cell strain–specific.
17. Cultures were considered senescent when 1 PD took longer than 2 weeks. Senescent cultures were >95% β-galactosidase–positive [G. P. Dimri *et al*., *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9363 (1995)] and could be maintained for over 2 months without a significant decrease in cell number (*15*).
18. Passage number after gene targeting was arbitrarily set to 0 for each cell strain when the culture was first expanded into a 10-cm culture dish.
19. Low-level mitotic activity coexisted with cell death; cells of variable size, irregular shape, and a significant number of large multinucleated cells were observed; cultures gradually declined over 6 to 8 weeks (*15*).
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Natural Behavior Polymorphism Due to a cGMP-Dependent Protein Kinase of *Drosophila*

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Naturally occuring polymorphisms in behavior are difficult to map genetically and thus are refractory to molecular characterization. An exception is the *foraging* gene (*for*), a gene that has two naturally occurring variants in *Drosophila melanogaster* food-search behavior: rover and sitter. Molecular mapping placed *for* mutations in the *dg2* gene, which encodes a cyclic guanosine monophosphate (cGMP)–dependent protein kinase (PKG). Rovers had higher PKG activity than sitters, and transgenic sitters expressing a *dg2* complementary DNA from rover showed transformation of behavior to rover. Thus, PKG levels affected food-search behavior, and natural variation in PKG activity accounted for a behavioral polymorphism.

The molecular identities of genetic polymorphisms in behavior have been difficult to establish because these traits are usually inherited polygenically. One example of a single gene underlying a naturally occurring polymorphism is the *foraging* gene (*for*), which is involved in food-search behavior in the fruit fly *Drosophila melanogaster* (*1*, *2*). Individuals with a rover allele for^R move greater distances while feeding than do those homozygous for sitter alleles for^s (*3*). This difference in foraging behavior is observed during both the larval and adult stages (*4*). Rovers and sitters do not differ in general activity in the absence of food (*4*). Both rovers and sitters are wild-type forms that exist at appreciable frequencies (70% rover; 30% sitter) in natural populations (*1*, *5*). Several mutations (*6*) of the locus map with the naturally occurring alleles in the 24A3-5 region of the *D. melanogaster* polytene chromosomes (*2*, *7*). This region contains *dg2*, one of two cGMP-dependent protein kinase (PKG) genes in *Drosophila* (*8*). We report that (i) mutations in *for* mapped in or near *dg2*, (ii) excision of a P-element inserted into the *dg2* gene reverted the sitter phenotype to rover, (iii) wild-type for^s flies and all sitter mutants showed a decrease in PKG activity and level compared to the wild-type for^R, and (iv) *dg2* transgenes rescued rover larval behavior. These results demonstrate that *for* is *dg2*.

The *dg2* gene has three major transcripts, T1, T2, and T3 (*8*), and the *for* mutations are localized to this region (Fig. 1) (*9*). The P[GAL4] (*10*) transposable element in 189Y was inserted in the 5′ end of the *dg2* T2 transcript. This homozygous viable insertion identified a new *for* allele, because P-element excision reverted larval foraging behavior from a sitter to a rover phenotype (Table 1). As was the case with other sitter alleles, locomotion of the 189Y larvae was not reduced in the absence of food, indicating that the change in behavior was foraging-specific.

PKG enzyme assays were performed on adult heads of wild-type for^R and for^s, and mutant for^{s1} and for^{s2} strains (Table 2). for^R flies had significantly higher amounts of PKG enzyme activity than did for^s flies. Even greater reductions in enzymatic activity were seen in the mutants for^{s1} and for^{s2}. The amount of PKG in adult heads correlated with the adult foraging phenotypes of these strains (*4*).

To determine whether PKG is directly responsible for the foraging polymorphism in *Drosophila*, we overexpressed *dg2* in sitter larvae. This resulted in a change of behavior to the rover phenotype. The transgenic strain contained four copies of a heat shock– driven *dg2*-cDNA (*11*). The basal level of PKG expression in this transgenic strain (Fig. 2) was sufficient to rescue rover larval behavior, thus eliminating the lethal and sublethal effects of heat on the *dg2*-transgenic larvae (Table 3). As expected, the PKG enzyme activities of the dissected larval central nervous systems (CNSs) showed that without heat shock, the *dg2*-cDNA transgenic strain had levels of PKG similar to those of for^R and significantly higher than those of the sitter control strain (Table 3).

The basis for the *dg2* activity difference between for^R and for^s was further addressed by measurement of RNA levels and PKG protein. Northern (RNA) analysis revealed that for^s and for^{s2} showed a small but consistent (*12*) reduction in the abundance of T1 RNA relative to that in for^R (Fig. 3A). T2 and T3 RNA were also reduced in these strains, but to a lesser extent (*12*). To assess protein levels, we subjected extracts of adult heads to protein immunoblot analysis by probing with an antibody to bovine PKG, or the extracts were affinity-purified by chromatography on cGMP-sepharose, labeled, and electrophoresed (*13*). In both experiments, a prominent band at a molecular mass of 80,000 Daltons was found. This was the only band strongly induced by heat shock in the *dg2*-cDNA transgenic strain, and it was less intense in for^s than

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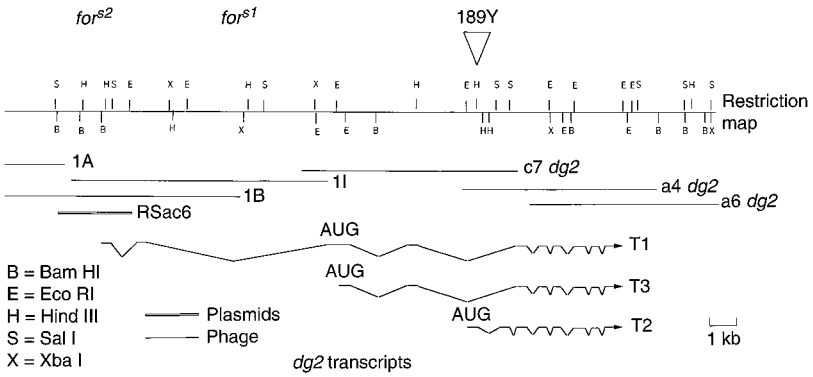

Fig. 1. Three major *dg2* transcripts are shown on the molecular map of *dg2*. They share a common 3′ region that encodes the kinase domain (*8*). Genomic clones used in the chromosome walk by D. Kalderon (*8*) are labeled a4, a6, c7, and RSac6, whereas those used in our walk are labeled 1I, 1A, and 1B. Mutants were mapped with restriction fragment length polymorphism analysis. Sitter *for* mutants cluster within *dg2*. for^{s1} and for^{s2} were generated in a for^R genetic background (*2, 4*), and 189Y is a P[GAL4] enhancer trap strain with an insert in *dg2*.

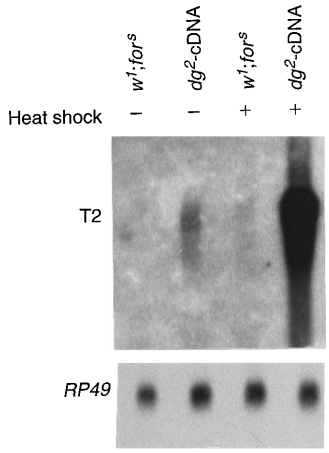

Fig. 2. The transgenic *dg2*-cDNA strain that carries T2 cDNA shows some expression in the absence of heat shock and strong overexpression under heat-shock conditions relative to the control w^1;for^s strain. Heat-shock protocol is in Table 3 legend. *RP49* probe was used as a control (*30*).

for^R (Fig. 3, B and C). (This band was also somewhat less intense in for^{s2} and nearly absent in 189Y homozygotes). Taken together, these results argue that the difference between the naturally occurring alleles for^R and for^s is in the level of expression of the enzyme.

The assignment of mutations in the *for* gene to the *dg2* locus not only establishes the identification of PKG mutations but also implicates the cGMP signal transduction pathway in the regulation of food-search behavior in *D. melanogaster*. Small but significant differences in the levels of this kinase affected the naturally occurring behavioral polymorphism. These small differences in PKG were even detectable in homogenates, indicating that the differences in PKG level in rovers and sitters might be larger in cells relevant to the expression of the foraging behavior. Our results suggest that the amount of kinase activity affects larval food-search behavior. Indeed, even modest quantitative changes in kinase activity affect behavior (*14*). Induced mutations that affect behavioral phenotypes often lie in signal transduction pathways (*15*). For example, the cyclic adenosine monophosphate (cAMP) system influences associative learning in flies (*16, 17*) and mice (*18*), and genetic variants in two other serine/threonine kinases, the calcium/calmodulin-dependent protein kinase II and protein kinase C,

Table 1. Foraging behavior and general locomotion of for^{189Y} and the excision strain E_1 measured as the mean ± SE (with sample size in parentheses) larval trail lengths in centimeters. The P-element in 189Y was excised with a source of transposase using the Δ*2-3* system (*27*). Excisions of the P-element were generated with the

w; $\frac{\text{Sp}}{\text{CyO}}$; $\frac{\text{ry } \Delta 2\text{-}3,\text{Dr}}{\text{TM6,Ubx}}$ strain.

Strain	Foraging behavior on yeast*	General locomotion on agar
189Y	4.3 ± 0.38 (39)	12.0 ± 0.66 (29)
E_1	9.1 ± 0.55 (40)	9.4 ± 0.65 (29)

*Locomotion of foraging third instar larvae was quantified as in (*5*). Strains differed in behavior on yeast [one-way analysis of variance (ANOVA), $F(\text{I}, 77) = 50.46$, $P < 0.0001$] and on agar [one-way ANOVA, $F(\text{I}, 56) = 7.77$, $P < 0.01$].

Table 2. Adult heads from flies with rover and sitter alleles show significant differences in their PKG activity (measured in pmol/min per milligram protein).

Genotype	Behavior	PKG kinase activity* (mean ± SE)	SNK†
Naturally occurring alleles			
for^R/for^R	Rover	12.6 ± 0.26	A
for^s/for^s	Sitter	11.4 ± 0.28	B
Sitter mutations induced on a for^R/for^R background			
for^{s1}/for^{s1}	Sitter	8.8 ± 0.26	C
for^{s2}/for^{s2}	Sitter	9.4 ± 0.22	C

*PKG enzyme assays were performed on adult head homogenates. Ten heads were homogenized in 25 mM tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM β-mercaptoethanol, PMSF (1 μg/ml), leupeptin (1 μg/ml), aprotinin (4 μg/ml), and 0.05% Triton X-100 and microcentrifuged for 5 min. The supernatant was removed and analyzed for cAMP-dependent protein kinase activity (*17*), and cGMP-dependent protein kinase activity (*28, 29*). A specific inhibitor of the cAMP dependent kinase (protein kinase inhibitor) was used (*17*). The reaction mixture contained (at final concentration): 25 mM tris (pH 7.4); 1 mM EDTA, 2 mM EGTA; 7 mM magnesium acetate; 0.5 mg/ml histone H2b; 1 nM of rabbit protein kinase inhibitor (PKI P-0300; Sigma), which is an inhibitor of cAMP kinase (K_i: 3 nM); 10 μM cGMP (Sigma); and 5μM adenosine 5′-triphosphate (ATP) with [∂-^{32}P]ATP at 5000 cpm/pmol in a final volume of 100 μl. [∂-^{32}P]ATP was supplied by New England Nuclear (NEG-002a, 3000 Ci/mmol). We performed cAMP kinase assays for each strain, using 10 μM ATP isotopically diluted at 1000 cpm/pmol, and the substrate of phosphorylation was the peptide Arg-Arg-Lys-Ser-Gly-Pro (A 3651, Sigma). cAMP kinase activities did not differ significantly between strains. Their means ± SD at $n = 3$ were 65.0 ± 5.0 pmol/min per milligram for for^R/for^R and for^s/for^s, 66.0 ± 5.8 for for^{s1}/for^{s1}, and 64.0 ± 4.5 for for^{s2}/for^{s2}. †ANOVA $F(4,49) = 26.18$, $P < 0.001$, $n = 12$ samples/strain, each sample was a mean of three measurements. Letters represent Student Neuman Keul's (SNK) test groupings at $P < 0.05$.

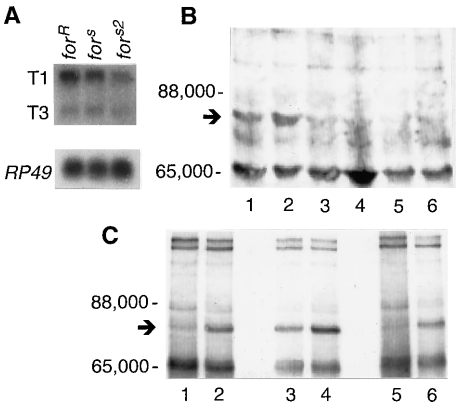

Fig. 3. (**A**) Northern analysis of polyadenylated RNA from homozygous adult flies probed with a 1.5-kb fragment specific to T1 and T3 (4.6 and 3.6 kb). The for^s naturally occurring allele and the for^{s2} mutation caused a reduction in the abundance of the T1 transcript relative to for^R. *RP49* probe used as a control (*30*). (**B**) Protein immunoblot of adult head extracts probed with antibody to bovine PKG (*13*). Lanes 1, for^R; lane 2, *dg2*-cDNA after heat-shock; lane 3, for^s; lane 4, for^{s2}; lane 5, w^1;for^s; lane 6, 189Y. (**C**) SDS-PAGE of enriched PKG fraction from adult head extracts after affinity chromatography on cGMP-sepharose and labeling with ^{125}I (*13*). Lanes 1, for^s; lane 2, for^R; lane 3, *dg2*-cDNA without heat shock; lane 4, *dg2*-cDNA after heat shock; lane 5, 189Y; lane 6, for^{s2}. Arrows in (B) and (C) indicate heat-inducible bands at ~80,000 Daltons recognized by PKG antibody and purified on cGMP-sepharose. Rovers have more intense bands than sitters.

Table 3. Larval foraging trail lengths (in centimeters) in *dg2*-cDNA transgenic flies and PKG enzyme activity assay levels (in pmol/min per milligram protein) in the larval CNS. Results are given as mean ± SE, with sample size in parentheses.

Strain	Foraging behavior*	PKG enzyme activity†
for^R (rover)	9.2 ± 0.53 (44)	11.2 ± 0.64 (13)
dg2-cDNA (transgenic)	8.9 ± 0.63 (44)	12.3 ± 0.56 (13)
w^1; for^s (host for transformation)	4.6 ± 0.29 (47)	10.0 ± 0.65 (12)

* *dg2*-cDNA transgenic strain had significantly longer mean trail lengths [one-way ANOVA, $F(1, 89) = 40.66$, $P < 0.0001$] and significantly higher PKG enzyme activities [ANOVA, $F(1, 23) = 7.15$, $P < 0.014$] than did the w^1; for^s strain. The for^R rover control and the *dg2*-cDNA transgenic larvae did not significantly differ in their foraging behavior and PKG enzyme activities; this demonstrated full rescue of rover behavior in the *dg2*-cDNA transgenic larvae (*11*). †The CNS of 25 third-instar larvae from *dg2*-cDNA transgenic strain and w^1; for^s were dissected and placed on ice in the extraction buffer [25 mM tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM β-mercaptoethanol, PMSF (1 μg/ml), leupeptin (1 μg/ml), aprotinin (4 μg/ml), and 0.05% Triton X-100]. Tissue samples were homogenized in 100 μl of extraction buffer and frozen at –70°C, thawed on ice, quickly centrifuged, and supernatants were immediately used for the assay. Three determinations gave mean ± SD. cAMP kinase activity of 69 ± 7 pmol/min/mg protein for *dg2*-cDNA transgenic strain and 65 ± 7 pmol/min per milligram for w^1; for^s. Under heat-shock conditions [two cycles of 15 min at 37°C and 15 min rest at 25°C, and 2.25 hours rest at 25°C], the PKG enzyme activities (mean ± SE) of dissected CNS of the *dg2*-cDNA transgenic strain = 19.51 ± 0.77 ($n = 10$) and w^1; for^s = 12.62 ± 0.67 ($n = 9$) differed [one-way, ANOVA, $F(1, 17) = 44.46$, $P < 0.0001$]; the for^R rover control PKG activity under heat shock was 14.35 ± 0.43 ($n = 3$).

affect learning and behavioral plasticity in flies (*14*) and mice (*19*). Our finding that *for* encodes a PKG shows that a naturally occurring genetic polymorphism in behavior involves these pathways.

PKG has a variety of pleiotropic cellular regulatory functions (*20*) that are also typical of signal transduction components (*15*). Electrophysiological studies have shown that injected kinase affects neuronal membrane conductance in snails and mammals (*21*), that inhibitors of PKG block long-term potentiation in mammalian hippocampus (*22*), and that PKG is involved in presynaptic long-term potentiation in cultured hippocampal neurons (*23*). Outside the nervous system, PKG has also been implicated in controlling proliferation of smooth muscle cells (*24*) and neutrophil degranulation (*25*). Our findings assign behavioral functions to this relatively scarce member of the serine/threonine kinases and show that subtle differences in PKG can lead to naturally occurring variation in behavior.

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12. This was quantified in five independent Northern analyses. T1 cDNA was digested with Xho I, which produced a 1.5-kb probe specific to T1 and T3. T2 RNA showed a small (25%) reduction in for^s and for^{s2} relative to for^R (K. A. Osborne and M. B. Sokolowski, data not shown).
13. Heads from 100 adult flies aged 4 to 7 days were homogenized in 2 ml of 40 mM tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 μg/ml phenylmethylsulfonyl fluoride (PMSF), 0.01% Triton X-100, then centrifuged at 10,000 rpm at 4°C for 10 min. For protein immunoblots, a sample of the supernatant was electrophoresed, blotted, and probed with a rabbit antibody to bovine PKG [T. L. Cornwell and T. M. Lincoln, *J. Biol. Chem.* **264**, 1146 (1989)] and detected with ^{125}I-labeled protein A (Dupont). For affinity chromatography, cGMP-sepharose [modified from J. D. Corbin *et al.*, *ibid.* **261**, 1208 (1986)] was incubated for 1 hour, by gentle resuspension at intervals of 5 min, with head homogenate prepared as described above, washed once in phosphate-buffered saline (PBS), incubated overnight at 4°C in 200 μl PBS with 10 μM cGMP, resuspended and centrifuged. For SDS–polyacrylamide gel electrophoresis (PAGE), 50 μl of affinity-purified supernatant was iodinated with ^{125}I and electrophoresed on a 7.5% acrylamide gel. Heat shock of the *dg2*-cDNA strain consisted of three treatments at 37°C of 15 min each, separated by 30-min intervals. To estimate the degree of enrichment from affinity chromatography, the eluted supernatants from each strain were assayed for PKG activity as described above, with the following modification to reduce nonspecific binding and background: the histone substrate was linked to *N*-hydroxysuccinimidyl-chloro-formate activated resin (Sigma) in 200 mM sodium bicarbonate (pH 8.0), with 1 M nonradiolabeled cGMP for 1 hour, followed by extensive washing in water. The activities measured (mean ± SD at $n = 3$) were: *dg2*-cDNA, 292 ± 50 pmol/min per milligram protein; w^1;for^s, 189 ± 33 pmol/min per milligram protein; and for^R, 201 ± 45 pmol/min per milligram protein.
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