Molecular Dissection of the 5’ Region of no-on-transientA of Drosophila melanogaster Reveals cis-Regulation by Adjacent dGpi1 Sequences

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ABSTRACT

The nonA gene of Drosophila melanogaster is important for normal vision, courtship song, and viability and lies ~350 bp downstream of the dGpi1 gene. Full rescue of nonA mutant phenotypes can be achieved by transformation with a genomic clone that carries ~2 kb of 5’ regulatory material that encodes most of the coding sequence of dGpi1. We have analyzed this 5’ region by making a series of deleted fragments, fusing them to yeast GAL4 sequences, and driving UAS-nonA expression in a mutant nonA background. Regions that both silence and enhance developmental tissue-specific expression of nonA and that are necessary for generating optomotor visual responses are identified. Some of these overlap the dGpi1 sequences, revealing cis-regulation by neighboring gene sequences. The largest 5’ fragment was unable to rescue the normal electroretinogram (ERG) consistently, and no rescue at all was observed for the courtship song phenotype. We suggest that sequences within the nonA introns that were missing in the UAS-nonA cDNA may carry enhancer elements for these two phenotypes. Finally, we speculate on the striking observation that some of the cis-regulatory regions of nonA appear to be embedded within the coding regions of dGpi1.

THE sex-linked no-on-transientA (nonA) gene in Drosophila encodes a putative RNA-binding protein (JONES and RUBIN 1990), and mutations in this gene affect viability (STANEWSKY et al. 1993), visual behavior (HOTTA and BENZER 1969), and the male fly’s courtship song (KULKARNI et al. 1988). The nonA transcript is present in the fertilized egg, but at a more advanced developmental stage its expression seems to decrease in all tissues except in the central nervous system (CNS), where it starts to decline only toward the end of embryonic development (RENDAHL et al. 1992). The expression pattern of the NONA protein is similar, but not identical, to that of its RNA. The protein seems to be present ubiquitously in the developing oocyte and the embryonic, larval, pupal, and adult stages. The protein is localized in the cytoplasm of the fertilized egg, but it enters the nuclei at the cellular blastoderm stage and remains nuclear in most tissues during embryonic and adult development (FRASH and SAUMWEBER 1989; RENDAHL et al. 1992). BESSER et al. (1990) also showed that the NONA protein is localized in several puffs on polytene chromosomes of Drosophila larvae, and its binding seemed correlated with active transcription within these puffs (FRASH and SAUMWEBER 1989). However, evidence for any specific in vivo role for NONA is equivocal (REIM et al. 1999).

Despite NONA’s almost ubiquitous adult expression pattern, mutations in the gene have specific effects on behavior. The first nonA mutants were isolated in screens for flies defective in phototaxis, were subsequently shown to lack both light-on and light-off transient spikes in the electroretinogram (ERG), and were impaired in their optomotor responses (HOTTA and BENZER 1969; PAK et al. 1970; HEISENBERG 1972; HEISENBERG and GOTZ 1975). In a screen for defective courtship songs, the nonA® mutant was identified by its abnormal pulse songs, which become increasingly polycyclic and very high in amplitude during a song burst (KULKARNI et al. 1988). Extensive mutational analysis of various domains of nonA have revealed that if a coding region mutation affects the song phenotype, then vision is always affected, but not vice versa, suggesting that the visual pathway is more sensitive to NONA disruption (REIM et al. 1992, 1996; STANEWSKY et al. 1996).

The phenotypic effects caused by an amorphic allele of the nonA locus have been studied by generating an X-linked deletion, T(1;4)9e2-10, which removed both nonA and the distal, partially overlapping, lethal locus l(1)91e (JONES and RUBIN 1990). Viable nonA− mutants
were then generated by transforming flies carrying the T(1;4)9e2-10 deletion with a clone encoding exclusively for the lethal gene’s product (Stanewsky et al. 1993). In hemizygous males, deletion of the nonA gene causes semilethality as only 10–30% of the expected number eclose from metamorphosis. If kept at a temperature below 25° no nonA males emerge. The nonA individuals are deficient in locomotor activity and flight and less than one-third of them survive to reproduce. In visual courtship behavior, nonA deficient males show similar, although more severe, defects to those of nonAdiss mutants, confirming that nonA is indeed the allele closest to an amorphic mutation. nonA encodes an arginine-to-cysteine substitution in a region that is rich in charged residues and lies C terminal to the two RNA recognition motif repeats, whereas the visually defective mutations nonA and nonA map, respectively, in the center of, or just outside, the second RRM repeat (Rendahl et al. 1996).

As yet, little is known about the regulatory regions of nonA. Here, we describe a series of 5′ deletions that remove progressively more of the sequences of a gene called dGpi1, which has been recently identified within the nonA promoter region and which almost certainly corresponds to the vital gene l(1)i19e (Jones and Rubin 1990; Campesan et al. 2001, accompanying article). dGpi1 lies 325 bp 5′ to the nonA transcription start and encodes a component used in the biosynthesis of glycosylphosphatidylinositol, which is used to anchor eukaryotic proteins to membranes (Campesan et al. 2001, accompanying article). The nonA promoter fragments were fused to the yeast transcriptional activator GAL4 (Fischer et al. 1988) and used to drive UAS-nonA expression in a nonA mutant background. By comparing wild-type nonA and the transformants’ developmental expression patterns, visual, and sexual behavior, we have correlated regions of the promoter, including sequences within dGpi1, with NONA function.

**MATERIALS AND METHODS**

**Fly stocks:** Flies were raised on a standard yeast-glucose-agar medium (Roberts and Standen 1998) and were maintained at 25°, 70% relative humidity, in 12-hr light/12-hr dark cycles. Adults were collected using CO2 anesthesia. Canton-S was used as the wild-type strain, and the dominantly marked, multiply inverted balancer chromosomes was use for the expression studies (Philips and Brand 1998). The stock nonA was originally received from J. Hall, Brandeis University, in 1994. Mutants were repeatedly outcrossed to Canton-S, and the song phenotype re-extracted via the closely linked forked bristle marker. This line, called nonA f (λ), provided the mutant background for analyzing the different KpnGAL4-UASnonA constructs with respect to the attempted rescue of the courtship song phenotype. The original nonA line, called nonA f (b), provided the mutant background for the attempted rescue of the ERG and optomotor responses. The T(1;4)9e2-10/In(I) FM7 balanced strain carries a reciprocal translocation T(1;4) that uncovers the nonA gene and the adjacent essential locus l(1)i19e (Stanewsky et al. 1993). Females crossed to males carrying an autosomal insertion of the Drosophila melanogaster transgene P(ry) 235R11, which encodes wild-type sequences for both nonA and l(1)i19e (Jones and Rubin 1990; Stanewsky et al. 1993), will generate viable F1 males. These male transformants were used as suitable nonA controls for the courtship song study.

**Generation of nonA promoter-GAL4 fusions (KpnGAL4):** Five 5′ progressively deleted fragments of the D. melanogaster nonA regulatory region were obtained by PCR amplification, fused with the yeast GAL4 gene, and cloned into P-element transformation vectors. Diagrams of the various gene fusions are shown in Figure 1. This was done by using six primers, one 3′, and five 5′, designed on the basis of the nonA sequence published by Jones and Rubin (1990). The primers introduced the restriction sites for KpnI and BamHI at the 5′ termini of the 5′ primers and 3′ primer, respectively. Initially, a 1866-bp region immediately upstream of the coding region of nonA, and known to contain the promoter and all the enhancers required for the correct expression of the NONA protein (Jones and Rubin 1990), was amplified via PCR employing the 5′ KpnI primer 5′-GGCGGGTACCTCTAGACTGAATCAACCA-3′ (positions 1–18 in sequence of Jones and Rubin 1990; boldface represents the restriction site), and the 3′ BamHI primer 5′-TATTGCCGCTTCAACTGGTTGCAAA-3′ (positions 1849–1866) with Vent-recombinant DNA polymerase (New England Biolabs, Beverly, MA). The 3′ end of this fragment ends 44 bp upstream of the initiating methionine of nonA and 45 bp downstream of the transcription start of nonA (Campesan et al. 2001, accompanying article). The nonA 235R11 fragment (Jones and Rubin 1990) was used as the DNA template. The 1866-bp region was cloned as a KpnI-BamHI fragment in front of the GAL4 gene in the pGATB construct (Brand and Perrimon 1993) to generate KpnGAL4. The KpnGAL4 fragment was excised from KpnGAL4-pGATB as a KpnI-Ndel fragment and subcloned into pWSΔB.
a pWs vector (Klemenz et al. 1987) in which the BamHI site had been previously deleted following the filling recessed 3′ termini method (Sambrook et al. 1989) to generate the transgene we call Kpn1GAL4.

A similar procedure was used to amplify a 1236-bp fragment using primer 5′-GGCGATCCACTCAGATTTTCTGAA-3′ (630–648, 5′ Kpn1), a 829-bp fragment using primer 5′-GCGGGGATCCAGGTGAAGCTCC-3′ (974–991, 5′ Kpn4), a 629-bp fragment using primer 5′-GGCCTTGACATAATCAATGTT-3′ (1237–1255, 5′ Kpn5), and a 179-bp fragment using primer 5′-GGCGATCCAGGACATTTAT-3′ (1687–1704, 5′ Kpn7) together with the 3′ BamHI primer, using KpnIpGAT as the template. The relevant Kpn region was then subcloned as a KpnI-BamHI fragment in front of the GAL4 gene in the pWs8 vector to generate the transgenes Kpn3GAL4, Kpn4GAL4, Kpn5GAL4, and Kpn7GAL4. All Kpn fragments were automatically sequenced (Turbo Catalyst, Perkin-Elmer, Norwalk, CT) to check for errors.

Generation of UASrons: The full-length 3.7-kb nonA cDNA was excised from the B5-6 clone (Beser et al. 1990) to a NotI-Kpn1 and subcloned downstream of the five UAS repeats in the pUAST construct (Brand and Perrimon 1993). This clone has ~1.2 kb of 3′ material downstream of the nonA transla- tion stop and includes at least two polyadenylation signals (Beser et al. 1990).

Generation of transgenic lines: Transgenic lines were generated by injection of DNA into embryos of strain y, w; /+; 5B, e, P, 2-3/TM6, Ubx at a concentration of 600 ng/μl using standard procedures (Rubin and Spradling 1982; Spradling and Rubin 1982). Three independently derived lines (21A, 28A, and 30G) were obtained for Kpn1GAL4; two (15A and 19G) for Kpn3GAL4; four (5C, 9A, 10D, and 18D) for Kpn4GAL4; two (15B and 41B) for Kpn5GAL4; three (15E, 24B, and 34A) for Kpn7GAL4; and three (9A, 20B, and 21C) for UASrons. For each line the chromosomal position of the various combinations of KpnGAL4, UAS-rons, and the transla- tion stop and includes at least two polyadenylation signals (Beser et al. 1990).

Behavioral and physiological analyses: The rescue of mutant behavior was examined in nonAΔA transgenic males carrying various combinations of KpnGAL4 and UASrons in trans on the second chromosome. These males were obtained as follows: female nonAΔA/+; nonAΔA k01486; In(2LR)O, Cy/Sco were crossed to Int(1)FMY/Y; transgene/In(2LR)O, Cy strain. nonAΔA/+; In(2LR)O, Cy males to produce an inverted chromosomes.

Walking optomotor test: The optomotor response was tested following the method described by Burnet and Beck (1968). Flies 3–6 days old were dark adapted for ~4 hr in food vials. Each fly was tested individually for its turning behavior in a moving visual field. This was created using a rotating Plexiglass drum (diameter 8 cm; height 9 cm) that had alternating black and white vertical stripes. The stripes subtended an angle of 12.4°. The drum was constantly rotated at 30 rpm. Each fly was placed in the middle arm of a T-shaped glass tube. This arm was painted black, so that the fly was forced to walk out into a choice point where it could turn into the right or the left arm. To test whether the turning behavior corresponded to the moving environment, the tube was placed in the middle of the rotating drum. A fly produced a correct response every time it turned out of the black arm in the same direction as the rotating stripes. Canton-S and nonAΔA b males were used as positive and negative controls, respectively. For each geno-type, at least 10 individuals were tested. Each fly was given 10 trials, and each time the rotating direction of the stripes was changed. A desk lamp (60 W) was placed above the drum to illuminate in a uniform way the center of the cylinder. All tests were performed at room temperature (22°C). The signifi-
cance of the difference between genotypes was determined by an ANOVA test and the Tukey-Kramer a posteriori test by using Statistica Statsoft 3.0 for Macintosh.

Electroretinograms: ERGs were recorded from adult flies
Figure 2.—Expression patterns of NONA in wild-type (Canton-S) and transgenic flies using anti-NONA antibodies, anti-β-gal antibodies, and X-gal staining (see text). Bars: A, 20 μm; B and C, 50 μm; D–G, 25 μm; H–J, 10 μm; K–R, 40 μm; S–Y, 100 μm. The acronym “KpnX” refers to flies carrying both KpnGAL4 and UAS-lacZ transgenes. (A) β-Gal expression in Kpn1 female ovaries. Activity was observed in the follicle epithelium and nurse cells. A similar expression pattern was observed in Kpn3, Kpn4, and Kpn5 female ovaries. fc, follicle epithelium; nc, nurse cells. (B) A weaker β-gal signal, restricted to the follicle epithelium, was observed in Kpn7 developing oocytes. (C) Control β-gal distribution pattern in a UAS-lacZ female ovary. (D) Localization of NONA protein with an anti-NONA antibody in a wild-type embryo at developmental stage 11. (E) Anti-β-galactosidase antibody localization of β-gal in a Kpn1 transgenic embryo (stage 11). Similar profiles of expression were observed in Kpn3, Kpn4, and Kpn5 embryos, while significantly weaker β-gal expression (F) was observed in Kpn7 embryos (stage 11). (G) β-Gal pattern of expression in a UAS-lacZ control transgenic embryo at developmental stage 9. (H) β-Gal expression in a Kpn1 third-instar larval brain. Similar profiles of expression were observed in Kpn3, Kpn4, and Kpn5 third-instar larvae. vg, ventral ganglion; ol, optic lobes. (I) A weaker β-gal expression was observed in Kpn7 third-instar larval brains. (J) Control β-gal expression pattern in a third-instar UAS-lacZ larval brain. (K) β-Gal expression in the eye-antennal imaginal disc of a Kpn1 third-instar larva. eye, eye; ant, antenna. (L) Significantly higher β-gal expression was observed in Kpn3 eye-antennal imaginal discs. A similarly high expression was observed in Kpn4 and Kpn5 third-instar eye-antennal imaginal discs. (M) β-Gal expression in the eye-antennal imaginal disc of a Kpn7 third-instar larva. β-Gal activity is here reduced and restricted to the disc posterior area. (N) A control β-gal expression pattern in a third-instar UAS-lacZ larval eye-antennal imaginal disc. (O) β-Gal expression in a wing imaginal disc of a Kpn1 third-instar larva. (P) β-Gal expression in a wing imaginal disc of a Kpn3 third-instar larva. Kpn3, Kpn4, and Kpn5 third-instar larval wing imaginal discs had a similar expression profile and showed more extensive β-gal expression than Kpn1. (Q) A significant reduction in expression was observed in Kpn7 compared to Kpn1 wing discs. (R) β-Gal expression in a control UAS-lacZ wing disc. (S) NONA protein localization, detected by anti-NONA antibody, in a frozen section (head and thorax) of a wild-type adult. ey, eye; la, lamina; me, medulla; lo, lobula; lp, lobula plate; mu, thoracic muscles. (T) β-Gal expression in head and thorax of Kpn3 transgenic fly. A similar expression pattern was observed in Kpn1 flies. (U) β-Gal expression in head and thorax of Kpn4 transgenic fly. A significant increase in β-gal expression was observed in the thoracic muscles with respect to that of Kpn1 and Kpn3 while the β-gal expression in the head was similar to that of Kpn1 and Kpn3. (V) β-Gal expression in a control UAS-lacZ adult fly. ey, eye; ol, optic lobe; mu, thoracic muscles. (W) β-Gal expression in a horizontal section through the head of an adult Kpn1 transgenic fly. ey, eye; la, lamina; me, medulla; lb, lobula; lp, lobula plate. A similar expression pattern was observed in Kpn3, Kpn4, and (X) Kpn5 adult heads. (Y) Reduced β-gal expression in a Kpn7 head.
(male and female Canton-S, \textit{nonA}°° and \textit{Kpn1-7GALA}/\textit{UAS-nonA} transgenic flies) using conventional electrophysiological techniques exactly as described in Campesan \textit{et al.} (2001, accompanying article).

\textbf{Courtship song:} Courtship songs were recorded as described in Ritchie and Kyriacou (1994). Briefly, \(\sim 10-15\) min of song were recorded from the relevant 3-5-day-old male in the presence of a 1-day-old virgin female using a specially constructed electret condenser microphone (Ritchie and Kyriacou 1994). The songs were recorded onto magnetic tape, filtered below 100 Hz and above 1000 Hz, and digitized with a CED (Cambridge, UK) analogue to digital converter. The mean value of cycles per pulse (CPP) for the first pulse from each song train was calculated using only song trains that had more than five pulses. A large number of song trains from each male were considered, and this CPP measure distinguishes clearly the mutant \textit{nonA}°° phenotype (CPP > 2) from the wild type (CPP < 1.5, see results). CPP was measured automatically by Spike2 (CED) software after setting a threshold level above and below any noise in the recording (Ritchie and Kyriacou 1994).

\textbf{RESULTS}

\textbf{Developmental expression:} For each \textit{Kpn1-7GALA} construct, the \(\beta\)-galactosidase distribution patterns were analyzed at various developmental stages in different transgenic lines to reduce the possibility of expression artefacts due to position effects. It became clear early on that \textit{UAS-lacZ} lines 1026 and 1027 gave almost identical expression levels, while line 1025 always gave lower levels compared to the others (data not shown). Consequently, only the results based on \textit{UAS-lacZ} line 1026 are presented throughout this section.

\textbf{Ovaries:} Figure 2A shows the \(\beta\)-gal expression in the \textit{Kpn1GALA}/\textit{UAS-lacZ} female ovary. Activity was observed in the follicle epithelium and nurse cells, and this pattern was similar to that of the NONA protein in wild-type flies, as reported by Frash and Saumweber (1989) and Rendahl \textit{et al.} (1992). A similar expression pattern was observed in \textit{Kpn3-}, \textit{Kpn4-}, and \textit{Kpn5GALA}/\textit{UAS-lacZ} female ovaries. A weak \(\beta\)-gal signal was observed in the \textit{Kpn7GALA}/\textit{UAS-lacZ} developing oocyte, which showed some punctate staining at the level of the follicle epithelium (Figure 2B). Figure 2C shows an absence of \(\beta\)-gal expression in a control \textit{UAS-lacZ} female ovary.

\textbf{Embryos:} Figure 2D shows the localization of NONA protein, detected with an anti-NONA antibody in a wild-type (Canton-S) embryo at developmental stage 11: an ubiquitous distribution of NONA in all the embryonic tissues can be seen. All \textit{Kpn1-}, \textit{Kpn3-}, \textit{Kpn4-}, and \textit{Kpn5GALA}/\textit{UAS-lacZ} transgenic embryos show a very similar \(\beta\)-gal distribution profile to the NONA patterns in the wild-type controls (Figure 2E), except for one of the four \textit{Kpn4GALA} lines (5C), which never presented any expression at all (data not shown). In contrast, anti-\(\beta\)-gal staining in the \textit{Kpn7GALA}/\textit{UAS-lacZ} embryos was much weaker (Figure 2F) if compared with the NONA
staining in the wild type or the levels of expression in all the other KpnGAL4/UAS-lacZ embryos. Consequently, there appears to be an enhancer of embryonic expression at least in the region between Kpn5 and Kpn7 (~223 to ~673 bp from initiating nonA methionine codon) Figure 2G shows control β-gal distribution pattern in a UAS-lacZ transgenic embryo at developmental stage 9.

Larvae: β-Gal in the third instar larval brain of Kpn1-, Kpn3-, Kpn4-, and Kpn5GAL4/UAS-lacZ transfectants was expressed both in the optic lobes and ventral ganglia (Figure 2H). No detectable differences in expression levels between the different Kpn1-5 fragments were observed, and the β-gal distribution pattern was similar to that of NONA protein reported in the wild-type larval brain (RendaHl et al. 1992). However, in the larval brain of Kpn7GAL4/UAS-lacZ flies the β-gal activity was generally weak, both in the optic lobes and in the ventral ganglion (Figure 2I). Figure 2J shows a control β-gal distribution pattern in a UAS-lacZ transgenic embryo at developmental stage 9.

Imaginal discs: The β-gal expression in the eye-antennal imaginal disc of Kpn1GAL4/UAS-lacZ third-instar larvae was mainly localized in the region that will develop into the photoreceptor cells of the adult eye (Figure 2K). In the eye-antennal discs of Kpn3-, Kpn4-, and Kpn5GAL4/UAS-lacZ third-instar larvae, the β-gal activity was present not only in the photoreceptor region but also in the part that will develop into the antenna (Figure 2L). Figure 2M shows an eye-antennal imaginal disc of a Kpn7GAL4/UAS-lacZ transgenic larva, in which the β-gal activity was significantly reduced compared to the other transgenics and restricted to the most posterior area that will form the adult eye. Figure 2N shows a control β-gal distribution pattern in an UAS-lacZ eye-antennal imaginal disc from a third-instar larva. Thus the distal fragment appears to have silencers that downregulate nonA expression in the antennal region of the disk.

The β-gal expression in the wing imaginal discs of Kpn1GAL4/UAS-lacZ third-instar larvae was predominantly localized in the region that will develop into the epithelial layer (Figure 2O). In the corresponding discs of Kpn3-, Kpn4-, and Kpn5GAL4/UAS-lacZ larvae, β-gal was expressed more extensively, both in the epithelial region and in the portion that will originate part of the thoracic cuticle, suggesting the existence of silencers in the distal fragment (Figure 2P). In the wing discs of Kpn7GAL4/UAS-lacZ larvae, the β-gal activity was significantly reduced compared to that of the other transformants (Figure 2Q). Again, the distal fragment ~1198 to ~1827 bp appears to carry silencers for the expression of imaginal disc tissue. Figure 2R shows a control β-gal distribution pattern in a UAS-lacZ wing disc.

Adult: Figure 2S shows the wild-type localization of NONA protein, detected by using an anti-NONA antibody in a frozen section of an adult fly. Antigen was seen in most cells of all tissues examined including photoreceptors, lamina, medulla, lobula and lobula plate of the optic lobe, the central brain, and the thoracic ganglia. Also thoracic muscles and the gut were stained. The β-gal distribution pattern in the head of Kpn1-, Kpn3-, Kpn4-, and Kpn5GAL4/UAS-lacZ transgenic flies was similar to that of the NONA protein in wild-type flies (Figures 2W, 2T, 2U, and 2X, respectively). Figure 2Y shows a head section of Kpn7GAL4/UAS-lacZ flies with the β-gal expression significantly reduced. A weak activity was found in the central brain, the lobula, in the lobula plate, and in the medulla, whereas little expression was observed in the lamina and none in the photoreceptor cells. The β-gal expression pattern in the thorax of Kpn1- and Kpn3GAL4/UAS-lacZ individuals was similar to that reported for NONA protein in wild type (Figure 2T).

Interestingly, in the thoracic muscles of Kpn4GAL4/UAS-lacZ adults we observed an extensive increase in β-gal expression level (Figure 2U), suggesting the elimination of an adult muscle silencing factor(s) in the 344-bp fragment that delimits the Kpn3GAL4 from the Kpn4GAL4 construct. The β-gal expression level in the adult thoracic muscles of Kpn5GAL4/UAS-lacZ flies in two independently derived lines (15A and 41B) was different. Whereas 15A flies showed high β-gal levels, estimated to lie between those of Kpn1- and Kpn4GAL4/UAS-lacZ, the 41B flies showed no β-gal expression at all, indicating a position effect. The β-gal pattern in the thorax of Kpn7GAL4/UAS-lacZ flies was significantly reduced compared to all the other KpnGAL4/UAS-lacZ lines, and weak expression was observed in the thoracic ganglia, but none in muscle cells. Figure 2V shows a control β-gal distribution pattern in a section of an UAS-lacZ adult.

Optomotor test: Confronted with a binary choice as to whether to follow the direction of the stripe movement, nonA adults turn at random, giving a mean value for the genotype of 50.5%. Canton-S males turn in the direction of the movement >80% of the time (Figure 3). Homozygous nonA males carrying the five different KpnGAL4 and UAS-nonA transgenes gave graded responses that reflected the amount of nonA promoter material carried in the insert—from 72.1% correct responses in Kpn1 to 50.2% in Kpn7GAL4/UAS-nonA flies (Figure 3). ANOVA gave highly significant differences between these values (F = 28.29; P = 0.0001). There are no significant differences (Tukey-Kramer test) between the wild-type (Canton-S) and Kpn1-, Kpn3-, and Kpn4GAL4/UAS-nonA genotypes. Similarly, the mean value obtained for Kpn7GAL4/UAS-nonA was not significantly different from that of nonA negative controls. Kpn5GAL4/UAS-nonA has a significantly poorer response compared to wild type (Canton-S) and Kpn1GAL4/UAS-nonA, but significantly higher than nonA and Kpn7GAL4/UAS-nonA (all P < 0.001). These results suggest that enhancers for the optomotor response must lie in
Figure 3.—Optomotor response of Canton-S (CS), \textit{nonA}^{disss} (diss), and hemizygous \textit{nonA}^{disss} males carrying \textit{KpnGAL4} and \textit{UAS-nonA} transgenes (Kp). The mean optomotor response of single male flies is expressed as percentage of turns in direction of the stripe movement (correct turns, see MATERIALS AND METHODS). From 20 to 70 flies were analyzed for each genotype. Mean values plus SDs are given.

ERGs: As reported in Table 1, almost all the transgenic individuals tested showed an ERG response characterized only by the sustained component and lacking the transient light-on and light-off components. Only in transgenic individuals with the 21A \textit{Kpn1GAL4/UAS-nonA} genotype was a wild-type ERG response observed in five out of six flies (Figure 4). Surprisingly, the light-on and light-off transient amplitudes in these individuals were significantly higher than those observed in wild-type (Canton-S) individuals ($P < 0.001$; Table 1).

\textbf{Courtship song:} Between three and six songs were analyzed for various lines of \textit{Kpn1-7GAL4/UAS-nonA} transgenic flies. This included two lines that carried only the \textit{UAS-nonA} transgene and the 235R11 \textit{nonA} control transformants (see Table 1).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Genotype & \textit{N} & \textit{+} & Light-on & Light-off \\
\hline
\textit{30G Kpn1/UAS-nonA} 20B & 7 & 0 & & 7 \\
\textit{30G Kpn1/UAS-nonA} 9A & 10 & 0 & & 10 \\
\textit{21A Kpn1/UAS-nonA} 20B & 5 & 0 & & 5 \\
\textit{21A Kpn1/UAS-nonA} 9A & 6 & 5 & 3.8 ± 0.2 (24) & 5.0 ± 0.9 (22) \\
\textit{28A Kpn1/UAS-nonA} 20B & 6 & 0 & & 6 \\
\textit{28A Kpn1/UAS-nonA} 9A & 3 & 0 & & 3 \\
\textit{15A Kpn3/UAS-nonA} 20B & 7 & 0 & & 7 \\
\textit{15A Kpn3/UAS-nonA} 9A & 10 & 0 & & 10 \\
\textit{18D Kpn4/UAS-nonA} 20B & 4 & 0 & & 4 \\
\textit{18D Kpn4/UAS-nonA} 9A & 4 & 0 & & 4 \\
\textit{41B Kpn5/UAS-nonA} 20B & 8 & 0 & & 8 \\
\textit{41B Kpn5/UAS-nonA} 9A & 6 & 0 & & 6 \\
\textit{15B Kpn5/UAS-nonA} 20B & 1 & 0 & & 1 \\
\textit{15B Kpn5/UAS-nonA} 9A & 10 & 0 & & 10 \\
\textit{34A Kpn7/UAS-nonA} 20B & 2 & 0 & & 2 \\
\textit{34A Kpn7/UAS-nonA} 9A & 2 & 0 & & 2 \\
\textit{24B Kpn7/UAS-nonA} 20B & 3 & 0 & & 3 \\
\textit{24B Kpn7/UAS-nonA} 9A & 3 & 0 & & 3 \\
\hline
\hline
\textit{Canton-S} & 4 & 4 & 2.8 ± 1.0 (16) & 2.4 ± 1.2 (14) \\
\hline
\end{tabular}
\caption{ERG response in wild-type (Canton-S) and \textit{Kpn1-7GAL4/UAS-nonA} transgenic flies}
\end{table}

\textit{N}, number of flies tested; +, wild-type ERGs (with light-on and light-off transients); −, mutant ERGs (without light-on and light-off transients). Mean, SD, and number (parentheses) of light-on and light-off amplitudes (mV) in Canton-S and in rescued 21A \textit{Kpn1/UAS-nonA} 9A transgenic flies are also reported.
The two groups of mutant nonA\textsuperscript{ths} songs (a and b, see materials and methods) were also recorded. We observed from preliminary analyses that in both these mutant lines, the average CPP value for the very first pulse in the song burst was >2. This value was thus significantly higher (see below) than the value for the 235R11 nonA\textsuperscript{+} control transformants, which was <1.5 (see Figure 5). Kulkarni \textit{et al.} (1988) also showed that the very first pulse of a nonA\textsuperscript{th} mutant train also had a higher CPP value than that of the (non-transformant) wild-type song. However, subsequent studies have used the significant and steady increase in CPP, which is observed during a nonA\textsuperscript{ths} mutant song train and which is not observed in the wild-type songs, to discriminate between the two genotypes (e.g., Renda\textit{ahl} \textit{et al.} 1992, 1996). We avoided this extremely labor-intensive approach, which requires logging all the pulses in each train, and replaced it by simply scoring the relevant pulse characteristics from the very first pulse of each train. This modified and shortened procedure revealed that neither the KpnGALA\textsubscript{U}UAS-nonA nor the UAS-nonA transformants showed any evidence for rescue of the nonA\textsuperscript{ths} song defect as measured by the high mean CPP values of the very first pulse in a train that ranged from 2.2 to 3.0 (Figure 5). The two nonA\textsuperscript{ths} lines also showed high CPP values as expected from the preliminary results. In contrast, the songs of 235R11 transformants, which carry the transgene that rescues all mutant phenotypes of nonA\textsuperscript{ths}, gave a mean CPP value of <1.5 (Figure 5). ANOVA gave a significant F-ratio ($F = 13.2$, d.f. 12, $P \ll 0.00001$), due mainly to the difference between 235R11 and the other groups. Post hoc tests also revealed a few marginal significant differences between some of the other groups. We conclude that the 5' region of nonA does not carry sequences that permit the normal expression of the wild-type song phenotype.

\textbf{Does the Kpn1GAL4 fragment rescue l(1)i19e lethality?} The 5' end of the large Kpn1GAL4 fragment encodes the sequences of dGpi1 from residue 149 onward (Campesan \textit{et al.} 2001, accompanying article). The dGpi1 transcription unit almost certainly corresponds to l(1)i19e, so the Kpn1GAL4 fragment should not rescue the l(1)i19e mutation because the N-terminal third of the dGPI1 will be absent. Heterozygous l(1)i19e/In(1) FM7, w females were crossed to X/Y; Kpn1GAL4/balancer males. If the Kpn1GAL4 fragment rescues l(1)i19e lethality, there should be non-In(1) FM7, w males among the progeny. We obtained 317 F1 males carrying In(1) FM7, w but not a single l(1)i19e/Y; Kpn1GAL4/+ male.

\textbf{DISCUSSION}

An overview of the spatial distribution of putative regulatory regions within the nonA promoter is shown in Figure 6. It is clear that the 451-bp fragment between Kpn5 and Kpn7GAL4 contains upregulating elements for all of the anatomical and one of the behavioral phenotypes. Residual contributions toward expression...
in the larval brain, eye and antennal discs, and adult CNS can be detected with the Kpn7GAL4 transgene. The restricted eye-antennal disc expression driven from this 5' fragment is limited to differentiating cells. The posterior ommatidia of the eye-antennal disc are formed first, and the anterior last (Lawrence 1992), so the proximal element could guarantee the early expression of nonA in these developing photoreceptors. More complex patterns of regulation are found for the eye-antennal and wing imaginal discs. In the more distal region located between Kpn1GAL4 and Kpn3GAL4, silencers appear to be present whose elimination leads to increased expression in these imaginal discs (Figure 6). In the adult, the Kpn7 region also drives low levels of transgene expression in the adult CNS. Moreover, silencing signals that downregulate expression in the adult thoracic muscles appear to be localized in the region present in Kpn3 but absent in Kpn5GAL4. Finally, sequences that drive nonA expression in the follicle epithelium and nurse cells of the female ovary are again localized in the proximal 450-bp region (between Kpn5GAL4 and Kpn7GAL4).

The data obtained from the walking optomotor test for Kpn1-, Kpn3-, and Kpn4GAL4/UAS-nonA flies that carried the larger promoter fragments revealed that the proportion of correct turning responses was lower, but not significantly different from the wild-type control. The optomotor behavior of Kpn5GAL4 flies was significantly poorer, but we did not see a correlated change in reporter gene activity in the optic lobes, even though a relationship between NONA protein levels and the optomotor phenotype has been reported (Rendahl et al. 1992; Stanewsky et al. 1993). Only in the Kpn7GAL4 transformants was there an association between the reduced lacZ reporter gene activity in the optic regions, with the further deterioration in optomotor performance. The significant decrease in the optomotor values of Kpn5 and Kpn7 transformants highlights the role of the 613-bp proximal element located between Kpn4 and Kpn7GAL4, which we presume may determine nonA levels in the lobula plate, the region in the optic lobes believed to play an important role in the control of the optomotor responses (Heisenberg et al. 1978).

Given the results of the optomotor response, it is perhaps surprising that only flies from line 21A Kpn1GAL4/UAS-nonA 9A showed rescue of the ERG. The progressive deletion of the nonA promoter region clearly has different effects on the two visual phenotypes. However, mutants defective in the ERG, but not in optomotor responses, have been described (Heisenberg 1972) and various nonA transformants show a similar dissociation of the two visual phenotypes (Rendahl et al. 1992). Thus the neural foci generating the ERG transients that are localized in the synapses within the first optic ganglion (lamina) are different from those that mediate the optomotor response (Heisenberg et al. 1978; Coombe 1986). Because the genomic 235R11 transgene rescues nonA mutant ERGs (Rendahl et al. 1992, 1996), we suspect that the introns that are absent in the UAS-nonA cDNA may be carrying enhancers for this phenotype. Intron enhancers are a common feature of Drosophila genes. For example, a 32-bp region within the second intron of proboscipedia acts as a labial disk enhancer (Kapoun and Kaufman 1995), whereas a 212-bp intron region within eyeless acts as an enhancer for both embryonic eye promordial and in the eye disks (Hauck et al. 1999). The rescue of ERGs in 21A Kpn1GAL4/UAS-nonA 9A individuals could reflect a position effect in which the Kpn1GAL4 transgene has come under the control of a local visual system “upregulating” enhancer. This view is supported by the peculiarly high-amplitude light-on and light-off transients observed in these rescued individuals when compared with the corresponding values in the control flies. However, it is still difficult to explain why the same Kpn1GAL4 transgene, when crossed to other independent UAS-nonA inserts, fails to rescue the ERG without advocating a peculiar interaction specific to those two lines.

Unlike the ERG results, where one line did rescue the mutant phenotype, no rescue of normal courtship song pulses was obtained even in the Kpn1GAL4 lines. This is unlikely to be due to the insert positions of all of these lines, so an explanation must be sought elsewhere. Once again we are forced to conclude that the introns of nonA may contain elements for enhancing the song phenotype. A hsp-nonA (cDNA) transgene rescues both song and visual defects of nonA94 mutants, even when the transgene is activated only in adulthood (Rendahl and Hall 1996). In the rescued transformants, the levels of heat-shock NONA detected by Western blots were almost twice those of wild type (Rendahl and Hall 1996). Therefore the high, promiscuous levels of NONA expression from the heat-shock promoter may have compensated for any enhancement problems stemming from the absence of introns. The first large >1.2-kb nonA intron in D. melanogaster reveals many putative binding sites for transcription factors that are detected by various algorithms (see Campesan et al. 2001, accompanying article; S. Campesan, unpublished results). Comparisons with the much larger 2.4-kb D. virilis first intron reveal conservation of a number of transcription factor sites including those for Even-skipped, Fushi-tarazu, Giant, Zerknullt, and Zeste (S. Campesan, unpublished results). In the 40–60 million years since these two species had a common ancestor, the neutral nucleotide substitution rate would have eliminated any sequence similarity that is not under selection (Moriyama 1987; Moriyama and Gojobori 1992; Schlotterer et al. 1994). Consequently, conservation of these putative intronic binding sites between the two species may represent functional regulatory regions.

The recently identified dGpiI gene overlaps with a large fraction of the nonA 5' sequences and almost cer-
tantly corresponds to l(1)i19e (Campesan et al. 2001, accompanying article). We could not rescue l(1)i19e lethality by crossing in the largest Kpn1GAL4 fragment, and so our results are not inconsistent with this view. From Figure 6 it can be observed that for every phenotype examined, anatomical or behavioral, upregulators for nonA expression are to be found in the region between the 5' ends of fragments Kpn5 and Kpn7. The transcription unit for dGpi1 ends in this region, 424 bp upstream of the initiating methionine of nonA in D. melanogaster (Campesan et al. 2001, accompanying article). This leaves ~200 bp within this Kpn5-7 region that is downstream of the dGpi1 transcription unit and upstream of the Kpn7 fragment, where we might predict that the majority of the enhancer elements might lie and thus not overlap with dGpi1 sequences. Within this intergenic region are found a putative Broad-Complex, Kruppel, and a heat-shock factor binding site (Campesan et al. 2001, accompanying article). However, the distal part of the Kpn5 fragment that does correspond to dGpi1 sequences (from the end of exon 7; see Figure 6) has a much larger number of putative transcription factor binding elements including Deformed, Antennapedia, Dorsal, and Broad-Complex (see Figure 6 in Campesan et al. 2001, accompanying article). The silencers of nonA expression for the wing and antennal disks and adult thoracic muscles, plus enhancers for the optomotor response, must lie within this region of the dGpi1 transcription unit. Future work will aim at elucidating the relative roles of the intergenic region vs. the dGpi1 sequence in their contribution to nonA regulation in this apparently congested area that defines Kpn5 from Kpn7.

To our knowledge this is the first time that coding sequences of a gene have been implicated as regulatory elements for its neighbor, at least in Drosophila. Overlapping genes are common in prokaryotes because of the constraints imposed by their small genome sizes. They are also found in eukaryotes and include genes inserted into the introns of other genes, genes that share a bidirectional promoter, 3' overlapping genes in which 3' exons are encoded by the same DNA fragment, and genes that share regulatory regions (reviewed in Bonnelaye and Laudet 1994). In Drosophila, the janusB (janB) transcription unit is encoded within the 3' untranslated exon of the adjacent janA gene (Yanicostas and Lepesant 1990). Promoter-reporter janB fusions reveal that the presence of the janA 3' sequences leads to reduced expression of the hybrid mRNA during spermatogenesis, suggesting that transcriptional interference may downregulate janB when both genes are being transcribed (Yanicostas and Lepesant 1990).

It is therefore intriguing that all the sequences that downregulate expression of nonA in the wing and antennal disks and in the adult thoracic muscles are to be found embedded in the dGpi1 transcription unit. Study of the temporal and spatial expression patterns of dGpi1 could illuminate the possibility of transcriptional interference between these two adjacent genes. In any case, the implications for the evolution of these regulatory sequences that are present within dGpi1 and in the intergenic spacer between dGpi1 and nonA have been explored by Campesan et al. (2001, accompanying article). These authors observed that putative binding sequences for transcription factors in the intergenic region showed a significant excess of fixed changes relative to polymorphisms compared to sites within dGpi1 sequences, suggesting a selective constraint within the latter region.

In conclusion, the promoter fragment we studied contains sequences that enhance and silence developmental and behavioral expression of nonA. All of the silencers are found within the dGpi1 transcription unit, suggesting a novel type of regulation of a downstream gene by the coding regions of its neighbor. We further suggest that there may be other regulatory sequences relevant for the ERG and courtship song phenotypes, and these may be located within the four introns of nonA that range from the smallest, 80 bp, to the largest, >1.2 kb (Jones and Rubin 1990). If the introns are not involved, then it would suggest that either the absence of the 45 bp of untranslated material in the nonA first exon may be responsible or that the GAL4 transactivation paradigm interferes with the normal expression of nonA. This could happen if spatial relationships between enhancers in the 5' region and within the nonA transcription unit itself were critical for some features of nonA expression. There is some evidence that using promoter GAL4 fusions can generate expression patterns that are not usually observed with the native sequences. For example, fusions of the timeless (tim) promoter to GAL4 produced ectopic expression of UAS-GFP in larval brain cells that were not observed using TIM immunohistochemistry (Emery et al. 1998). Such additional ectopic patterns might be expected if GAL4 stability is greater than that of the native protein that it replaces.

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