The *nonA* Gene in Drosophila Conveys Species-Specific Behavioral Characteristics

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ABSTRACT

The molecular basis of species-specific differences in courtship behavior, a critical factor in preserving species boundaries, is poorly understood. Genetic analysis of all but the most closely related species is usually impossible, given the inviability of hybrids. We have therefore applied interspecific transformation of a single candidate behavioral locus, *no-on-transient A* (*nonA*), between *Drosophila virilis* and *D. melanogaster,* to investigate whether *nonA*, like the *period* gene, might encode species-specific behavioral information. Mutations in *nonA* can disrupt both visual behavior and the courtship song in *D. melanogaster.* The lovesong of $nonA^{diss}$ mutant males superficially resembles that of *D. virilis*, a species that diverged from *D. melanogaster* 40–60 mya. Transformation of the cloned *D. virilis nonA* gene into *D. melanogaster* hosts carrying a synthetic deletion of the *nonA* locus restored normal visual function (the phenotype most sensitive to *nonA* mutation). However, the courtship song of transformant males showed several features characteristic of the corresponding *D. virilis* signal, indicating that *nonA* can act as a reservoir for species-specific information. This candidate gene approach, together with interspecific transformation, can therefore provide a direct avenue to explore potential speciation genes in genetically and molecularly tractable organisms such as Drosophila.

THE results of many years of experiments, in which closely related species have been crossed together to detect the genetic architecture of species-specific characters, have usually detected an underlying polygenic system (reviewed in Coyne 1992). For obvious reasons, interspecific hybridization is difficult and seldom leads to the production of segregating generations. However, in some cases, a donor species can be transformed with the DNA of another, effectively bypassing the commonly associated sterility and inviability of hybrids. In the behavioral literature, this kind of study has been performed twice, in both cases with the *period* (*per*) gene in Drosophila (Petersen *et al.* 1988; Wheeler *et al.* 1991). These two studies clearly showed that *per* genes of the two donor Drosophila species were able to transfer the species-specific characteristics of their rhythmic phenotypes (circadian locomotor activity and ultradian courtship song cycles, respectively) to their *Drosophila melanogaster* hosts. This switch in the behavior of the transformants was determined by interspecific differences in the coding region of *per*, as opposed to the regulatory regions. Furthermore, in the case of the ultradian lovesong cycle, the species-specific sequences that were responsible resided in a small fragment that contained a limited number of interspecific amino acid substitutions (Wheeler *et al.* 1991).

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A single gene is therefore capable of acting as a reservoir for species-specific information and raises the question of how general this phenomenon might be. Stripped of the cumbersome burden of interspecific genetics, transformation of single genes between species could reveal, as in the case of *per*, a rather simpler deterministic picture of species-specific behavior than otherwise believed. Consequently, we have decided to extend this approach to another "candidate" locus.

The *no-on-transientA* (*nonA*) gene from Drosophila encodes a putative RNA-binding protein, but its function at the biochemical level is unknown (Besser *et al.* 1990; Jones and Rubin 1990). The *nonA* locus is sex linked and overlaps partially with the lethal locus *l(1)i19e* (Jones and Rubin 1990; Campesan *et al.* 2001; Sandrelli *et al.* 2001). Synthetic deletions of *nonA* are poorly viable (Stanewsky *et al.* 1993). Other mutant alleles of *nonA* produce visual abnormalities (Hotta and Benzer 1969, 1970; Pak *et al.* 1970), but the $nonA^{diss}$ male is, in addition, defective in its courtship song (Kulkarni *et al.* 1988; Rendahl *et al.* 1992). Interestingly, the latter's mutant song phenotype shows similarities to the wild-type songs of the *D. virilis* group of species, which has song pulses that are more polycyclic than those of *D. melanogaster* (Hoikkala and Lumme 1984, 1987 and see Figure 1). This situation with $nonA^{diss}$ mutants is somewhat analogous to that of the *per* mutants, per^s and ΔTG, whose males have song rhythms that are characteristic of wild-type *D. simulans* (Kyriacou and Hall 1980, 1986, 1989; Yu *et al.* 1987; Alt *et al.*

1998). Thus, like *per*, this raises the possibility that *nonA* might also carry species-specific song information.

The cloning and sequencing of the *D. virilis nonA* orthologue has been described, as has the transformation of the *D. virilis nonA* transgene ($nonA^{vir}$) into *D. melanogaster* $nonA^{-}$ mutants and the associated full rescue of the mutants' poor viability and abnormal electroretinogram (ERG; Campesan *et al.* 2001; Sandrelli *et al.* 2001). In this study, we investigate the courtship song and another visual phenotype in $nonA^{vir}$ transformants. Our results suggest that the *D. melanogaster* transformant hosts may have taken on some of the *D. virilis* song characteristics.

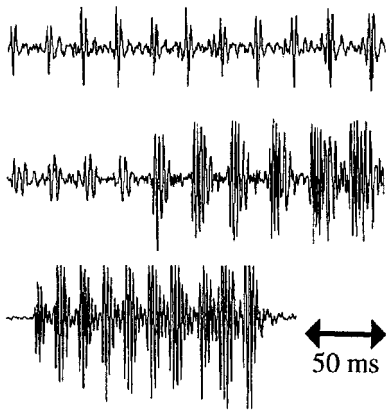

Figure 1.—Courtship song bursts from *D. melanogaster* transformant male hemizygous for the wild-type *nonA* transgene (top), $nonA^{diss}$ mutant male (middle), and a *D. virilis* male (bottom).

MATERIALS AND METHODS

Fly strains: *T(1:4)9e2-10/FM7:* This strain carries a reciprocal translocation *T(1:4)*, which uncovers the *nonA* gene and the adjacent essential locus *l(1)i19e* (Stanewsky *et al.* 1993) and is balanced with *FM7* (Lindsley and Zimm 1992). Females crossed to males carrying an autosomal *D. melanogaster* or *D. virilis nonA* transgene generate viable males carrying *T(1:4)9e2-10* only if the transgene also encodes the neighboring lethal gene *l(1)i19e* (Campesan *et al.* 2001).

P[(ry) 235R11]: This is a transformant strain carrying an 11-kb *Eco*RI fragment (homozygous on the third chromosome) that encodes both the *D. melanogaster nonA* locus and the adjacent lethal gene *l(1)i19e* (Jones and Rubin 1990). This fragment rescues all *nonA* mutant phenotypes and the associated lethality due to *l(1)i19e* (Jones and Rubin 1990; Rendahl *et al.* 1992; Campesan *et al.* 2001). Hemizygous males that carry a single autosomal copy of this *nonA* encoding fragment, *T(1:4)9e2-10/Y; P[(ry)235R11]/+* (abbreviated to *mel/d*), were generated and served as the *melanogaster* control. The particular *235R11* transformant line we used is one of two, which, when crossed to *T(1:4)9e2-10* to generate hemizygous $nonA^{+}$ males, gives identical song phenotypes (Stanewsky *et al.* 1993; Rendahl *et al.* 1996).

The negative control was provided by $nonA^{diss}/Y$ males, which were taken from a $nonA^{diss}$*/FM7a ; ry 506/MKRS* strain, which was repeatedly backcrossed to Canton-S, and the $nonA^{diss}$ mutants re-extracted by selecting for males with the mutant song phenotype. A similar high level of congenicity was maintained between all the genotypes to be compared by prior crossing of all transformant individuals (see below) to a Cantonized *w* strain.

Transformant lines: A number of lines were generated that carried a large genomic fragment from the *D. virilis nonA* region (Campesan *et al.* 2001). This transgene included the corresponding *D. virilis* sequences of the adjacent lethal gene *l(1)i19e*, which lies in the promoter region of *nonA* (Campesan *et al.* 2001; Sandrelli *et al.* 2001). Lines *113*, *67-4*, and *168-8* have lost some 3′ untranslated material from the transgene, but rescue completely the $nonA^{-}$ ERG and viability defects (Campesan *et al.* 2001). Males carrying more than one copy of *nonA* were generated with appropriate crosses.

Courtship song: Males were recorded for 10 min while in the presence of virgin females at a temperature of 25°–26°, using an electret condenser microphone (Ritchie and Kyriacou 1994). For *D. melanogaster*, the wings of virgin females were removed with sharp forceps on collection, and when 1 or 2 days old, they were placed with 2- to 4-day-old males. For *D. virilis*, a single 15-day-old male and virgin female were placed into the recording chamber. Songs were filtered and analyzed using SPIKE2 software as described previously (Ritchie and Kyriacou 1994). In addition, the peaks and troughs corresponding to the cycles within each song pulse were detected and marked automatically for each song using one of the SPIKE2 subroutines (see Figure 1). Manual editing was used mainly to remove events caused by extraneous loud noises or to add or remove peaks and troughs that had not been marked properly in the automated analysis. All transformant songs were coded and analyzed "blind" by the experimenter. Further programming allowed the following parameters to be extracted from each song: the intrapulse frequency (PF) for each pulse and the slope of PF (bPF), as calculated by regression of PF against sequential pulse position in a song burst; the peak-to-peak mean interpulse intervals (IPI); the cycles per pulse (CPP) and the slope of CPP (bCPP; measured in the same way as bPF); and the sine song frequency. Only song bursts with 5 or more pulses were analyzed in this way. CPP or PF values at each sequential position in the burst were averaged for each male before performing least-squares regression and ANOVA. A minimum of 200 pulses per male were analyzed in this way, but more vigorous courtships produced >1000 pulses.

Optomotor test: Three- to 8-day-old flies were dark-adapted for 4 hr and each fly tested individually for its turning behavior in a moving visual field of alternating black and white stripes (Burnet and Beck 1968). Ten flies per genotype were tested by placing each in a T-shaped tube in which the arm is painted black, so that the fly is forced to walk out of the opaque tube into a choice point facing the moving stripes. Once outside, it can turn into the right or left arm, and the fly produces the correct response when it turns in the direction of the moving environment. *D. virilis* flies are much larger than *D. melanogaster,* so we modified our methods so that flies were placed individually in an inverted empty glass vial positioned at the center of the rotating drum. When the fly reached the top, its movement was scored as the drum rotated. A correct optomotor response was obtained each time a fly performed at least five rotations in the same direction as the moving stripes. Each fly *(melanogaster* and *virilis)* was given 20 trials in which the direction of the stripe movement was randomly switched, with either narrow or broad stripes, each subtending an angle of 13.3° or 36°, respectively, from the center of the drum. The drum was rotated at a constant speed of 46 rpm, and a 60-W desk lamp was placed above the drum to uniformly illuminate the center of the cylinder. All tests were performed at room temperature (20°–22°).

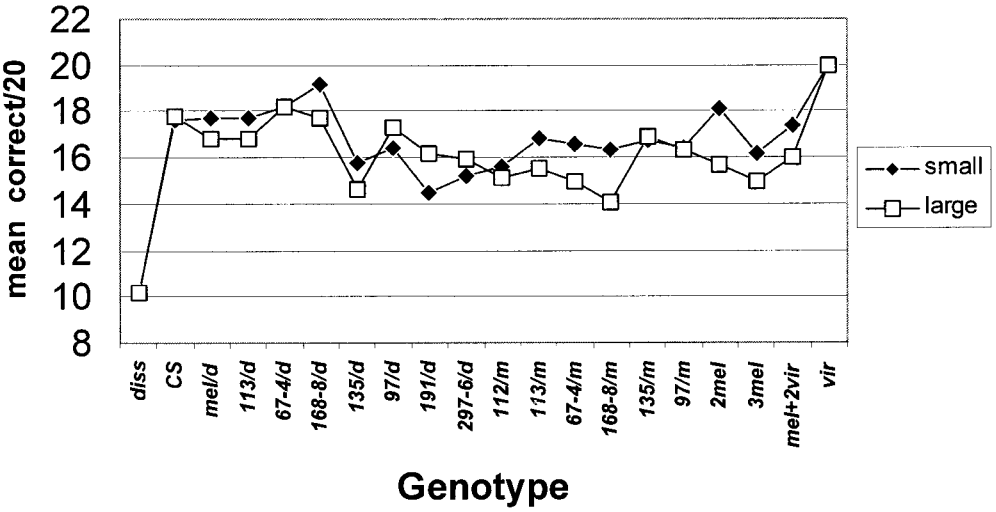

FIGURE 2.—Optomotor responses. The mean number out of 20 of correct turning responses (plus SEM and SD) for broad stripes (open squares) and narrow stripes (solid diamonds) is shown for each genotype/line. #-/*d* represents a line carrying the relevant species hemizygous transgene in a *nonA*$^-$ background; #-/*m* represents the transgene in a wild-type *melanogaster nonA*$^+$ background (see text).

RESULTS

Optomotor behavior: The walking optomotor response is a sensitive test of the fly's capacity to follow movements of the visual environment and reflects more central aspects of the functioning of the visual system than the ERG (HEISENBERG and BUCHNER 1977). Hemizygous males carrying one copy of *nonA*^{vir} on the double deletion *T(1:4)9e2-10* background (these transformants are collectively referred to as *vir/d*) were compared with their corresponding hemizygous *nonAmel* male transformant counterparts (*mel/d*), Canton-S males, partial hybrids carrying the endogenous *melanogaster nonA* gene plus *nonA*^{vir} (collectively termed *vir/m*), partial hybrids carrying two copies of *nonA*^{vir} plus the endogenous *melanogaster nonA* gene (*mel + 2vir*), along with males carrying two or three copies of *melanogaster nonA* (*2mel, 3mel*), *nonAdiss*, and *D. virilis* males.

Figure 2 reveals that the wider (larger) stripes generally gave more turning errors. Analysis of variance revealed a significant genotype effect ($F = 5.96$, $P \ll 0.0001$, d.f. 17,324), stripe width ($F = 10.33$, $P = 0.0014$, d.f. 1324), and a marginally significant genotype × stripe interaction ($F = 1.71$, d.f. 17,324, $P = 0.039$). Each *vir/d* transformant line gave a significantly higher number of correct turning responses than the *nonAdiss* mutants, which turn at random, generating an average of 10 correct responses out of 20 ($P \ll 0.0001$, Newman-Keuls *a posteriori* procedure). The *mel/d* transformant males rescue the optomotor response to a level similar to that of Canton-S with only lines *191/d* and the partial hybrids *168/m* having significantly poorer scores than *mel/d* ($P = 0.038$, 0.007, respectively). Consequently, the overall conclusion is that the optomotor defect associated with *nonA* mutants appears to be rescued in the great majority of *vir/d* lines to a level indistinguishable from that of *mel/d*.

Song analysis: All mutant *nonA* transgenes so far examined either affect vision only, or vision and song, but never song only (RENDAHL *et al.* 1992, 1996; STANEWSKY *et al.* 1996). Both the ERG (CAMPESAN *et al.* 2001) and optomotor responses studied above suggest that *nonA*^{vir} gives a full rescue of *nonA*-mutant phenotypes. It therefore follows that any alterations of the song in a *nonA*^{vir} transformant are unlikely to reflect incomplete rescue (because we would expect an accompanying visual defect) and could instead indicate species-specific transfer of song information (see DISCUSSION).

It became clear during song recording that the vigor with which flies sang during the 10-min observation period was very different, one fly producing 15 sec of song and another 171 sec, representing the two extremes. A correlation matrix was generated between each song parameter, CPP, bCPP, PF, bPF, and IPI, and the amount of song generated (pulse plus hum song), but this did not reveal any consistent relationship for any of the 19 genotypes, in that the correlation could be positive or negative and usually not significant (data not shown). However, the correlations between the amount of song produced and PF were always negative, but only in one case (*mel + 2vir*) was it significant. Nevertheless, PF was corrected for the different amounts of song by analysis of covariance (ANCOVA).

CPP distributions: One way of examining whether the *nonA*^{vir} transformants showed a complete rescue of the *nonA* mutant song phenotype is to examine overall CPP distributions. Figure 3A shows the results of the CPP distributions for males carrying a single copy of *nonA*; the *nonA*^{diss} males have a long tail in CPP frequency distribution but their modal value is 2, reflecting the mutants' developing polycyclicity as the song burst progresses (see also Figure 1). *D. virilis* males, on the other hand, have a distribution that is more normally distributed around a modal value of 4.5 CPP. The *mel/d* males have 95% of their pulses spread between 1, 1.5, and 2 CPP, with 35% having 1 CPP (see Figure 3A). Of the seven *vir/d* lines examined, six had modal values of 2 or 2.5, with one (*97/d*) having a modal value of 1.5 (Figure 3A). We examined the skew and kurtosis values

for CPP for these genotypes by grouping together individual lines of *vir/d* after first checking the homogeneity of these lines with a Kruskal-Wallis test. Kolmogorov-Smirnov comparisons revealed that for kurtosis, the *vir/d* distributions differed significantly from *mel/d* ($P < 0.05$) and from *nonA*^{diss} ($P < 0.001$), but not from *D. virilis* (Figure 3A). In contrast, kurtosis in the partial hybrid *vir/m* group did not differ from control line *me1/d*, but was significantly different from *D. virilis* ($P < 0.05$) and from *nonA*^{diss} ($P < 0.001$). Therefore, this initial analysis of CPP distributions shows the *vir/d* lines to be more similar to *D. virilis* than to *D. melanogaster* (*mel/d*) and with no evidence for mutant CPP (>4) in any of the *vir/d* lines.

CPP regression on pulse position: Another way of describing song characteristics is to examine the CPP regression in song bursts. Mutant *nonA*^{diss} songs generally show a steep positive slope (bCPP) in longer song bursts with pulses become increasingly polycyclic (Rendahl *et al.* 1992; Stanewsky *et al.* 1996). For each genotype, the number of songs out of 10 (only 6 for line *67-4/m*) that had significant regression lines was calculated, and the significant slopes were scored as positive (increasing CPP with pulse position) or negative (decreasing CPP). The slope (bCPP) was calculated for each song and the mean value for bCPP for each transformant line is plotted in Figure 3B and excludes the *nonA*^{diss} and *D. virilis* values, which are off the scale of the *y*-axis. ANOVA of these data (excluding *D. virilis* and *nonA*^{diss}) gave a significant genotype effect ($F = 4.26$, d.f. 16,149, $P \ll 0.001$). Figure 3B reveals that the *vir/d* transformants have significantly higher bCPP values than *mel/d* (*vir/d* = 0.0158, *mel/d* = −0.0022; $F = 17.3$, d.f. 1149, $P \ll 0.001$). As expected (Rendahl *et al.* 1992; Stanewsky *et al.* 1996), the *nonA*^{diss} songs all had highly significant positive slopes (mean bCPP value = 0.257, off scale in Figure 3B). Of the 10 *me1/d* males, 4 gave significant regressions, 1 positive and 3 negative, with a mean slope of −0.0022. All individual *vir/d* lines, however, had between 7 and 8 songs with significant slopes, nearly all of which were positive. Partial hybrid lines (*vir/m*) had fewer significant slopes and this is reflected in their generally lower average bCPP values (Figure 3B). All *D. virilis* songs had significant positive slopes with a mean bCPP value of 0.186 (off scale in Figure 3B). This aspect of *D. virilis* songs had not been reported previously. Therefore, as in *nonA*^{diss}, *D. virilis* pulses become increasingly polycyclic during a song burst. Consequently, the positive bCPP values of the *vir/d* lines means that either rescue of this character is incomplete, or that this component of *D. virilis* songs has been transferred to the *melanogaster* hosts.

From the regressions we calculated the value of CPP at the first pulse (CPP1 in Figure 3C. Analysis of the transformant lines only revealed a highly significant genotype effect ($F = 15.5$, d.f. 16,149, $P \ll 0.001$). Planned comparison of *mel/d vs.* the *vir/d* values was highly sig-

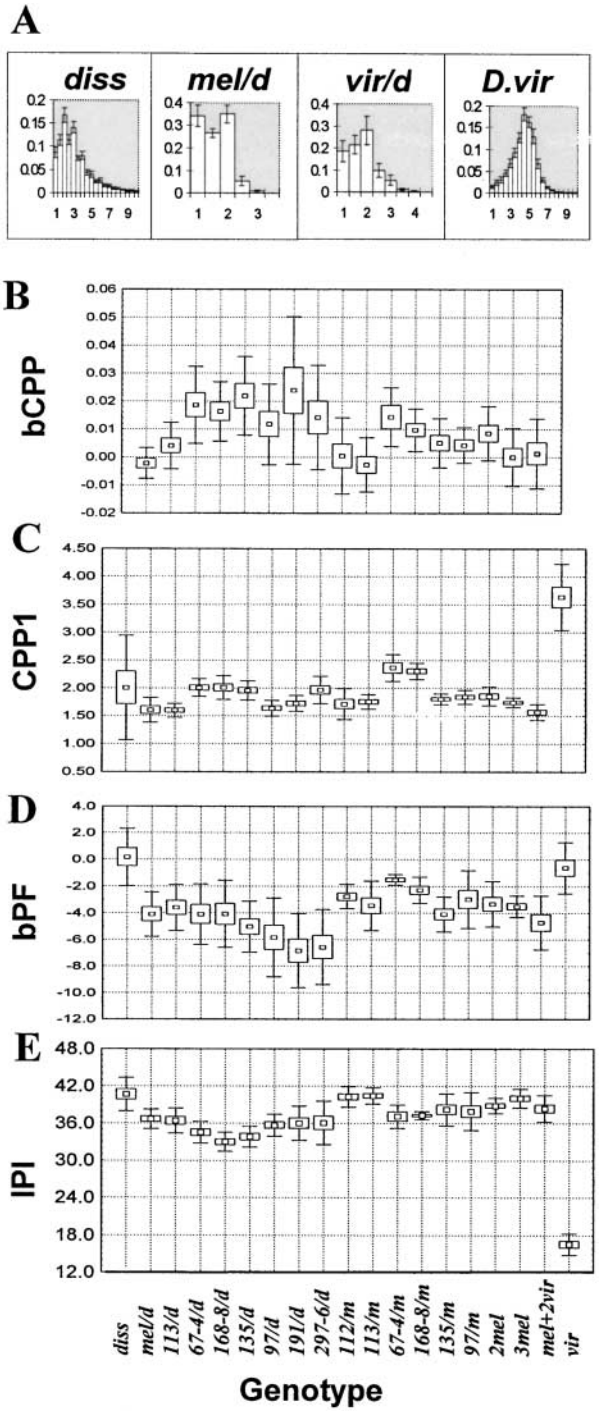

Figure 3.—Results of univariate analyses for courtship songs of all lines/genotypes. (A) Frequency histograms for CPP for males carrying a single *nonA* gene. *diss, nonA*^{diss}; *mel/d*, hemizygous *nonA*^{mel} transformants; *vir/d*, hemizygous *nonA*^{vir} transformants; *D.vir, D. virilis.* (B–E) Mean values, SEM and SD, are shown for (B) bCPP (slope of regression of CPP) for all lines except *nonA*^{diss} and *D. virilis*; (C) CPP at the first pulse (CPP1) of a pulse train taken from the regression (D) mean value of bPF (slope of pulse frequency); and (E) mean IPIs.

nificant (*mel/d* = 1.607, *vir/d* = 1.826; $F = 16.4$, d.f. 1149, $P \ll 0.001$). From Figure 3C it is clear that CPP1 values are significantly higher for most of the *vir/d* lines compared with *me1/d* (Newman-Keuls tests showed significant differences for lines *67-4/d*, *168-8/d*, *135/d*, and *297-6/d* compared with *mel/d*). Furthermore, partial hybrids *vir/m* also have significantly higher CPP1 values compared with *vir/d* lines (*vir/m* = 1.93; $F = 14.87$, d.f. 1149, $P \ll 0.001$). In general (with the exception of line *135*), each individual partial *vir/m* hybrid line bears a higher CPP1 value than its *vir/d* corresponding counterpart (Figure 3C), possibly suggesting a dosage effect caused by carrying two *nonA* genes. However, comparing *mel/d* scores with *2mel* and *3mel* does not reveal any linear effect on CPP with increasing *nonA* dose (Figure 3C). Thus the *vir/m* values suggest a semidominant effect of adding the *virilis* copy of *nonA* to that of *D. melanogaster* (see DISCUSSION).

The three analyses of CPP described above reveal no convincing evidence that the *vir/d* transformants show characteristics that may reflect an incomplete rescue of the song phenotype. In contrast, they appear to share features of the *D. virilis* song.

PF regression on pulse position: As a possible further indicator of the completeness of the rescue by $nonA^{vir}$ we examined PF using a least-squares regression as with CPP. Five out of 10 $nonA^{diss}$ mutant males showed a significant regression, 2 positive but 3 negative, with a mean slope (bPF) of 0.155 (Figure 3D). *D. virilis* songs were similar to the mutants in this respect, with 1 out of 10 males having a significant negative slope, giving a mean slope for the group of −0.63 (Figure 3D). The vast majority of flies in the other genotypes gave significant negative slopes, revealing that intrapulse frequency is systematically decreased during a song burst (RENDAHL *et al.* 1992). One-way ANOVA revealed a significant difference between the genotypes in bPF even when the $nonA^{diss}$ and *D. virilis* values were excluded ($F = 4.77$, d.f. 16,149, $P \ll 0.001$). This difference is caused mainly by each *vir/d* line having a lower negative slope than its corresponding *vir/m* partial hybrid counterpart (Figure 3D, planned comparison, $F = 41.08$, d.f. 1167, $P < 0.001$). The *vir/d* lines have similar values to *mel/d* ($F = 2.465$, d.f. 1167, $P = 0.118$), revealing that these subtle changes in PF detected during a song burst are observed in both *vir/d* and *mel/d* songs, with no evidence for the mutant $nonA^{diss}$, nor the *D. virilis*-like phenotype.

As a further measure of intrapulse frequency, we calculated PF at the first pulse position in a burst (PF1) using the regression equation, then performed ANCOVA using the amount of song as covariate (see MATERIALS AND METHODS), which revealed a highly significant effect ($F = 18.8$, d.f. = 18,166, $P \ll 0.001$). The adjusted PF1 values revealed that $nonA^{diss}$ has significantly lower frequencies of 250 Hz compared to the *D. virilis* value of 410 Hz ($P < 0.01$ by Newman-Keuls), whereas all the other genotypes gave adjusted PF1 values between 290 and 360 Hz that were significantly higher than $nonA^{diss}$ ($P < 0.05$), but significantly lower than *D. virilis* ($P < 0.05$). We conclude that the *vir/d* transformants show no evidence for a lowered PF that might be indicative of incomplete rescue of the song phenotype.

IPI: Mean IPI values of $nonA^{diss}$ males are 41 msec and higher than those of *mel/d* males by ~4 msec (Figure 3E). *D. virilis* males have very short IPIs of ~17 msec. The *vir/d* lines showed significantly lower IPIs than *mel/d* (*vir/d* = 35.06 msec, *mel/d* = 36.67 msec; $F = 5.3$, d.f. 1167, $P = 0.023$), although in only two individual lines, *168-8/d* and *135/d*, was this significant by *a posteriori* comparison. Each partial hybrid line also showed higher IPIs than its *vir/d* counterpart (Figure 3E, $F = 83.3$, d.f. 1167, $P \ll 0.001$). There was an apparent dosage effect in that *3mel* had significantly higher IPI than *mel/d* (Figure 3E, $P < 0.01$), with *2mel* males having intermediate scores. These results were unexpected and suggest that the $nonA^{vir}$ gene may have shortened IPI in the transformants, raising the possibility that *nonA* might carry species-specific IPI information. IPI values, as measured peak to peak, will be affected by the pulse pattern, which includes the variables that have been examined (CPP, bCPP, PF, and bPF). Consequently, we performed ANCOVA for IPI in which these four characteristics were covariates. The resultant *F*-ratio was also significant ($F = 22.1$, d.f. 18,163, $P \ll 0.001$) with the only major change from the previous analysis being that the adjusted IPI value for $nonA^{diss}$ was slightly, but not significantly, lower that that of *mel/d*. Therefore, if degeneration of pulse pattern of the mutant is taken into account (reflected in its abnormal values of CPP, bCPP, PF, and bPF), the $nonA^{diss}$ IPI is actually very similar to that of the wild type. However, every *vir/d* line still maintained a lower IPI than *mel/d*, which is surprising because *vir/d* males have higher values of CPP and bCPP, lower PF1 values, and generally more negative bPF. All these factors will serve to *increase* the duration of a pulse and thereby should indirectly *increase* IPI because of the way it was measured (peak to peak). Thus the lower IPIs of *vir/d* compared to *mel/d* suggest that the *virilis* transgene is playing an active role in reducing IPIs.

Multivariate analysis: So far, all the analyses have used a single variable, compared between genotypes. To compare genotypes using all the data simultaneously, a multivariate method, discriminant analysis, was performed with the song characters bCPP, CPP, bPF, PF, and IPI using SYSTAT 7.0 software. All transformant lines were treated individually, and the results for all 19 groups are shown in Figure 4A. An extremely low probability of misclassification of groups is provided by Wilks' λ of 0.002 ($F = 21.7$, d.f. 90,790, $P \ll 0.00001$). Factor 1 accounts for >80% of the variance, which is primarily generated by the difference between *D. virilis* songs and the rest. Factor 2 (nearly 10%) is generated largely by

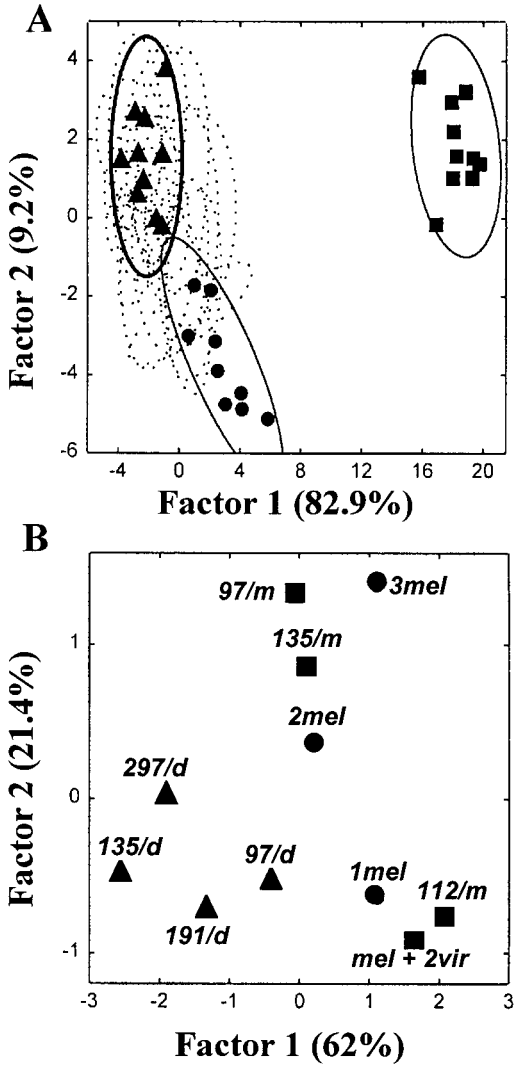

FIGURE 4.—Results of discriminant analysis for song characters. (A) Individual values within *D. virilis* (squares), *nonA*^{diss} (circles), and *mel/d* (triangles) are represented together with their corresponding ellipses, giving the 95% confidence intervals. For clarity, all the other individuals from each genotype are not shown. (B) Analysis of transformant lines only (see text). Mean values of individual *vir/d* strains are represented as triangles, partial hybrids (either *vir/m* or *mel + 2vir*) as squares, and lines carrying one, two, or three copies of the *melanogaster nonA* gene, *mel/d* (*1mel*), *2mel*, and *3mel*, respectively, are shown as circles.

the difference between *nonA*^{diss} males and the other genotypes. All five song characters contribute significantly ($P \ll 0.0001$) to the discrimination between groups.

Clearly, the difference between *D. virilis* and the other genotypes is so large that any differences between the transformants are "swamped." We therefore analyzed the transformants only. Lines *113*, *67-4*, and *168-8*, which may have lost some 3′ regulatory material (CAMPESAN *et al.* 2001), did not appear any different from the "intact" lines in the univariate analyses. However, to be conservative and for simplicity, we removed these and their corresponding partial hybrids from the subsequent discriminant analysis, which nevertheless proved highly significant (Wilks' $\lambda = 0.108$, $F = 5.49$, d.f. 50,436, $P \ll 0.0001$), with all song characters contributing significantly to the discrimination ($P < 0.0001$). Figure 4B reveals that the *vir/d* lines (*297/d*, *135/d*, *191/d*, and *97/d*) cluster away from the other lines on factor 1, which contributes 62% of the variance. A dosage effect is observed on factor 2 (21.4% of variance) for those flies carrying one, two, or three doses of *D. melanogaster nonA*, which is not reflected in the *vir/m* and *mel + 2vir* comparison. As the *vir/m* partial hybrids (lines *97/m*, *135/m*, *112/m*, and *mel + 2vir*) have factor 1 values similar to the *melanogaster nonA* carriers (*mel/d* or *1mel*, *2mel*, and *3mel*), the *melanogaster nonA* gene appears to be dominant with regard to factor 1.

Finally, we took each *vir/d* transformant line and compared it directly with its corresponding partial *vir/m* hybrid. In each of the five comparisons, involving lines *113*, *135*, *168-8*, *67-4*, and *97*, there was almost no overlap between each pair of genotypes on factor 1. Wilks' λ was significant for all comparisons (P between 0.029 and $\ll 0.0001$). All CPP values contributed significantly to the discrimination of each pair, as did IPI in all but one line (line *97*, $P = 0.06$). The other song characters, bCPP, PF, and bPF, contributed sporadically in only one or two pairings. To sum up, the multivariate analyses have buttressed the results of the univariate methods, in that they clearly show a difference between the song characteristics of the *vir/d* transformants and those flies carrying *melanogaster nonA* genes.

DISCUSSION

The courtship song of the *nonA*^{diss} mutant male shows some similarities to that of *D. virilis*, in that pulses are polycyclic. Our study has shown that this superficial similarity runs somewhat deeper, in that the *D. virilis* song burst also shows the same pattern of progressive polycyclicity as the mutant. The site of the *nonA*^{diss} mutation, in which an asparagine is substituted by cysteine (RENDAHL *et al.* 1996), although conserved in *D. virilis*, nevertheless falls within a seven-residue region in *D. melanogaster*, KREVDNE (residues 547–553; R is the *nonA*^{diss} site) that is altered to KRESDNE in *D. virilis*. This V to S substitution generates potential cyclic nucleotide-dependent and casein kinase II protein kinase phosphorylation sites in *D. virilis* (CAMPESAN *et al.* 2001). In addition, the *nonA* gene from *D. virilis* shows considerable divergence from its *D. melanogaster* orthologue, particularly in the N-terminal encoding regions (CAMPESAN *et al.* 2001). Consequently, it is logical to suggest that *D. virilis nonA* may have the potential to encode species-specific song characteristics.

Mutational analysis of *nonA* has revealed that the first RNA recognition domain (RRM1) in *nonA* is absolutely

necessary for all the known functions of NONA, whereas mutations such as $nonA^{diss}$, which lie in the C-terminal charged region, not only cause severe defects in both visual and song phenotypes, but also invariably reduce the viability of the affected flies (Rendahl *et al.* 1996; Stanewsky *et al.* 1996). Further *in vitro* mutations in the second RRM2 domain left unaltered all the visual, courtship, and viability phenotypes tested or produced defects of visual behavior only (Rendahl *et al.* 1996; Stanewsky *et al.* 1996). It was inferred that NONA visual functions are particularly sensitive to alterations in the RRM domains, with courtship song also being affected whenever the charged region is mutated. An alternative interpretation would be that the array of phenotypes observed in *nonA* mutants results from increasingly severe, nonspecific alterations of the protein. Therefore, relatively mild *nonA* mutations would disrupt visual behavior only, while more severe protein alterations would also affect courtship song.

This pattern of defects, observed in *nonA* mutants described above, is thus extremely helpful for interpreting the behavioral results from the $nonA^{vir}$ transformants. If $nonA^{vir}$ is indeed generating a slightly mutant song phenotype in *melanogaster* hosts due to incomplete rescue, rather than a species-specific transfer of behavioral information, then we would predict that vision should be disrupted, as "vision is the first to go" in mutants (Rendahl *et al.* 1992, 1996; Stanewsky *et al.* 1996). In the optomotor responses measured here, broad and narrow stripes were used, the former representing a more challenging stimulus than the latter (Heisenberg and Buchner 1977), but the $nonA^{vir}$ transformants nonetheless appeared completely normal in this aspect of visual behavior. These transformants also have a normal ERG, underscoring the full rescue of the *nonA* mutant visual phenotype by the $nonA^{vir}$ transgene (Campesan *et al.* 2001). In contrast, the song characteristics that we have measured consistently show *virilis*-like features while maintaining sensitive indicators of "normal" *melanogaster* song ouput such as the subtle change in PF during a song burst (bPF). We also find evidence that $nonA^{vir}$ appears to confer a *virilis*-like shorter IPI on the hosts, even though the other characteristics of the transformants' song pulses that have been measured should, indirectly, lead to a longer IPI.

These modest effects of the $nonA^{vir}$ transgene in heterospecific hosts may occur because *nonA* is genuinely one of a number of genes that are involved in species-specific behavioral differences. Any $nonA^{vir}$-determined adaptive changes that have occurred in the courtship song during the 40–60 million years since the two species have been separated (Moriyama 1987) may require compensating changes in *trans*-acting factors for their full effects to be seen in the heterospecific *D. melanogaster* host transformants. In contrast, the $nonA^{vir}$ transgene rescues basic visual functioning in transformants perhaps because there is little adaptive species-specific variation in this phenotype, so the relevant vision-specific *trans*-acting factors have not diverged significantly between the two species. Reciprocal interspecific transformations involving *nonA* transgenes from two very closely related species with very different song patterns, for example, *D. virilis* and *D. littoralis*, could conceivably generate more dramatic song phenotype exchanges because any relevant *trans*-acting factors would have had less time to diverge (Hoikkala *et al.* 2000). Recent developments with interspecific transformation technology and gene knockouts could make such transformation experiments feasible (Handler *et al.* 1998; Misquitta and Paterson 1999). It is further encouraging that the genetic mapping of song differences between *D. virilis* and *D. littoralis* has implicated the X chromosomal interval that carries the *nonA* locus (Hoikkala *et al.* 2000).

We thus conclude that *nonA* carries species-specific song information for both pulse pattern and IPIs. This situation differs from that of the *per* gene, whose coding region controls the species specificity of both locomotor activity patterns and lovesong cycles in an all-or-none manner (Petersen *et al.* 1988; Wheeler *et al.* 1991). However, for the lovesong phenotype, only one species-specific component between the two closely related species *D. melanogaster* and *D. simulans* is determined by *per* (Kyriacou and Hall 1986; Wheeler *et al.* 1991), whereas $nonA^{vir}$ appears to move several song features toward a *virilis*-like pattern. Thus, *nonA* could be one of several genes that can evolve to generate novel patterns of courtship song, a species characteristic that shows enormous diversity. The transduced $nonA^{vir}$ fragment that we employed also encoded the promotor regions of $nonA^{vir}$, so that any influences on species behavior could, unlike *per*, also be due to these regulatory regions, with which the host's transcription factors would interact. Clearly, chimeric *nonA* genes carrying both *virilis* and *melanogaster* material would be helpful in dissecting out whether these species-specific effects are due to coding *vs.* regulatory sequences.

How might the $nonA^{vir}$ gene be mediating these effects in the transformants? The NONA protein does have characteristics of a "housekeeping" gene in that it is expressed ubiquitously during development and some *nonA* mutations can produce an almost lethal phenotype (Rendahl *et al.* 1992; Sandrelli *et al.* 2001). It could thus be imagined from its mutational spectra that NONA regulates mRNAs that are involved in the development of the visual system, the central nervous system, or musculature responsible for song production, as well as other tissues that are important for viability. However, the behavioral specificity of the defects in some of the *nonA* mutations mentioned earlier belie the housekeeping gene scenario painted above. The interspecific results reported here further suggest that *nonA* may play a more instructive role in the species-specific song pattern

than simply providing the permissive conditions for courtship songs to be generated. A heat-shock-inducible $nonA^+$ cDNA can rescue normal vision and song patterns in *nonA* mutants even if activated during the early adult stages (Rendahl and Hall 1996). This would suggest that NONA is not required for directing the development of the relevant neuronal and muscular pathways toward the proper structures required for song production. Alternatively, a more dynamic instructive role for NONA might be reflected in the way the neuromuscular system stimulates song production, perhaps by altering responsiveness via changes in ion channel characteristics.

Such a speculative but seductive scenario would have *nonA* as a regulator (perhaps via splicing) of a downstream gene, such as the *Dmca1A* locus, which encodes a voltage-gated calcium channel and displays a complicated pattern of alternative splicing (Smith *et al.* 1996). The mutant alleles of *Dmca1A* produce an array of courtship songs and visual and viability defects that are similar but distinguishable from *nonA* alleles (Heisenberg and Gotz 1975; von Schilcher 1977; Perrimon *et al.* 1984; Kulkarni and Hall 1987). Thus the phenotypic parallels between *nonA* and *Dmca1A* are striking and suggest a functional interaction between the two loci. The apparent semidominant effects of the *D. virilis nonA* gene on CPP suggest that a qualitative and quantitative comparative survey of the transcripts from the *Dmca1A* locus of *D. virilis*, as well as those from $nonA^{diss}$ mutants, could prove worthwhile. This could be extended to other ion channel-encoding genes that, when mutant, generate defective lovesongs in *D. melanogaster* (Peixoto and Hall 1998).

In summary, the interspecific transformation of candidate genes is a more direct method of investigating species-specific characteristics than the reliance on hybridization between closely related species, the subsequent genetic analysis (if possible), and the subsequent laborious molecular work for identifying the relevant loci. Focusing on closely related species that are able to hybridize also limits the analysis, because only recent genetic variation will be scanned. In the case of *nonA*, transformation of the gene from *D. virilis* to *D. melanogaster*, two species that cannot hybridize, reveals the subtle behavioral effects of ancient genetic changes in the locus that must have occurred up to 60 mya.

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