the remaining noncoding sequence in *y* is not well conserved (see Munte *et al.* 1997). Mutations shown in gray have no effect on male mating success, and mutations shown with black-and-yellow stripes significantly lowered male noncompetitive mating success. A square on the outer edge of a mutational effect region indicates that the effect of the insulator mutation extends past the drawing, to the extent of the transgene sequence, or in the case of the y^2 mutation at the endogenous location, beyond the *y* locus to intergenic sequence. For clarity, and because the omitted mutations provide redundant mapping information, not all mutations phenotyped in this study are shown. The dashed lines extending from insertions y^{3d} and y^{2S} indicate that the precise sites of insertion are unknown, although they have been localized to the −300 to +1 bp region. The sizes of insertions y^{3d} and y^{2S} are ∼7 and ∼4.5 kb, respectively. Partial sequencing of insertion y^{a77} revealed 357 bp of one end of a typical *P* element.

site. [We note that while a Fisher's exact test applied to the y^{-893} data resulted in a marginally significant difference of $P = 0.10$, both chi-square analyses had *P*-values greater than this (Table 2).] Combined with the nonsignificant results from analysis of the other alleles affecting the same general region of the *y* locus, for example, $y^{-300gin}$, we parsimoniously conclude that the y^{-893} lesion caused no meaningful decrease of MMS. It may be the case that there is a position effect of the transgene in the y^{-893} strain that lowers MMS (see discussion).

Third, males carrying the y^{-91} mutant, a deletion of bases −2873 to −91 in the noncoding region upstream of the *y* transcription start within a transgene (Geyer and Corces 1987), had a significant reduction in MMS relative to matched control males (Table 2, Figure 3). This result suggests that the 2782-bp deletion contains a sequence required for normal male mating success. On the basis of this and results for other *y* mutants already presented, we infer that the 209-bp sequence between −300 and −91 bp of the *y* locus is required for normal MMS.

Fourth, three insertion mutations upstream of the first *y* exon reduce MMS. The reduced MMS of y^{-91} males predicts that insertional mutations in or near the −300- to −91-bp region should reduce MMS. Three mutations of the endogenous *y* locus—y^{3d}, y^{a77}, and y^{2S}—each were previously shown to be large DNA insertions in the ∼700-bp region just upstream of *y* transcription start (Morris *et al.* 1999). These mutants all display a reduction of pigmentation and are classified as hypomorphic because in each case at least one body part with *y*-dependent pigmentation has normal pigment levels (see Table 3 in Morris *et al.* 1999). The fact that males carrying each of these *y* lesions had significantly lower mating success than control males did (Table 2, Figure 3) suggests that these insertions disrupt normal MRS function. They appear to do so to different degrees, however (see below).

The reduced MMS caused by y^{3d}, y^{a77}, and y^{2S} predicts that the insertions, before this study known only to be within ∼700 bp upstream of the *y* transcription start, should be located between −300 bp and the start of transcription at +1 bp. Using PCR analysis the location of each of these insertions was narrowed to the −300- to 0-bp region that contains the MRS (not shown). Partial sequencing indicated that the y^{a77} allele had an insertion at −4 bp (see materials and methods). Comparison of this y^{a77} insertion DNA to sequences in public databases using BLASTn (Altschul *et al.* 1990) revealed 94% identity to the 5′-end of a typical *P* element, including the requisite, conserved, perfect 31-bp terminal inverted repeat region. We conclude that y^{a77} is a *P*-element insertion in reverse orientation to *y* at −4 bp in the *y* locus.

Adult male pigmentation is not predictive of mating success: The most conspicuous trait that *y* mutant adult

TABLE 3

Adult pigmentation phenotypes caused by *yellow* mutants are not predictive of male mating success

yellow allele	Wing[a,b]	Body	Bristles	Aristae	Tarsal claws	Sex combs	Overall mating success[d]
y^{1}	–	–	–	–	–	+/–[c]	–
$Df(1)y\text{-}ac^{22}$	–	–	–	–	–	+/–	–
y^{59b}	–	–	–	–	–	+/–	–
y^{-893}	–	–	+	+	+	+	+
y^{2}	–	–	+	+	+	+	+
y^{3P}	+/–	–	+	+	+	+	+
$y^{-300gin}$	+	–	+	+	+	+	+
y^{bl}	+	+/–	–	–	+	+	+
y^{-91}	–	–	+	+	+/–	+	–
y^{3d}	+	–	–	–	+/–	+	–
y^{2S}	–	+/–	+/–	+	+	+/–	–
y^{a77}	–	–	–	–	+/–	+/–	–
$y^{intronless}$	+	+	+/–	–	–	+/–	+
y^{+660R}	+	+	–	–	–	+	+
y^{+1310}	+	+	+	+	–	+	+

[a] Pigmentation data are a summary of personal observations by M. D. Drapeau and P. K. Geyer, the primary references in Table 1, Lindsley and Zimm (1992), and FlyBase.

[b] Wing, body, bristles, aristae, tarsal claws, and sex combs are adult pigmented structures.

[c] "+/–" indicates that the pigmentation is neither completely wild type (dark) nor mutant (yellow), but rather intermediate.

[d] The male mating success data are from this report.

males have is reduced pigmentation resulting in a yellow body color (Lindsley and Grell 1968; Lindsley and Zimm 1992). Indeed, we utilized pigment differences between mutant and control male cuticles as a neutral marker to distinguish them for experimentation. However, changes in male cuticular coloration may not be neutral and could in fact reduce mating success, an hypothesis that our data allow us to test. One way in which cuticular pigmentation could influence male mating success is if the female responds to visual cues from the male's body pigmentation during courtship. If she receives less "pigment signal," she will be less stimulated toward a receptivity threshold, thereby lowering the mating success of males with reduced pigmentation. Wing coloration is of particular interest, because wing colors, patterns, and movements in many insects, including Drosophila, are used as visual courtship signals (*e.g.*, Singh and Chatterjee 1987; True *et al.* 1999; Kopp and True 2002; Gompel *et al.* 2005; Robertson and Monteiro 2005).

Our data do not support the hypothesis that variation in adult male cuticle features changes MMS (Table 3). Males carrying the pigment-mosaic (normal pigment in some body parts/tissues, and mutant in other) y^{2}, y^{-893}, and $y^{-300gin}$ alleles had greatly reduced pigmentation throughout their entire body and wings, yet their mating success was not meaningfully reduced in comparison to control males (Tables 2 and 3, Figure 3). Furthermore, the pigmentation of other, smaller adult male body parts that females might visually assess during courtship is unrelated to male mating success. For example, mutants y^{bl}, y^{+600R}, and $y^{intronless}$ all have reduced pigmentation in the aristae yet exhibited normal MMS (Tables 2 and 3, Figure 3). These data suggest that females are not using reduced cuticle color as a visual signal of poor male quality and subsequently reducing their receptivity.

There is a second mechanism by which pigmentation of adult male body parts could affect male mating success. Because *y* null mutant males have reduced melanin in their wings, and most likely reduced sclerotin (a hardening compound found in the cuticle and metabolized from tyrosine in the same biochemical pathway as melanin; Burnet and Connolly 1974; Wright 1987), some physical properties of the wing might be altered such that these flies are less efficient at transmitting auditory (song) or olfactory (pheromone) signals to females while courting. Our data allowed us to test, and reject, the hypothesis that wing cuticle structure variation among *y* mutants is predictive of male mating success. The normal MMS of *y* alleles conferring yellow-colored wings (y^{2}, y^{-893}, and $y^{-300gin}$) provides evidence against this hypothesis (Tables 2 and 3, Figure 3). Conversely, y^{3d} males have normal-colored (gray) wings yet severely reduced mating success (Tables 2 and 3, Figure 3). In total, our data suggest that the adult pigmentation and male sexual behavior phenotypes that *y* confers are independent of each other.

The MRS overlaps a sequence controlling larval pigmentation: The ~300-bp MRS overlaps a regulatory sequence previously characterized as a control region

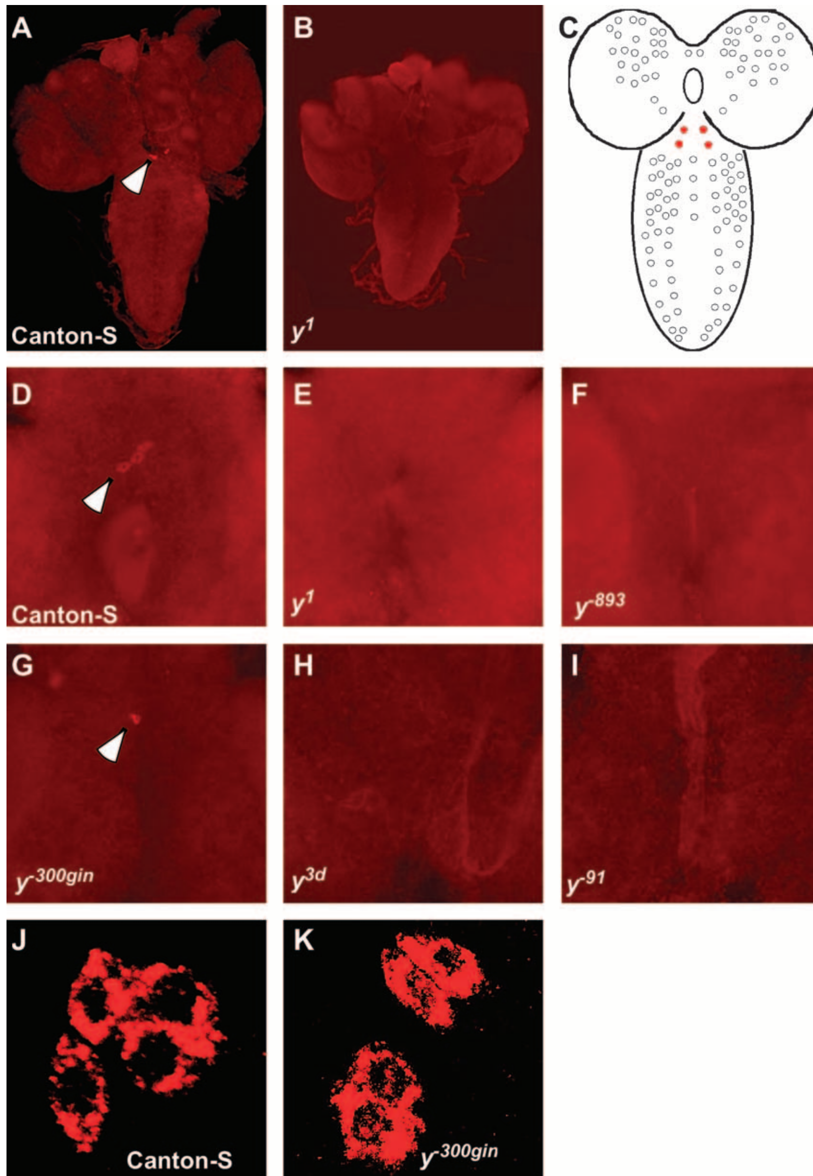

Figure 5.—Presence of Yellow protein in third instar CNS neural cells is associated with adult male mating success. In all images, anti-Yellow antibody fluorescence is shown in red. Shown are fluorescent microscope (A, B, D–I) or confocal microscope (J and K) images of the third instar larval CNS. (A) Canton-S wild-type positive-control CNS, showing anti-Yellow staining in four CNS cells. (B) The y^1 loss-of-function negative-control CNS, showing no anti-Yellow staining. (C) Schematic of the third instar CNS, after Truman *et al.* (1993, their Figure 7), showing approximate locations of dividing neuroblasts at this developmental stage. The Yellow$^+$ cells may be the four neuroblasts in red (see also Drapeau *et al.* 2003). (D–I) Shown are the CNSs from positive- and negative-control flies, and four hypomorphic *y* mutants, at higher magnification than that in A and B. (D) Canton-S CNS showing anti-Yellow cells. (E) y^1 CNS showing no anti-Yellow cells. (F) The y^{-893} mutant has moderately low MMS and no anti-Yellow cells in the CNS at this stage. (G) The $y^{-300gin}$ mutant has normal MMS and normal anti-Yellow cells in the CNS at this stage. (H and I) Both y^{3d} and y^{-91} mutants have severely reduced MMS and no anti-Yellow cells in the CNS at this stage. (J and K) Confocal images of anti-Yellow cells from Canton-S positive-control flies, and the $y^{-300gin}$ hypomorph whose males exhibit normal MMS. Yellow appears to be largely, if not completely, cytoplasmic in these cells.

for larval pigmentation (Geyer and Corces 1987; Martin *et al.* 1989; Figure 4). Not surprisingly, the hypomorphic mutants that disrupt this region (y^{-91}, y^{3d}, y^{a77}, and y^{2S}) reduce both MMS (this article) and larval mouth-part pigmentation (Biessmann 1985; Chia *et al.* 1986; Geyer and Corces 1987; Harrison *et al.* 1989; FlyBase 2003; M. D. Drapeau and P. K. Geyer, unpublished observations; see also Martin *et al.* 1989). Interestingly, as with MMS, these mutants reduce larval mouth-part pigmentation to different degrees, with colors ranging from brown, to golden-brown, to yellow. The larval pigment phenotype and the adult male sexual behavior phenotype may therefore be developmentally linked. However, we reason that the pigmentation state of the larval mouth parts is unlikely to have any direct bearing on female mate choice decisions.

Expression of *y* in the third instar CNS of wild-type and mutant flies: Previous work suggested that expression of *y*, and hence presence of the Yellow protein product, is necessary and sufficient in the third instar CNS for normal male courtship and subsequent mating success (Drapeau *et al.* 2003; M. D. Drapeau, M. Suster, A. Piccin, and A. D. Long, unpublished data). Using an anti-Yellow antibody applied to wild-type flies, we were able to detect Yellow presence in the cytoplasm of a limited number of cells at this stage (Figure 5). This is generally consistent with our earlier work on the third instar CNS (Drapeau *et al.* 2003), the detection of cytoplasmic Yellow in cuticle cells (Figure 3 in Wittkopp *et al.* 2002a), and the fact that Yellow is a secreted protein (Kornezos and Chia 1992; Wittkopp *et al.* 2002a; Drapeau 2003; Drapeau *et al.* 2003). We did not observe Yellow in these cells in the loss-of-function y^1

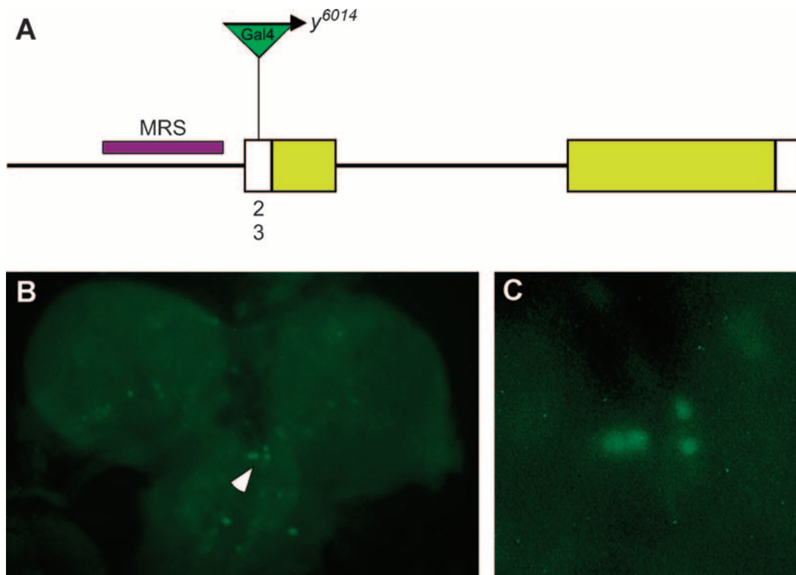

FIGURE 6.—Evidence that the MRS drives *y* expression in a small number of cells in the third instar CNS. (A) Diagram of the *y* locus in the newly described mutant y^{6014}. The y^{6014} mutation is caused by a *P*{GawB} insertion at +23 bp relative to the start of *y* transcription. This *P*-element insertion contains the Gal4 sequence, allowing sequences upstream of the element, including the MRS, to control expression of a UAS-GFP reporter in F_1 offspring. (B) Fluorescent microscope image of a third instar CNS from the genotype y^{6014}; *UAS-nu.GFP/+* with the *P*{GawB} driving nuclear-localized GFP. We observed expression in four cells in the same location as the Yellow$^+$ cells shown in Figure 5 (open arrowhead). Ectopic expression of GFP in a few cells throughout the CNS was also seen (see RESULTS). Generally, the strongest GFP expression was seen in the four putative Yellow$^+$ cells. (C) Magnification of the four cells next to the white arrow in B.

mutant (Figure 5, B and E). Therefore the fluorescence of the anti-Yellow antibody is representative of *y* expression in this tissue at this developmental stage.

To test the hypothesis that Yellow presence in these cells is relevant to adult male mating success, we examined Yellow in the third instar CNS of four hypomorphs with varying degrees of MMS (Figures 3 and 5). Mutants y^{-91} and y^{3d} have greatly reduced MMS, $y^{-300gin}$ has normal MMS, and y^{-893} has nonsignificant but moderately low MMS. Yellow presence was associated with MMS levels (Figure 5). We detected a strong cytoplasmic Yellow signal in the $y^{-300gin}$ mutant, which has normal MMS, as expected (Figure 5, G and K). In contrast, the mutants with severely reduced MMS, y^{-91} and y^{3d}, had no Yellow in the third instar CNS, as we predicted (Figure 5, H and I). We found that the y^{-893} mutant, with moderately low MMS compared to control males ($P = 0.10$, Fisher's exact test; see also Table 2), had no detectable Yellow in the CNS at this developmental stage (Figure 5F), although this may be caused by a transgene position effect (see DISCUSSION). In total, the data suggest that the Yellow that we detected in the third instar CNS is most likely related to adult MMS.

The Yellow protein data presented above, in combination with our behavior genetic mapping data, predict that the MRS controls *y* expression in these third instar CNS cells. To test this, we utilized a novel *y* mutant, which we named y^{6014} (see MATERIALS AND METHODS). This mutation is caused by a *P{Gal4}* element inserted ~23 bp downstream of the endogenous MRS and the *y* transcription start site (Figure 6A). Combined with a UAS-GFP responder element, we can use y^{6014} as a reporter of *y* expression controlled by the MRS and sequences upstream of it. As predicted, we found y^{6014}-controlled GFP expression in the identical location and cell number as that seen when viewing Yellow using immunocytochemistry (Figure 6, B and C). We also saw a small amount of GFP expression in other cells, in no distinct or obvious pattern (Figure 6B; M. D. DRAPEAU and S. A. CYRAN, unpublished results). Unfortunately, because y^{6014} appears to be a null mutant (see MATERIALS AND METHODS), we could not easily colocalize Yellow and y^{6014}-controlled GFP expression in these flies. These data nonetheless strongly suggest that the MRS controls *y* expression in a small number of third instar CNS cells and that Yellow action in these cells is required for normal adult MMS.

Natural genetic variation within the *D. melanogaster* MRS: To identify naturally occurring genetic variation of potential relevance to the evolution of courtship behavior, we sequenced the MRS of 85 wild-type *D. melanogaster* strains that have diverse origins and ages since laboratory domestication. The sequenced isolates were the well-characterized laboratory strains Oregon-R and Canton-S (isolated many decades ago in Oregon and Ohio, respectively), Ives (isolated ~35 years ago in Massachusetts), and strains from Zimbabwe, North Carolina, and Napa Valley, California (isolated within the last decade).

We found no common single nucleotide polymorphism (SNP) within the 209-bp sequence that is strictly required for normal MMS, as defined by the y^{-91} mutant. There was only a single common genetic variant within the longer 300 bp MRS, an A/C SNP at −36 bp relative to the start of *y* transcription (C in 22/85 lines = 25.9%). In addition to the single common polymorphism, we identified three rare polymorphisms (<5%) within the MRS. They were an A/G site at −67 [G in two strains (2.4%), both from Napa Valley], a C/T site at −141 [T in three strains (3.5%), all from Zimbabwe], and an A/G site at −225 [G in one strain (1.2%) from Zimbabwe].

Molecular evolution of the MRS within the genus Drosophila: Males of most Drosophila species perform courtship behavior whose purpose is to stimulate

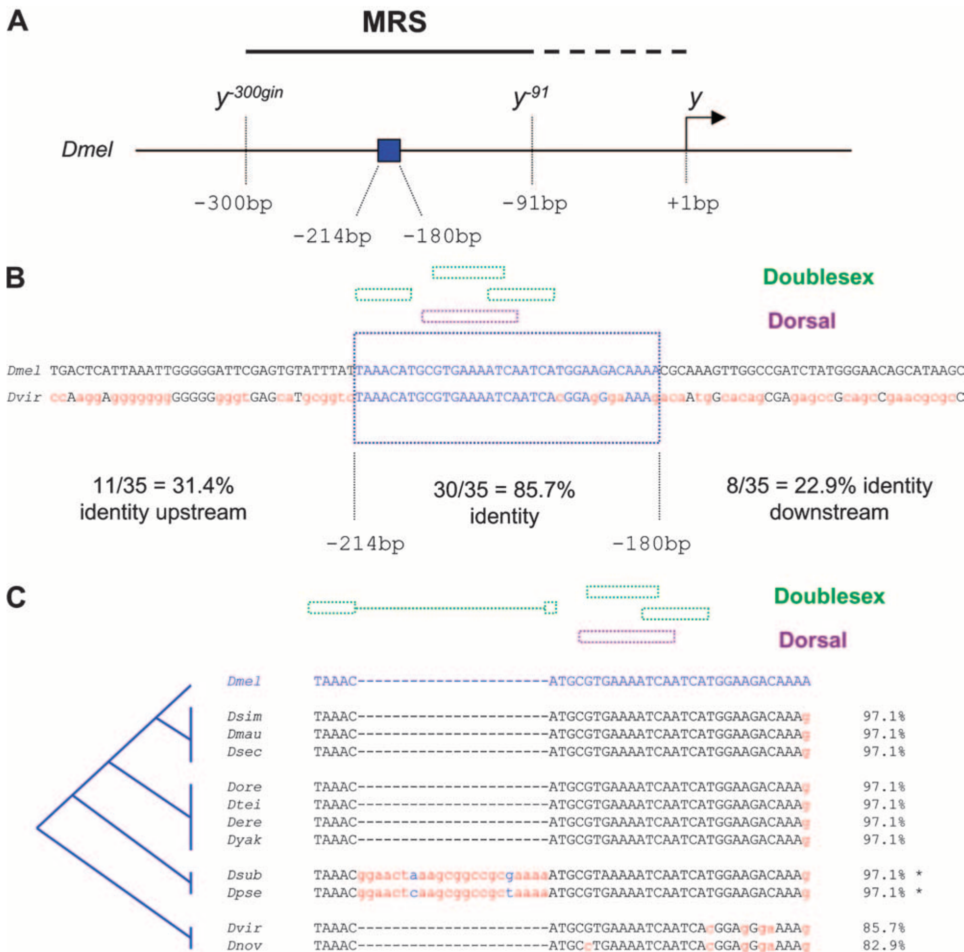

Figure 7.—Molecular evolutionary analysis of the MRS. (A) Map of the *yellow* locus of *D. melanogaster* showing the start of transcription at +1 bp, and *y* mutant landmarks at −300 and −91 bp. Deletion of the region between −300 and −91 bp (solid black MRS line) reduces male mating success to null-allele levels. Insertions within the region between −300 and +1 bp (solid plus dashed MRS line) significantly reduce male mating success to varying degrees. The blue square demarcates a highly conserved 35-bp sequence from −214 to −180 bp. (B) The 105-bp sequence from *D. melanogaster* and *D. virilis*, which shows the 35-bp conserved sequence in detail. This sequence is 85.7% identical between the species. The representative 35 bp immediately upstream and downstream of the sequence of interest is poorly conserved with 31.4% and 22.9% identity, respectively. The purple rectangle demarcates a putative Dorsal transcription factor binding sequence. This consensus sequence was identified using the web-based program TFSEARCH, which utilizes the TRANSFAC database (see Thisse *et al.* 1991; Akiyama 1998; Heinemeyer *et al.* 1998). The three green rectangles demarcate three putative DSX-binding sites, two of which overlap the putative Dorsal site. Consensus DSX sequences are from Erdman *et al.* (1996). (C) Alignment of the conserved 35-bp sequence across 12 Drosophila species. The right column indicates percentage identity of sequence. The (*) indicates that the percentage identity calculated does not include the 22-bp insertion in the marked species. Across the Drosophila species that we analyzed, there is only a single base change in the putative Dorsal-protein-binding site, in *D. novamexicana*. The putative Doublesex-protein-binding sites are perfectly conserved across all the species in our analysis.

females to be receptive to copulation (Spieth 1952, 1974). While the precise details of the male courtship rituals have diverged among species, similar characteristics such as courtship song have been retained in many cases (see, for example, Table 1 in Spieth 1974). Since there have been numerous reports of males in various Drosophila species carrying null or nearly null mutations at the *yellow* locus with reduced mating success (Rendel 1944, *D. subobscura*; Tan 1946, *D. pseudoobscura*; Frias and Lamborot 1970, *D. gaucha*; Pruzan-Hotchkiss *et al.* 1992, *D. pseudoobscura*; da Silva *et al.* 2005, *D. willistoni*; W. J. Etges, unpublished data, *D. mojavensis*), the general requirement for *y* in the development of courtship behavior is fairly conserved within the genus. It could be that small changes in the regulation of *y* have resulted in minor changes in male courtship signals in different species (*e.g.*, compare the sibling species *D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana*, Cobb and Ferveur 1996). Molecular evolution within the 300-bp MRS of the *y* locus may have consequences for the evolution of courtship behavior within species and reproductive isolation between species.

Noncoding sequence conservation among divergent species can indicate conserved regulatory function (*e.g.*, Moses *et al.* 2003; Sinha *et al.* 2004). We determined the patterns of constraint and divergence of the MRS region in Drosophila species with divergent patterns of wing usage during courtship. These species were: *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. pseudoobscura*, *D. subobscura*, *D. virilis*, and *D. novamexicana*, the most divergent of which has been separated from *D. melanogaster* for ∼65 million years (MY). Generally, the MRS is poorly conserved across wide species boundaries within Drosophila (*e.g.*, Figure 7B). However, a striking feature of MRS evolution is an extremely well-conserved 35-bp sequence, positioned in the center of the region that we have shown to be required for normal male mating success: the region between −300 and −91 bp (Figure 7, A and B). This 35 bp shows 100% identity among

wild-type inbred laboratory *D. melanogaster* strains Oregon-R, Canton-S, and Ives ("iso-female line 2"), and also 82 wild-type strains recently collected from Napa Valley, North Carolina, and Zimbabwe (see above). There is 100% identity between *D. melanogaster* and its sibling species *D. simulans*, *D. mauritiana*, and *D. sechellia* along the first 34 bases, and a shared polymorphism among the three sibling species at base 35 (Figure 7C). In the more diverged species *D. subobscura* and *D. pseudoobscura* (∼35 MY), there is also 100% identity along the 34 bp, with the caveat of a 22-bp insertion at the sixth base position in each species (Figure 7C). There are two SNPs between these two species within the 22-bp insertion sequence. Base 35 is identical between *D. subobscura* and *D. pseudoobscura* and the three sibling species, but is different from *D. melanogaster*. Finally, the most diverged species that we investigated are 85.7% (in the case of *D. virilis*) and 82.9% (*D. novamexicana*) identical to *D. melanogaster* and do not share the 22-bp insertion with *D. subobscura* and *D. pseudoobscura* (Figure 7C). Both species share base 35 with every species except *D. melanogaster*, suggesting a recent base change at that site in the *D. melanogaster* lineage. Additional previously published sequence data from the *D. ananassae*, *D. willistoni*, and *D. mojavensis* genome projects also show perfect or nearly perfect conservation within this 35-bp region, but not outside it (data not shown). This 35-bp sequence may have a conserved *cis*-regulatory function with respect to male sexual behavior, which the 22-bp inserts in *D. subobscura* and *D. pseudoobscura* could modify. No *D. melanogaster* SNP variation was found within this 35-bp region (see above).

Candidate transcription factors for regulation of *y* via the MRS: The male-specific sexual differentiation transcription factor FRU^{M} may regulate *y* with regard to courtship behavior and MMS (Radovic *et al.* 2002; Drapeau *et al.* 2003). This regulation may occur via the MRS, although currently there is no direct evidence for this. In any case, given that temporal, spatial, and sexual regulation of *y* expression is likely to be more complicated than FRU^{M} acting in isolation, we used a bioinformatic approach to identify additional transcription factors that might regulate *y* in the CNS by binding to sequences within the MRS.

The web-based program TFSEARCH allowed us to search the MRS for consensus sequences potentially binding known Drosophila transcription factors in the TRANSFAC database (Akiyama 1998; Heinemeyer *et al.* 1998). A significant match for the Dorsal (DL) binding sequence (see Thisse *et al.* 1991) was found within the MRS, a 10/11 nt match to the consensus of (A/C/T)(C/G)(A/C/G)G(A)$_4$(A/C/T)C(A/C/G), and a 9/11 nt match to the "high-affinity" binding site of GGG(A/T)$_5$CC(C/A) described by Markstein *et al.* (2002). Remarkably, this putative DL consensus sequence falls within the highly conserved 35-bp region of the 300-bp MRS (Figure 7, B and C).

Giot *et al.* (2003) found that Dorsal binds to the Doublesex (DSX) protein in a yeast two-hybrid assay (see also the Fly GRID database located at http://biodata.mshri.on.ca/fly_grid/servlet/searchpage). DSX is a transcription factor that is crucial for proper sexual differentiation, and it has sex-specific products DSX^{M} and DSX^{F} that compete for the same binding sites (*e.g.*, Hildreth 1965; Baker and Ridge 1980; Burtis and Baker 1989; Coschigano and Wensink 1993; Zhu *et al.* 2000). Function of *dsx* appears to be required mainly for nonbehavioral sexual differentiation outside the CNS (for example, sex-specific pigmentation for which Yellow is necessary and sufficient; Jursnich and Burtis 1993; Kopp *et al.* 2000), although DSX is found in the CNS at the same stage as Yellow and FRU^{M} (Lee *et al.* 2002) and is required for some aspects of normal sexual behavior (Villella and Hall 1996). Because of the *in vitro* link between DL and DSX, We utilized information about binding sites/affinities for DSX from Burtis *et al.* (1991) and Erdman *et al.* (1996) to search the entire 300-bp MRS for potential DSX-binding sites. The only sequence within the MRS to resemble the DSX consensus binding sequence (most important portion underlined) of CT<u>ACAAAGT</u> from Burtis *et al.* (1991) or (G/A)NN<u>AC(A/T)A(T/A)GT</u>NN(C/T) from Erdman *et al.* (1996) was, again, within the 35-bp highly conserved region (Figure 7, B and C). Three potential DSX-binding sequences are located within the subregion of the 35 bp that is conserved among all species, except a single nucleotide substitution in *D. novamexicana*. Two of these are exact matches at 6/7 bases, and the third is an exact match but for a 2-nt insert two bases from one end. One of these potential DSX-binding sites is disrupted by the 22-bp inserts in *D. subobscura* and *D. pseudoobscura* (Figure 7C). The close proximity of the DL- and DSX-binding sites, combined with the *in vitro* two-hybrid results of Giot *et al.* (2003), suggest that DL and DSX might bind to each other and also within the MRS to regulate *y* transcription.

DISCUSSION

Courtship genetics—major issues: Animal courtship in nature is of obvious adaptive importance. Laboratory courtship often recapitulates what is found in the wild, and it can be quantified and manipulated (*e.g.*, Hebets and Uetz 1999; Hebets 2003). Therefore its genetic basis can be uncovered. The rationale for examining the genetic basis of instinctual courtship behavior is threefold. First, a fundamental and understudied question of developmental biology is: How does gene action in the immature nervous system build circuitry specifically dedicated to adult behaviors? Second, a major problem in the field of evolutionary neurobiology is: How do genes change the structure and properties of nervous systems, to allow the evolution of complex behaviors? Third, from a quantitative genetic viewpoint, we can

ask: Is the genetic architecture of rapidly evolving courtship behavior different from that of other behaviors whose genetic architecture is more slowly evolving (*e.g.*, circadian-regulated activity; Allada *et al.* 2001; Panda *et al.* 2002)?

Drosophila courtship can be utilized as a model system with which to answer these questions. Many genes that are required for normal *D. melanogaster* male courtship have been identified, including two key transcription factors, which are upstream of at least a subset of these genes: *fru* and *dsx* (Hall *et al.* 1982; Hall 1994a; Greenspan 1997; Yamamoto *et al.* 1997, 1998; Yamamoto and Nakano 1998, 1999; Goodwin 1999; Gaines *et al.* 2000; Orgad *et al.* 2000; Romanova *et al.* 2000; Baker *et al.* 2001; Greenspan and Ferveur 2000; Sokolowski 2001; Boll and Noll 2002; Dauwalder *et al.* 2002; Subocheva *et al.* 2003; Grosjean *et al.* 2004). Besides *fru* and *dsx*, most of these genes have not been well-characterized from the standpoint of their transcriptional regulation, biochemical function, or evolution with regard to male-specific behaviors.

The *y* gene of *D. melanogaster* is an excellent candidate for study with regard to understanding the origins of innate sexual behavior in insects. Reasons for this include: (1) Genetic mutations, antibodies, and other sophisticated tools (*e.g.*, the ability to make somatic mosaics via Gal4-mediated UAS misexpression and RNA interference) are available for studying the effects of changing *y* gene regulation (*e.g.*, Calleja *et al.* 1996; Piccin *et al.* 2001; FlyBase 2003); (2) the *y* gene is under male-specific regulation by the sex determination pathway (Radovic *et al.* 2002; Drapeau *et al.* 2003); (3) mutations at *y* disrupt a specific subset of the behaviors performed during the male courtship ritual (in this case, wing extension) (Bastock 1956; Burnet *et al.* 1973; Drapeau *et al.* 2003); and (4) the male sexual behavior function of *y* appears to be conserved, at least among Drosophila species (Rendel 1944, *D. subobscura*; Tan 1946, *D. pseudoobscura*; Frias and Lamborot 1970, *D. gaucha*; Pruzan-Hotchkiss *et al.* 1992, *D. pseudoobscura*; da Silva *et al.* 2005, *D. willistoni* ; W. J. Etges, unpublished data, *D. mojavensis*).

Here, we investigated the regulation of *y* with regard to male sexual behavior. The research presented is part of a larger investigation of *yellow* with regard to the three fundamental questions posed above. With two exceptions (Burnet and Wilson 1980; Robertson 1982), the many studies involving the requirement of *y* for normal *D. melanogaster* male mating success levels have exclusively involved the y^1 allele or, in a minority of cases, undesignated pigmentation-null ("type-1," *e.g.*, Green 1961; Chia *et al.* 1986; Lindsley and Zimm 1992) alleles of *y* (Sturtevant 1915; Spett 1931; Diederich 1941; Merrell 1949; Mayr 1950; Meyers 1953; Bastock 1956; Barker 1962; Schroeck 1971; Burnet *et al.* 1973; Threlkeld *et al.* 1974; Dow 1975, 1977a,b,c,d; Tracey and Espinet 1976, 1977; Wilson *et al.* 1976; Oakeshott and Hayman 1979; Bradman *et al.* 1981; Mizuguchi and de Almeida 1983; Heisler 1984; Hamerlynck 1994; Drapeau *et al.* 2003). In this article, we extended these earlier results in numerous ways.

The y^1 allele is a null with regard to male mating success: We unambiguously showed that y^1 is a null allele for male mating success. Our consideration of this point is nontrivial, since reputable literature discussion of *y* function with respect to sexual behavior (*e.g.*, Hall 1994a, 2002) is based on the studies cited above that were conducted with y^1. This mutant allele has been previously characterized as an A-to-C transversion in the first translated codon, resulting in an altered mRNA product (Geyer *et al.* 1990; Morris *et al.* 1999). Therefore, before this study, with respect to male sexual behavior the hemizygous y^1 allele could possibly have been a null allele, a hypomorph, or even a neomorph, if the mutated mRNA had a unique behavioral function unrelated to the normal purpose of the *y* gene (Muller 1932). Our data (Table 2, Figure 2) suggest that the mating success phenotype of y^1 is equivalent to that of males carrying either of two null alleles caused by the deletions $Df(1)y\text{-}ac^{22}$ and y^{59b} (Table 1). We have also shown that y^1 has a more severe MMS phenotype than two mild hypomorphs for mating behavior, y^{a77} and y^{2S} (Figures 2 and 3, Table 2). These behavior–genetic data, combined with immunocytochemical evidence that a new anti-Yellow antibody does not recognize any proteins in either y^1 or $Df(1)y\text{-}ac^{22}$ flies (Figure 5, B and E; see also Drapeau 2001b; Radovic *et al.* 2002; Wittkopp *et al.* 2002a; Drapeau *et al.* 2003), fulfill the requirements of a null hemizygous allele classification for y^1 with regard to male mating success (Muller 1932).

A regulatory sequence controlling male sexual behavior: We used *y* mutant alleles with known molecular lesions as a tool for mapping the regulatory sequence required for normal MMS (Figure 4). In addition to the behavioral data collected from males carrying *y* null alleles, the utilization of a y^+ minichromosome to control genetic and environmental backgrounds (Figure 1), combined with the fact that four hypomorphic *y* alleles with independent origins all significantly reduced male mating success (Table 2, Figure 3), is strong evidence for an overall requirement for *y* in the specification of adult male sexual behavior. Following the classic example of the per^s, per^L, and per^0 alleles shortening, lengthening, and abolishing behavioral circadian rhythms, respectively (Konopka and Benzer 1971), some of the most convincing evidence that a gene is directly involved in the control of a behavior is the detection of a range of phenotypic effects of different alleles at the locus. We found statistically significant variation in MMS among the four hypomorphic *y* alleles that each individually decreased MMS, y^{-91}, y^{3d}, y^{a77}, and y^{2S}. In addition, the nonsignificant MMS results for the remaining eight *y* hypomorphic alleles with mild-to-severe effects on pigmentation

imply that the positive results that we found were not spurious. Our molecular and behavioral data demarcate a region of ∼300 bp whose function is required for normal levels of male mating success.

Two past studies (Burnet and Wilson 1980; Robertson 1982) reported data from a small number of non-null alleles of *y* whose molecular underpinnings were not known at the time. These two studies reported data collected using a wide variety of experimental designs, varying the number of days the flies spent courting and mating, the size of the mating chamber and whether or not food was present in the chamber, the number of males and females simultaneously in the chamber, the crossing schemes to generate mutant and control males, and the level of inbreeding of the assayed flies. These varied designs yielded a spectrum of results. For example, both studies investigated the y^2 mutant, which we found to have no effect on male mating success (Table 2, Figure 3). Across all experiments performed by Burnet and Wilson (1980) and Robertson (1982), they measured MMS phenotypes ranging from a strong disadvantage to a slight advantage of y^2 relative to controls.

In this study, using a standardized, outbred genetic background across all mutants and all controls (Figure 1) and quantifying behavior of individual F_1 mutant or control males with single females ("single-pair matings") simultaneously in the same mating apparatus with moderate-sized 25-mm-diameter × 20-mm-deep behavioral chambers (Drapeau and Long 2000) allowed us to accurately and sensitively measure the effects of different *y* mutations on MMS. The minichromosome-rescue strategy employed in our study exquisitely controls genetic and environmental backgrounds between mutant and control subjects, allowing confident measurement of behavior in fully outbred flies. This strategy or similar ones should be useful in behavior–genetic studies on other loci.

We hypothesized that regulatory variants of *y* that reduced MMS would define a small region of the locus whose function is required for a complex, sex-limited adult behavioral performance. The behavior–genetic data that we collected define an ∼300-bp region upstream of the *y* transcription start site that contains a regulatory sequence required for normal MMS, the sequence we termed the MRS. Conservatively, the 5′ limit of the MRS was defined by the $y^{-300gin}$ mutation that carried the *gypsy* insulator at −300 bp relative to *y* transcription start and the 3′ limit by the y^{a77} insertion at −4 bp (Figure 4). It is likely that the 209-bp sequence between −300 and −91 bp contains the sequence(s) required for MMS, since males carrying the y^{-91} deletion mutant have severely decreased mating success, while $y^{-300gin}$ mutant males do not (Table 2, Figures 3 and 4). We propose that the mutants with insertions that are located in the 300-bp region upstream of the transcription start, y^{3d}, y^{a77}, and y^{2S}, reduce or eliminate the function of the 209-bp element, which in turn lowers male mating success to varying degrees (Table 2, Figures 3 and 4).

While this is the most parsimonious interpretation of the data, the true regulation of *y* with regard to MMS may be more complicated. The y^{2S} allele, for example, causes a slight reduction in MMS, and experiment-wide significance was detectable only with a large sample size (Table 2, Figure 3). Within trials, effects of this mutant allele were sometimes significantly different and sometimes not distinct from the control males (data not shown). The underlying cause of this variability is currently unclear. It is possible that the y^{2S} insertion stochastically recruits factors that can substitute for the MRS. Another complicated interpretation is data collected from males carrying the y^{-893} allele. These males had a nonsignificant difference in their overall MMS, but the percentage of success measured for this genotype was similar to that of y^{2S} males (45.3% *vs.* 44.9%). The overall sample sizes for these two genotypes also differed (123 *vs.* 335), giving us more statistical power to detect a difference in y^{2S} than in y^{-893} (Table 2). Our current interpretation is guided by the fact that four other mutant alleles affecting the same region of the *y* locus as y^{-893}, upstream of −300 bp, did not cause a significant reduction of MMS, and likewise, three mutants affecting the same region as y^{2S}, −300 to 0 bp, significantly reduced MMS (Table 2, Figures 3 and 4). The finding that y^{-893} flies do not have Yellow in their third instar CNS (Figure 5F, compare to Figure 5, A and B) further clouds our interpretation. It is possible that the MMS and Yellow protein reduction in y^{-893} flies is not caused by an intrinsic regulatory defect at *y*, but rather because of a position effect due to insertion of the transgene at an ectopic site. Alternatively, it may be the case that while these Yellow$^+$ third instar neural cells are associated with adult MMS, they are part of a more complex story. Only a careful series of behavior–genetic experiments with Gal4-mediated somatic neural y^+ mosaic flies, complementing data in this study, will shed light on these issues.

To put our mapping resolution (*i.e.*, a 209-bp *cis*-regulatory region required for normal behavior) into perspective, earlier investigations of sequences within the *y* locus controlling pigmentation defined these regulatory regions as ranging in size from ∼130 to 1200 bp (Geyer and Corces 1987, 1992; Martin *et al.* 1989). One recent study of *D. melanogaster* courtship genetics had a focus similar to ours. Boll and Noll (2002) mapped two adjacent *cis*-regulatory regions necessary for normal male mating speed/time to copulation within a *Pox neuro* gene to one region of ∼2 kb and one of ∼10 kb. This behavioral phenotype appears to be correlated with expression of the *Pox neuro* transcription factor in the adult brain. It is not presently clear what the relationship is, if any, between *Pox neuro* and other courtship genes such as *fru* and *y*.

Within the broadly defined 300-bp MRS, we identified four SNP variants, one common and three rare, among a group of 85 wild-type *D. melanogaster* strains. These SNPs may contribute to naturally occurring variation in male sexual behavior. However, unlike the mutants in Table 1 that had severe (or no) effects on MMS, any effect of these SNP variants on male sexual behavior is expected to be minor. Such small effects can be difficult to detect because of the greater behavioral scrutiny required to find slight differences between mutants and controls, the large sample sizes needed to detect small phenotypic effects, and genetic epistasis and environmental influences that can obscure the primary effect of the *y* mutation. Furthermore, because the *y* locus is on the *X* chromosome, the SNPs cannot be used in a simple quantitative complementation test (Long *et al.* 1996). Alternatively, these SNPs may be neutral mutations with respect to male sexual behavior.

Adult male pigmentation and behavior are not correlated: Another way in which we have extended previous work on the *y* gene and male sexual behavior is by demonstrating that there is no obvious relationship between two adult phenotypes controlled by *y*. We found that cuticle pigmentation in various adult tissues was not predictive of *y* mutant male mating success (Table 3). This is an interesting negative result, because there are numerous mechanisms by which pigment/color alteration can cause differences in male mating success (*e.g.*, Burnet and Connolly 1974; Singh and Chatterjee 1987). In total, our data indicate that the adult pigmentation and male sexual behavior phenotypes are developmentally regulated independently. Indeed, the separate, modular control of *y* transcription with regard to adult pigmentation (*e.g.*, Geyer and Corces 1987; Martin *et al.* 1989; Gompel *et al.* 2005) and adult behavior (this study) suggests that individual *cis*-regulatory modules within a pleiotropic gene provide a mechanism by which multiple evolutionarily important traits can separately evolve.

Molecular function of the mating-success regulatory sequence: The data presented in this report provide a starting point for understanding the transcriptional regulation of the *y* gene with respect to male sexual behavior. The MRS now defined, we ask, what is its function? An important clue comes from previous studies of *cis*-regulatory sequences of *y*-controlling pigmentation. The sequence between −300 and −91 bp is required for normal levels of larval pigmentation (Geyer and Corces 1987; Martin *et al.* 1989), implying that this sequence is required for upregulating *y* transcription in specific larval tissues that normally are pigmented (*e.g.*, Walter *et al.* 1991). Larval pigmentation is unlikely to be behaviorally relevant in the adult male, since these pigmented structures disappear during metamorphosis. (Furthermore, y^1 larvae act normally in at least one larval behavioral paradigm; see Mazzoni *et al.* 2005.) However, at a molecular level, the 209-bp region probably contains enhancer sequences that bind transcription factors required for normal development of both larval pigmentation and adult male behavior. Enhancers are (1) often identified with selective gene expression in particular tissues, (2) distance independent, and (3) orientation independent. While the MRS/larval pigment control region DNA appears to direct *y* expression in specific tissues, distance independence and orientation independence remain to be tested for this *y* regulatory region. Following the approach of Geyer and Corces (1987), future work can focus on determining whether the MRS region functions as a transcriptional enhancer.

Candidate transcription factors for regulation of *y* via the MRS: Our working model is that the MRS binds factors that activate *y* transcription in CNS cells relevant to male sexual behavior (Radovic *et al.* 2002; Drapeau *et al.* 2003). One factor that might bind within the MRS is the male-specific FRU isoform BM, notated $FRU^{M(BM)}$, which we have shown by temporal, spatial, and sexual misexpression to be sufficient for Yellow accumulation, as detected by immunohistochemistry and Western blotting (Drapeau *et al.* 2003). Experiments with fru^1, fru^3, and fru^4 mutants suggest that the zinc-finger regions of FRU are required for Yellow production in these cells (Drapeau *et al.* 2003). On the basis of the results reported here, we hypothesize that $FRU^{M(BM)}$ or a downstream target of $FRU^{M(BM)}$ binds within the MRS to activate *y* transcription in larval neural cells crucial for the development of adult male behaviors.

The specific function of the 60-kDa Yellow protein is unknown, but there are hypotheses about its requirement in pigmentation and behavior. Yellow is secreted from the cells where the *y* gene is expressed (Hannah 1953; Geyer *et al.* 1986; Kornezos and Chia 1992; Radovic *et al.* 2002; Wittkopp *et al.* 2002a; Drapeau *et al.* 2003). Assuming that Yellow is diffusable, it may then influence the properties of nearby cells in the CNS via a hormone- or growth-factor-like mechanism (Drapeau 2003). An alternative but nonmutually exclusive hypothesis is that Yellow crosslinks dopamine derivatives during melanization (Geyer *et al.* 1986). This function requires the amino acid cysteine (Cys), and indeed Yellow is Cys-rich, unlike other cuticle proteins. If Yellow has this function in the CNS, it could exert its effect through a melanin-signaling pathway. Either of these proposed Yellow functions could be relevant to the construction of a neural circuit subserving male courtship behavior (Baker *et al.* 2001; Stockinger *et al.* 2005).

In this report, we presented evidence that the transcription factors Dl and DSX may bind within a highly conserved 35-bp region within the MRS. DSX is an excellent candidate to be a direct regulator of *y* expression. First, as previously mentioned, it is a member of the sex determination cascade and is expressed in the CNS, and mutations in *dsx* alter sex-specific

behaviors. Second, the normal function of the *takeout* gene with regard to normal male courtship is dependent on the function of both *fru* and *dsx* (Dauwalder *et al.* 2002), and therefore this may reasonably apply to *y* as well. (We did not investigate regulation by *dsx* during our previous work with *fru* and *y*.) Third, the sex-specific DSX^M and DSX^F proteins that share binding sites suggest a mechanism by which *y* would be upregulated in males and suppressed in females (see, for example, Waterbury *et al.* 1999; Dauwalder *et al.* 2002). While this discussion could stem from mere coincidence, our bioinformatic data provide fruitful avenues for future molecular-genetic and biochemical research on the MRS. We speculate that the well-studied transcription factors Dorsal and Doublesex bind within a small, highly conserved sequence in the *y* locus to, in collaboration with $FRU^{M(BM)}$, control behavioral phenotypes.

Functional analysis of the MRS: Using genetic and biochemical methods, we presented evidence that the MRS directs expression of *y* in a small number of cells in the third instar CNS and that Yellow protein in these cells is associated with normal MMS. On the basis of this, we hypothesize that the presence of Yellow in these third instar neural cells is required for the development of normal male courtship behavior (specifically, WE), with normal mating success as a consequence. Our earlier demonstration that a global third instar CNS Gal4 driver combined with UAS-y^+ in a y^1 background is sufficient to rescue low levels of WE and MMS supports this idea (Drapeau *et al.* 2003). However, with our current data we cannot exclude that *y* expression in the CNS at other stages of development may play a role, nor can we exclude that *y* expression in other tissues is relevant, with the exception of the adult body cuticle (see results).

The next step in this line of research is to study the MRS in isolation and understand its properties with regard to transcriptional regulation. In this work, we look to the case of the "E-box" sequence and its role in the generation of circadian molecular rhythms and overt rhythmic behavior. A 69-bp regulatory sequence was mapped, then shown to act as an enhancer of *period* transcription, and subsequently dissected further using molecular and biochemical techniques to demonstrate that the shorter E-box sequence within the original 69 bp was sufficient to generate rhythms (*e.g.*, Hao *et al.* 1997, 1999; Darlington *et al.* 1998). Future work on the MRS within the *y* locus should shed light on the molecular-genetic mechanisms underlying a complex adult instinct with relevance to reproductive isolating mechanisms and speciation.

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LITERATURE CITED

Akiyama, Y., 1998 TFSEARCH: searching transcription factor binding sites, http://www.rwcp.or.jp/papia/ (http://www.cbrc.jp/research/db/TFSEARCH-notice.html).

Allada, R., P. Emery, J. S. Takahashi and M. Rosbash, 2001 Stopping time: the genetics of fly and mouse circadian clocks. Annu. Rev. Neurosci. **24:** 1091–1119.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. **15:** 403–410.

Arthur, B. I., Jr., J.-M. Jallon, B. Caflisch, Y. Choffat and R. Nöthiger, 1998 Sexual behavior in *Drosophila* is irreversibly programmed during a critical period. Curr. Biol. **8:** 1187–1190.

Baker, B. S., and K. A. Ridge, 1980 Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster.* Genetics **94:** 383–423.

Baker, B. S., B. J. Taylor and J. C. Hall, 2001 Are complex behaviors specified by dedicated regulatory genes? Reasoning from *Drosophila.* Cell **105:** 13–24.

Barker, J. S. F., 1962 Studies of selective mating using the *yellow* mutant of *Drosophila melanogaster.* Genetics **47:** 623–640.

Bastock, M., 1956 A gene mutation which changes a behavior pattern. Evolution **10:** 421–439.

Bastock, M., 1967 *Courtship: An Ethological Study.* Aldine, Chicago.

Bastock, M., and A. Manning, 1955 The courtship of *Drosophila melanogaster.* Behaviour **8:** 85–111.

Belote, J. M., and B. S. Baker, 1987 Sexual behavior—its genetic control during development and adulthood in *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA **84:** 8026–8030.

Biessmann, H., 1985 Molecular analysis of the *yellow* gene (*y*) region of *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA **82:** 7369–7373.

Biessmann, H., and J. M. Mason, 1988 Progressive loss of DNA sequences from terminal chromosome deficiencies in *Drosophila melanogaster.* EMBO J. **7:** 1081–1086.

Boll, W., and M. Noll, 2002 The *Drosophila Pox neuro* gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers. Development **129:** 5667–5681.

Bradbury, J. W., and S. L. Vehrencamp, 1998 *Principles of Animal Communication.* Sinauer Associates, Sunderland, MA.

Bradman, S. R., S. A. Espinet and M. L. Tracey, 1981 Normal and *T(X;Y) Drosophila melanogaster* male courtship success. Genetics **97:** s14.

Brand, A. H., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

Burnet, B., and K. Connolly, 1974 Activity and sexual behaviour in *Drosophila melanogaster,* pp. 201–258 in *The Genetics of Behaviour,* edited by J. H. F. van Abeelen. American Elsevier, New York.

Burnet, B., and R. Wilson, 1980 Pattern mosaicism for behaviour controlled by the *yellow* locus in *Drosophila melanogaster.* Genet. Res. **36:** 235–247.

Burnet, B., K. J. Connolly and B. Harrison, 1973 Phenocopies of pigmentary and behavioral effects of the *yellow* mutant in *Drosophila* induced by α-dimethyltyrosine. Science **181:** 1059–1060.

Burtis, K. C., and B. S. Baker, 1989 Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell **56:** 997–1010.

Burtis, K. C., K. T. Coschigano, B. S. Baker and P. C. Wensink, 1991 The *doublesex* proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. EMBO J. **10:** 2577–2582.

Calleja, M., E. Moreno, S. Pelaz and G. Morata, 1996 Visualization of gene expression in living adult Drosophila. Science **274:** 252–255.

Campuzano, S., L. Carramolino, C. V. Cabrera, M. Ruiz-Gomez, R. Villares, 1985 Molecular genetics of the *achaete-scule* complex of *D. melanogaster.* Cell **40:** 327–338.

Carew, T. J., 2000 *Behavioral Neurobiology.* Sinauer Associates, Sunderland, MA.

Chia, W., G. Howes, M. Martin, Y. B. Meng, K. Moses *et al.*, 1986 Molecular analysis of the *yellow* locus of *Drosophila.* EMBO J. **5:** 3597–3605.

Cobb, M., and J. F. Ferveur, 1996 Evolution and genetic control of mate recognition and stimulation in *Drosophila.* Behav. Processes **35:** 35–54.

Coschigano, K. T., and P. C. Wensink, 1993 Sex-specific transcriptional regulation by the male and female *doublesex* proteins of Drosophila. Genes Dev. **7:** 42–54.

Crabbe, J. C., D. Wahlsten and B. C. Dudek, 1999 Genetics of mouse behavior: interactions with laboratory environment. Science **284:** 1670–1672.

Darlington, T. K., K. Wager-Smith, M. F. Ceriani, D. Staknis, N. Gekakis *et al.*, 1998 Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim.* Science **280:** 1599–1603.

Darwin, C., 1874 *The Descent of Man, and Selection in Relation to Sex*, Ed. 2. Hurst, New York.

Da Silva, L. B., D. F. Leite, V. L. S. Valente and C. Rohde, 2005 Mating activity of *yellow* and *sepia Drosophila willistoni* mutants. Behav. Processes **70:** 149–155.

Dauwalder, B., S. Tsujimoto, J. Moss and W. Mattox, 2002 The Drosophila *takeout* gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. Genes Dev. **16:** 2879–2892.

de Belle, J. S., and M. Heisenberg, 1996 Expression of Drosophila mushroom body mutations in alternative genetic backgrounds: a case study of the *mushroom body miniature* gene (*mbm*). Proc. Natl. Acad. Sci. USA **93:** 9875–9880.

de Belle, J. S., A. J. Hilliker and M. B. Sokolowski, 1989 Genetic localization of *foraging* (*for*), a major gene for larval behavior in *Drosophila melanogaster.* Genetics **123:** 157–163.

Demir, E., and B. J. Dickson, 2005 *fruitless* splicing specifies male courtship behavior in Drosophila. Cell **121:** 785–794.

Diederich, G. W., 1941 Non-random mating between *yellow-white* and wild type *Drosophila melanogaster.* Genetics **26:** 148.

Dow, M. A., 1975 Environmental modification of a genetically based behaviour pattern in *D. melanogaster.* Nature **255:** 172.

Dow, M. A., 1977a Density and the mating success of *yellow* mutant males. Dros. Inf. Serv. **52:** 70.

Dow, M. A., 1977b Artificial selection for *yellow* mating success. Dros. Inf. Serv. **52:** 76.

Dow, M. A., 1977c Light regime and temperature effects on mating success. Dros. Inf. Serv. **52:** 104.

Dow, M. A., 1977d Multiple male courtship in *D. melanogaster.* Dros. Inf. Serv. **52:** 116.

Drapeau, M. D., 2001a Beyond heritability: the future of behavioral genetics. Trends Genet. **17:** 561–562.

Drapeau, M. D., 2001b The family of Yellow-related *Drosophila melanogaster* proteins. Biochem. Biophys. Res. Commun. **281:** 611–613.

Drapeau, M. D., 2003 A novel hypothesis on the biochemical role of the Drosophila Yellow protein. Biochem. Biophys. Res. Commun. **311:** 1–3.

Drapeau, M. D., and A. D. Long, 2000 The Copulatron, a multi-chamber apparatus for observing *Drosophila* courtship behaviors. Dros. Inf. Serv. **83:** 194–196.

Drapeau, M. D., A. Radovic, P. J. Wittkopp and A. D. Long, 2003 A gene necessary for normal male courtship, *yellow,* acts downstream of *fruitless* in the *Drosophila melanogaster* larval brain. J. Neurobiol. **55:** 53–72.

Erdman, S. E., H. J. Chen and K. C. Burtis, 1996 Functional and genetic characterization of the oligomerization and DNA binding properties of the Drosophila Doublesex protein. Genetics **144:** 1639–1652.

FlyBase, 2003 The FlyBase database of the Drosophila genome projects and community literature. Nucleic Acids Res. **31:** 172–175 (http://flybase.bio.indiana.edu/).

Frias, D., and M. Lamborot, 1970 Reproductive isolation between the *yellow, white,* and "wild" stocks of *D. gaucha* at two temperatures (in Spanish). Arch. Biol. Med. Exp. **7:** 67.

Gaines, P., L. Tompkins, C. T. Woodard and J. R. Carlson, 2000 *quick-to-court*, a Drosophila mutant with elevated levels of sexual behavior, is defective in a predicted coiled-coil protein. Genetics **154:** 1627–1637.

Geyer, P. K., and V. G. Corces, 1987 Separate regulatory elements are responsible for the complex pattern of tissue-specific and developmental transcription of the *yellow* locus in *Drosophila melanogaster.* Genes Dev. **1:** 996–1004.

Geyer, P. K., and V. G. Corces, 1992 DNA position-specific repression of transcription by a *Drosophila* zinc-finger protein. Genes Dev. **6:** 1865–1873.

Geyer, P. K., C. Spana and V. G. Corces, 1986 On the molecular mechanism of *gypsy*-induced mutations at the *yellow* locus of *Drosophila melanogaster.* EMBO J. **5:** 2657–2662.

Geyer, P. K., M. M. Green and V. G. Corces, 1990 Tissue-specific transcriptional enhancers may act in *trans* on the gene located in the homologous chromosome: the molecular basis of transvection in *Drosophila.* EMBO J. **9:** 2247–2256.

Giot, L., J. S. Bader, C. Brouwer, A. Chaudhuri, B. Kuang *et al.*, 2003 A protein interaction map of *Drosophila melanogaster.* Science **302:** 1727–1736.

Gompel, N., B. Prud'homme, P. J. Wittkopp, V. A. Kassner and S. B. Carroll, 2005 Chance caught on the wing: *cis*-regulatory evolution and the origin of pigment patterns in Drosophila. Nature **433:** 481–487.

Goodwin, S. F., 1999 Molecular neurogenetics of sexual differentiation and behavior. Curr. Opin. Neurobiol. **9:** 759–765.

Green, M. M., 1961 Complementation at the *yellow* locus in *Drosophila melanogaster.* Genetics **46:** 1385–1388.

Greenspan, R. J., 1997 *Fly Pushing: The Theory and Practice of Drosophila Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Greenspan, R. J., and J. F. Ferveur, 2000 Courtship in Drosophila. Annu. Rev. Genet. **34:** 205–232.

Grosjean, Y., M. Savy, J. Soichot, C. Everaerts, F. Cezilly *et al.*, 2004 Mild mutations in the pan neural gene *prospero* affect male-specific behaviour in *Drosophila melanogaster.* Behav. Processes **65:** 7–13.

Hall, J. C., 1979 Control of male reproductive behavior by the central nervous system of Drosophila: dissection of a courtship pathway by genetic mosaics. Genetics **92:** 437–457.

Hall, J. C., 1994a The mating of a fly. Science **264:** 1702–1714.

Hall, J. C., 2002 Courtship lite: a personal history of reproductive behavioral neurogenetics in Drosophila. J. Neurogenet. **16:** 135–163.

Hall, J. C., R. J. Greenspan and W. A. Harris, 1982 *Genetic Neurobiology.* The MIT Press, Cambridge, MA.

Hamerlynck, M. J., 1994 The effect of egg load on female mating decisions in *Drosophila melanogaster.* M.S. Thesis, Department of Biology, University of Oregon, Eugene, OR.

Hannah, A., 1953 Non-autonomy of *yellow* in gynandromorphs of *Drosophila melanogaster.* J. Exp. Zool. **123:** 523–560.

Hao, H., D. L. Allen and P. E. Hardin, 1997 A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster.* Mol. Cell. Biol. **17:** 3687–3693.

Hao, H., N. R. J. Glossop, L. Lyons, J. Qiu, B. Morrish *et al.*, 1999 The 69 bp circadian regulatory sequence (CRS) mediates *per*-like developmental, spatial, and circadian expression and behavioral rescue in *Drosophila.* J. Neurosci. **19:** 987–994.

Harrison, D. A., P. K. Geyer, C. Spana and V. G. Corces, 1989 The *gypsy* retrotransposon of *Drosophila melanogaster*: mechanisms of mutagenesis and interaction with the *suppressor of Hairy-wing* locus. Dev. Genet. **10:** 239–248.

Hebets, E. A., 2003 Subadult experience influences adult mate choice in an arthropod: exposed wolf spiders prefer males of a familiar phenotype. Proc. Natl. Acad. Sci. USA **100:** 13390–13395.

Hebets, E. A., and G. W. Uetz, 1999 Female responsiveness to isolated signals from multimodal male courtship displays in the wolf spider genus Schizocosa (Araneae: Lycosidae). Anim. Behav. **57:** 865–872.

Heinemeyer, T., E. Wingender, I. Reuter, H. Hermjakob, A. E. Kel *et al.*, 1998 Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL. Nucleic Acids Res. **26:** 364–370.

Heinrichs, V., L. C. Ryner and B. S. Baker, 1998 Regulation of sex-specific selection of *fruitless* 5′ splice sites by *transformer* and *transformer-2*. Mol. Cell. Biol. **18:** 450–458.

Heisler, I. L., 1984 Inheritance of female mating propensities for *yellow* locus genotypes in *Drosophila melanogaster.* Genet. Res. **44:** 133–149.

Hildreth, P. E., 1965 *doublesex*, a recessive gene that transforms both males and females of Drosophila into intersexes. Genetics **51:** 659–678.

Ito, H., K. Fujitani, K. Usui, K. Shimizu-Nishikawa, S. Tanaka *et al.*, 1996 Sexual orientation in *Drosophila* is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. Proc. Natl. Acad. Sci. USA **93:** 9687–9692.

Judson, O., 2002 *Dr. Tatiana's Sex Advice to All Creation*. Metropolitan Books, Henry Holt, New York.

Jursnich, V. A., and K. C. Burtis, 1993 A positive role in differentiation for the male Doublesex protein of Drosophila. Dev. Biol. **155:** 235–249.

Konopka, R. J., and S. Benzer, 1971 Clock mutants of *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA **68:** 2112–2116.

Kopp, A., and J. R. True, 2002 Evolution of male sexual characters in the oriental *Drosophila melanogaster* species group. Evol. Dev. **4:** 278–291.

Kopp, A., I. Duncan, D. Godt and S. B. Carroll, 2000 Genetic control and evolution of sexually dimorphic characters in Drosophila. Nature **408:** 553–559.

Kornezos, A., and W. Chia, 1992 Apical secretion and association of the Drosophila *yellow* gene product with developing larval cuticular structures during embryogenesis. Mol. Gen. Genet. **235:** 397–405.

Lee, G., and J. C. Hall, 2001 Abnormalities of male-specific FRU protein and serotonin expression in the CNS of *fruitless* mutants. J. Neurosci. **21:** 513–526.

Lee, G., M. Foss, S. F. Goodwin, T. Carlo, B. J. Taylor *et al.*, 2000 Spatial, temporal, and sexually dimorphic expression patterns of the *fruitless* gene in the Drosophila central nervous system. J. Neurobiol. **43:** 404–426.

Lee, G., J. C. Hall and J. H. Park, 2002 *doublesex* gene expression in the central nervous system of *Drosophila melanogaster.* J. Neurogenet. **16:** 229–248.

Lindsley, D. L., and E. H. Grell, 1968 *Genetic variations of Drosophila melanogaster,* Pub. 627. Carnegie Institute, Washington, DC.

Lindsley, D. L., and G. G. Zimm, 1992 *The Genome of Drosophila melanogaster.* Academic Press, San Diego.

Long, A. D., S. L. Mullaney, T. F. Mackay and C. H. Langley, 1996 Genetic interactions between naturally occurring alleles at quantitative trait loci and mutant alleles at candidate loci affecting bristle number in *Drosophila melanogaster.* Genetics **144:** 1497–1510.

Manoli, D. S., M. Foss, A. Villella, B. J. Taylor, J. C. Hall *et al.*, 2005 Male-specific *fruitless* specifies the neural substrates of Drosophila courtship. Nature **436:** 395–400.

Markow, T. A., 1981 Genetic and sensory aspects of mating success of phototactic strains of *Drosophila melanogaster.* Behav. Genet. **11:** 273–279.

Markow, T. A., and M. Manning, 1982 Female olfaction, vision, and reproductive isolation between *D. melanogaster* females and *D. simulans* males. Dros. Inf. Serv. **58:** 104–105.

Markstein, M., P. Markstein, V. Markstein and M. S. Levine, 2002 Genome-wide analysis of clustered Dorsal binding sites identifies putative target genes in the Drosophila embryo. Proc. Natl. Acad. Sci. USA **99:** 763–768.

Martin, M., Y. B. Meng and W. Chia, 1989 Regulatory elements involved in the tissue-specific expression of the *yellow* gene of *Drosophila*. Mol. Gen. Genet. **218:** 118–126.

Mayr, E., 1950 The role of the antennae in the mating behavior of female *Drosophila*. Evolution **4:** 149–154.

Mazzoni, E. O., C. Desplan and J. Blau, 2005 Circadian pacemaker neurons transmit and modulate visual information to control a rapid behavioral response. Neuron **45:** 293–300.

Merrell, D. J., 1949 Selective mating in *Drosophila melanogaster.* Genetics **34:** 370–389.

Meyers, H. L., Jr., 1953 Natural selection against the sex-linked gene for yellow body color in *Drosophila melanogaster.* M.S. Thesis, Michigan State College of Agriculture and Applied Science, East Lansing, MI.

Mizuguchi, Y., and J. R. de Almeida, 1983 Effect of *yellow* mutant on mating speed and duration of copulation in *Drosophila melanogaster* Meigen, 1830. Anais de Reuniao Anual. Sociedade Brasiliera de Entomol. **12:** 145–150.

Morris, D. M. F., 1970 *Patterns of Reproductive Behaviour: Collected Papers by Desmond Morris.* McGraw-Hill, New York.

Morris, J. R., J.-l. Chen, S. T. Filandrinos, R. C. Dunn, R. Fisk *et al.*, 1999 An analysis of transvection at the *yellow* locus of *Drosophila melanogaster.* Genetics **151:** 633–651.

Moses, A. M., D. Y. Chiang, M. Kellis, E. S. Lander and M. B. Eisen, 2003 Position-specific variation in the rate of evolution in transcription factor binding sites. BMC Evol. Biol. **3:** 19.

Muller, H. J., 1932 Further studies on the nature and causes of gene mutations. Proceedings of the Sixth International Congress of Genetics, Vol. 1, pp. 213–255.

Munté, A., M. Aguadé and C. Segarra, 1997 Divergence of the *yellow* gene between *Drosophila melanogaster* and *D. subobscura*: recombination rate, codon bias and synonymous substitutions. Genetics **147:** 165–175.

Oakeshott, J. G., and D. L. Hayman, 1979 Effects of genotype and light on mating preference in *Drosophila melanogaster.* Aust. J. Biol. Sci. **32:** 607–614.

Orgad, S., G. Rosenfeld, R. J. Greenspan and D. Segal, 2000 *Courtless*, the Drosophila UBC7 homolog, is involved in male courtship behavior and spermatogenesis. Genetics **155:** 1267–1280.

Panda, S., J. B. Hogenesch and S. A. Kay, 2002 Circadian rhythms from flies to humans. Nature **417:** 329–335.

Parkhurst, S. M., and V. G. Corces, 1986 Interactions among the *gypsy* transposable element and the *yellow* and *Suppressor of Hairy wing* loci in *Drosophila melanogaster.* Mol. Cell. Biol. **6:** 47–53.

Parnell, T. J., M. M. Viering, A. Skjesol, C. Helou, E. J. Kuhn *et al.*, 2003 An endogenous Suppressor of Hairy-wing insulator separates regulatory domains in *Drosophila*. Proc. Natl. Acad. Sci. USA **100:** 13436–13441.

Piccin, A., A. Salameh, C. Benna, F. Sandrelli, G. Mazzotta *et al.*, 2001 Efficient and heritable knock-out of an adult phenotype in Drosophila using a GAL4-driven hairpin RNA incorporating a heterologous spacer. Nucleic Acids Res. **29:** E55.

Pruzan-Hotchkiss, A., K. Sato and J. F. Thompson, 1992 Genetic and behavioral studies on *yellow Drosophila*. Behav. Genet. **22:** 747.

Radovic, A., P. J. Wittkopp, A. D. Long and M. D. Drapeau, 2002 Immunohistochemical colocalization of Yellow and male-specific Fruitless in *Drosophila melanogaster* neuroblasts. Biochem. Biophys. Res. Commun. **293:** 1262–1264.

Rendel, J. M., 1944 Genetics and cytology of *Drosophila subobscura.* II. Normal and selective matings in *Drosophila subobscura*. J. Genet. **46:** 287–302.

Robertson, H. M., 1982 Studies on the mating behaviour of some species of Zygoptera (Odonata) and Drosophila (Diptera). Ph.D. Thesis, University of the Witwatersrand, Johannesburg, South Africa.

Robertson, K. A., and A. Monteiro, 2005 Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. Proc. R. Soc. Lond. Ser. B **272:** 1541–1546.

Romanova, L. G., N. I. Romanova, E. A. Subocheva and A. I. Kim, 2000 Mating success and courtship ritual in strains of *Drosophila melanogaster* carrying mutation *flamenco* (in Russian). Genetika **36:** 500–504.

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of *Drosophila* with transposable element vectors. Science **218:** 348–353.

Ryner, L. C., S. F. Goodwin, D. H. Castrillon, A. Anand, A. Villella *et al.*, 1996 Control of male sexual behavior and sexual orientation in *Drosophila* by the *fruitless* gene. Cell **87:** 1079–1089.

Schroeck, J. A., 1971 Influence of the *yellow* allele on the mating behavior of *Drosophila melanogaster.* M.S. Thesis, Mankato State College, Mankato, MN.

Singh, B. N., and S. Chatterjee, 1987 Greater mating success of *Drosophila biarmipes* males possessing an apical dark black wing patch. Ethology **75:** 81–83.

Sinha, S., M. D. Schroeder, U. Unnerstall, U. Gaul and E. D. Siggia, 2004 Cross-species comparison significantly improves genome-wide prediction of *cis*-regulatory modules in Drosophila. BMC Bioinformatics **5:** 129.

Sokal, R. R., and F. J. Rohlf, 1995 *Biometry,* Ed. 3. W. H. Freeman, New York.

Sokolowski, M. B., 2001 Drosophila: genetics meets behavior. Nat. Rev. Genet. **2:** 879–890.

Spett, G., 1931 Gibt e seine partielle sexuelle isolation unter den mutationen und der grundform von *Drosophila melanogaster* Meig.? Z. Indukt. Abstamm. Vererbungsl. **60:** 63–83.

Spieth, H. T., 1952 Mating behavior within the genus Drosophila (Diptera). Bull. Am. Mus. Nat. Hist. **99:** 395–474.

Spieth, H. T., 1974 Courtship behavior in Drosophila. Annu. Rev. Entomol. **19:** 385–405.

Stockinger, P., D. Kvitsiani, S. Rotkopf, L. Tirian and B. J. Dickson, 2005 Neural circuitry that governs Drosophila male courtship behavior. Cell **121:** 795–807.

Sturtevant, A. H., 1915 Experiments on sex recognition and the problem of sexual selection in *Drosophila.* J. Anim. Behav. **5:** 351–366.

Subocheva, E. A., N. I. Romanova, N. N. Karpova, A. O. Iuneva and A. I. Kim, 2003 Male reproductive behavior in *Drosophila melanogaster* strains with different alleles of the *flamenco* gene (in Russian). Genetika **39:** 675–681.

Takano-Shimizu, T., 1999 Local recombination and mutation effects on molecular evolution in Drosophila. Genetics **153:** 1285–1296.

Tan, C. C., 1946 Genetics of sexual isolation between *Drosophila pseudoobscura* and *Drosophila persimilis.* Genetics **31:** 558–573.

Thisse, C., F. Perrin-Schmitt, C. Stoetzel and B. Thisse, 1991 Sequence-specific transactivation of the Drosophila *twist* gene by the *dorsal* gene product. Cell **65:** 1191–1201.

Threlkeld, S. F. H., R. A. Procwat, K. S. Abbott and A. D. Yeung, 1974 Genetically based behaviour patterns in *Drosophila melanogaster.* Nature **247:** 232–233.

Tower, J., G. H. Karpen, N. Craig and A. C. Spradling, 1993 Preferential transposition of Drosophila *P* elements to nearby chromosomal sites. Genetics **133:** 347–359.

Tracey, M. L., and S. A. Espinet, 1976 Sex chromosome translocations and speciation. Nature **263:** 321–323.

Tracey, M. L., and S. A. Espinet, 1977 Evidence of a *yellow* position-effect altering male mating advantage in X.Y^L-Y^S *Drosophila melanogaster.* Can. J. Genet. Cytol. **19:** 581–582.

True, J. R., K. A. Edwards, D. Yamamoto and S. B. Carroll, 1999 Drosophila wing melanin patterns form by vein-dependent elaboration of enzymatic prepatterns. Curr. Biol. **9:** 1382–1391.

Truman, J. W., B. J. Taylor and T. A. Awad, 1993 Formation of the adult nervous system, pp. 1245–1275 in *The Development of Drosophila melanogaster,* edited by M. Bate and A. Martinez-Arias, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Villella, A., and J. C. Hall, 1996 Courtship anomalies caused by *doublesex* mutations in *Drosophila melanogaster.* Genetics **143:** 331–344.

Villella, A., D. A. Gailey, B. Berwald, S. Oshima, P. T. Barnes *et al.*, 1997 Extended reproductive roles of the *fruitless* gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. Genetics **147:** 1107–1130.

von Schlicher, F., and J. C. Hall, 1979 Neural topography of courtship song in sex mosaics of *Drosophila melanogaster.* J. Comp. Physiol. **129:** 85–95.

Walter, M. F., B. C. Black, G. Afshar, A. Y. Kermabon, T. R. Wright *et al.*, 1991 Temporal and spatial expression of the *yellow* gene in correlation with cuticle formation and dopa decarboxylase activity in Drosophila development. Dev. Biol. **147:** 32–45.

Walters, M. J., 1988 *Courtship in the Animal Kingdom.* Anchor Books, Doubleday, New York.

Waterbury, J. A., L. L. Jackson and P. Schedl, 1999 Analysis of the *doublesex* female protein in *Drosophila melanogaster*: role in sexual differentiation and behavior and dependence on *intersex.* Genetics **152:** 1653–1667.

Wilson, R., B. Burnet, L. Eastwood and K. Connolly, 1976 Behavioural pleiotropy of the *yellow* gene in *Drosophila melanogaster.* Genet. Res. **28:** 75–88.

Wittkopp, P. J., J. R. True and S. B. Carroll, 2002a Reciprocal functions of the *Drosophila* Yellow and Ebony proteins in the development and evolution of pigment patterns. Development **129:** 1849–1858.

Wittkopp, P. J., K. Vaccaro and S. B. Carroll, 2002b Evolution of *yellow* gene regulation and pigmentation in Drosophila. Curr. Biol. **12:** 1547–1556.

Wittkopp, P. J., B. L. Williams, J. E. Selegue and S. B. Carroll, 2003b Drosophila pigmentation evolution: divergent genotypes underlying convergent phenotypes. Proc. Natl. Acad. Sci. USA **100:** 1808–1813.

Wright, T. R. F., 1987 The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster.* Adv. Genet. **24:** 127–222.

Yamamoto, D., and Y. Nakano, 1998 Genes for sexual behavior. Biochem. Biophys. Res. Commun. **246:** 1–6.

Yamamoto, D., and Y. Nakano, 1999 Sexual behavior mutants revisited: molecular and cellular basis of Drosophila mating. Cell. Mol. Life Sci. **56:** 634–646.

Yamamoto, D., J. M. Jallon and A. Komatsu, 1997 Genetic dissection of sexual behavior in *Drosophila melanogaster.* Annu. Rev. Entomol. **42:** 551–585.

Yamamoto, D., K. Fujitani, K. Usui, H. Ito and Y. Nakano, 1998 From behavior to development: genes for sexual behavior define the neuronal sexual switch in Drosophila. Mech. Dev. **73:** 135–146.

Zhu, L., J. Wilken, N. B. Phillips, U. Narenda, G. Chan *et al.*, 2000 Sexual dimorphism in diverse metazoans is regulated by a novel class of intertwined zinc fingers. Genes Dev. **14:** 1750–1764.

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