Mechanism of Activation of the *Caenorhabditis elegans ras* Homologue *let-60* by a Novel, Temperature-Sensitive, Gain-of-Function Mutation

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ABSTRACT

The *Caenorhabditis elegans let-60* gene encodes a Ras protein that mediates induction of the hermaphrodite vulva. To better understand how mutations constitutively activate Ras and cause unregulated cell division, we have characterized *ga89*, a temperature-sensitive, gain-of-function mutation in *let-60 ras*. At 25°, *ga89* increases *let-60* activity resulting in a multivulva phenotype. At 15°, *ga89* decreases *let-60* activity resulting in a vulvaless phenotype in *let-60(ga89)/Df* animals. The *ga89* mutation causes a leucine (L) to phenylalanine (F) substitution at amino acid 19, a residue conserved in all Ras proteins. We introduced the L19F change into human H-Ras protein and found that the *in vitro* GTPase activity of H-Ras became temperature-dependent. Genetic experiments suggest that LET-60(L19F) interacts with GAP and GNEF, since mutations that decrease GAP and GNEF activity affect the multivulva phenotype of *let-60(ga89)* animals. These results suggest that the L19F mutation primarily affects the intrinsic rate of GTP hydrolysis by Ras, and that this effect may be sufficient to account for the activated-Ras phenotype caused by *let-60(ga89)*. Our results suggest that a mutation in a human *ras* gene analogous to *ga89* might contribute to oncogenic transformation.

RAS proteins are a conserved family of plasma membrane-associated GTPases that transduce signals from receptor tyrosine kinases during the growth and development of eukaryotic cells (BARBACID 1987; LOWY and WILLUMSEN 1993; PRONK and BOS 1994). Ras proteins cycle between a GTP-bound state in which they are active for signal transduction and a GDP-bound state in which they are inactive. The interconversion of Ras between the GDP- and GTP-bound states is under the control of two types of enzymes: guanine nucleotide exchange factors (GNEFs) and GTPase activating proteins (GAPs) (BOGUSKI and MCCORMICK 1993). GNEFs increase Ras signaling activity by facilitating the exchange of GDP for GTP, and include CDC25 in *Saccharomyces cerevisiae* and SOS in Drosophila and mammals. GAP proteins decrease Ras signaling activity by greatly increasing the rate of GTP hydrolysis by Ras, and include GAP-1 in Drosophila and *Caenorhabditis elegans* (A. HAJNAL and S. K. KIM, unpublished results) and p120-GAP and NF1 in mammals. Activation of receptor tyrosine kinases by binding to ligand leads to the recruitment of GNEFs to the plasma membrane where they increase the fraction of Ras that is in the GTP-bound, active state. When bound to GTP, Ras transmits signals by binding various effector proteins such as the protein serine/threonine kinase Raf, phosphitidyl-inositol-3-kinase, and others (PRONK and BOS 1994; MARSHALL 1996).

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Mutations in one of the three human *ras* genes (H-, K- and N-*ras*) are found in ~30% of all tumors, and in up to 90% of some types of carcinomas (BOS 1989). These mutations cause amino acid substitutions at residues 12, 13 or 61 of the 189 amino acid Ras protein. Biochemical characterization of the mutant Ras proteins encoded by Ras oncogenes has shown that these substitutions have three effects on Ras function and regulation (LOWY and WILLUMSEN 1993). First, they decrease the intrinsic GTPase activity of Ras. Second, they render the proteins insensitive to GTPase activation by GAP. Both of these effects are likely to cause an increase in the percentage of Ras that is in the GTP-bound state, leading to an excess of signaling. Third, these mutations appear to allow Ras signaling independently of GNEF activity, since they can cause cellular transformation in the presence of dominant-negative Ras mutant proteins that are believed to sequester GNEF (SIGAL *et al.* 1986; FEIG and COOPER 1988; BEITEL *et al.* 1990; HAN and STERNBERG 1990; STACEY *et al.* 1991). Therefore, these previously identified activating mutations appear to have multiple effects on Ras, making it difficult to dissect apart the relative contributions of each of these changes toward constitutive signaling leading to oncogenic growth. In particular, can a *ras* mutation that primarily affects only one of these biochemical properties (*e.g.*, the intrinsic GTPase activity) lead to a transformed cellular phenotype?

We have been able to address this question by studying how a Ras homologue functions in the induction of the *C. elegans* hermaphrodite vulva (reviewed in HOR-

VITZ and STERNBERG 1991). In the developing hermaphrodite, six vulval precursor cells (P3.p to P8.p) are competent to express vulval cell fates when induced by a signal from the anchor cell in the overlying somatic gonad. In wild-type animals, the anchor cell signal causes the closest vulval precursor cell (P6.p) to adopt the 1° vulval cell fate, which is to generate eight progeny cells that form the inner parts of the developing vulva. P6.p subsequently sends a lateral signal that causes the adjacent vulval precursor cells (P5.p and P7.p) to adopt the 2° vulval fate, which is to generate seven progeny cells that form the outer parts of the developing vulva (STERNBERG 1988; KOGA and OHSHIMA 1995; SIMSKE and KIM 1995). Under certain circumstances, low levels of anchor cell signal may directly induce P5.p or P7.p to adopt the 2° fate (STERNBERG 1988; KATZ *et al.* 1995). The remaining three vulval precursor cells (P3.p, P4.p and P8.p) are not induced by either the anchor cell or lateral signals and consequently adopt the 3° cell fate, which is to divide once and fuse with the surrounding hypodermal syncytium.

Signaling between the anchor cell and P6.p is mediated by a Ras signaling pathway (STERNBERG 1993; EISENMANN and KIM 1994; DUFFY and PERRIMON 1996). The components of the pathway relevant to this paper include the *lin-3* gene product [likely to be the anchor cell signal (HILL and STERNBERG 1992)], the *let-23* gene product [a receptor tyrosine kinase similar to EGF receptor (AROIAN *et al.* 1990)], the *let-60* gene product [a Ras homologue (HAN and STERNBERG 1990)] and the *mpk-1/sur-1* gene product [a MAP kinase homologue (LACKNER *et al.* 1994; WU and HAN 1994)]. Weak loss-of-function mutations in these genes result in a vulvaless phenotype in which too few or no vulval precursor cells express vulval cell fates. Null or strong loss-of-function mutations in these genes cause larval lethality, indicating that this Ras-mediated signaling pathway performs an essential function during development in addition to its role in vulval induction (reviewed in STERNBERG 1993; EISENMANN and KIM 1994; M. LACKNER, personal communication).

In addition to loss-of-function mutations, there are gain-of-function and dominant-negative mutations in *let-60 ras*. A gain-of-function *let-60* mutation, *n1046*, results in a multivulva phenotype in which excess vulval precursor cells express vulval cell fates (FERGUSON and HORVITZ 1985; BEITEL *et al.* 1990; HAN *et al.* 1990). The *n1046* mutation causes a glycine 13 to glutamic acid substitution (BEITEL *et al.* 1990). Missense mutations affecting residue 13 (G13V and G13D) in human N-Ras and K-Ras lead to transformation of NIH3T3 cells *in vitro* and contribute to oncogenesis *in vivo* (FASANO *et al.* 1984; BOS 1989). Dominant-negative mutations in *let-60 ras* lead to a vulvaless mutant phenotype, possibly by binding to and sequestering a factor required for normal LET-60 function, such as guanine nucleotide exchange factor (GNEF) (BEITEL *et al.* 1990; HAN *et al.* 1990; HAN and STERNBERG 1991).

Here we describe the identification and characterization of a temperature-sensitive mutation in *let-60 ras, let-60(ga89)*, which encodes a leucine to phenylalanine substitution at the conserved residue 19 (L19F). This mutation causes an activated *let-60 ras* phenotype (multivulva) at high temperature, and a loss-of-function *let-60 ras* phenotype (vulvaless) at low temperature. To determine how the *let-60(ga89)* mutation causes an increase or decrease in *let-60* signaling activity depending upon temperature, we analyzed the effect of this mutation on three separate functions of Ras; its intrinsic rate of GTP hydrolysis and its interactions with GAP and with GNEF. Biochemical analysis showed that this mutation (introduced into human H-Ras) decreased GTPase activity at high temperatures and increased it at low temperature relative to the activity of wild-type H-Ras. Genetic experiments suggested that the LET-60(L19F) protein produced by *let-60(ga89)* may retain its interactions with GAP and GNEF. Together these results suggest that the multivulva phenotype caused by *let-60(ga89)* may be caused primarily by effects on the intrinsic GTPase activity of LET-60. In addition, we determined that excess *let-60 ras* activity can perturb vulval development only during the late L2 and L3 stages, which may also identify the time during development that wild-type *let-60* activity is normally required.

MATERIALS AND METHODS

Genes, alleles and general procedures: Methods for culturing, handling and genetic manipulation of *C. elegans* were as described (BRENNER 1974). The animals described as wild-type were *C. elegans*, variety Bristol, strain N2. Individual cells were identified and Pn.p cell lineages were analyzed as described in SULSTON and HORVITZ (1977), using the criteria for the assignment of 1°, 2° and 3° cell fates described in STERNBERG and HORVITZ (1986). The following genes and alleles were used in this work; unless otherwise designated, the reference for a given allele is WOOD (1988): LGII: *let-23(sy1)* (AROIAN and STERNBERG 1991), *lin-7(e1413)* (SULSTON and HORVITZ 1981), *unc-52(e250)*; LGIII: *unc-79(e1068), mpk-1(n2521)* (LACKNER *et al.* 1994). LGIV: *unc-5(e53), unc-24(e138), lin-3(n378)* (FERGUSON and HORVITZ 1985), *dpy-20(e1282ts, e1362), let-60(ga89)* (this work), *(n1046)* (FERGUSON *et al.* 1987), *(n1876, n1531)* (BEITEL *et al.* 1990; HAN *et al.* 1990), *unc-22(e66), unc-30(e191), dpy-4(e1166), sDf8, nT1*. LGV: *him-5(e1490)*. LGX: *gap-1(n1329)* (A. HAJNAL and S. K. KIM, unpublished results), *dpy-3(e27), lon-2(e678), lin-2(n397)* (FERGUSON and HORVITZ 1985).

Isolation and mapping of *let-60(ga89)*: *let-60(ga89)* was isolated during a screen for EMS-induced mutations causing a protruding vulva (Pvl) phenotype (D. M. EISENMANN and S. K. KIM, unpublished results). *let-60(ga89)* hermaphrodites at 20° often have a weak multivulva phenotype due to either P3.p or P4.p adopting an induced vulval fate. The following genetic data from three-factor crosses indicated that the *ga89* mutation was within 0.05 map units of the *dpy-20* locus, which is <0.01 map units from the *let-60* locus: *unc-24(e138)* 12/25 *ga89* 13/25 *dpy-4(e1166)*; *unc-5(e53)* 50/53 *ga89* 3/53 *dpy-20(e1282ts)*; *dpy-20(e1282ts)* 0/48 *ga89* 48/48 *unc-30(e191)*.

Characterization of *let-60(ga89)* phenotypes: At 25°, *let-60(ga89)* hermaphrodites are multivulva, almost sterile and appear bloated or fluid-filled, and *let-60(ga89)* males do not mate. Strains were scored at different temperatures by transferring eggs from 15° to 20° or 25° and then by scoring the phenotypes of adults. *let-60(ga89)* multivulva (Muv) animals have ectopic pseudovulvae derived from inductions at P3.p and/or P4.p but not P8.p (Table 2). As described in the text, *let-60(ga89)* also causes a vulvaless phenotype that causes eggs or larvae to accumulate inside the mother.

The *let-60(ga89)* sterile phenotype is temperature-sensitive; the average number of progeny from *let-60(ga89)* hermaphrodites is 122 at 15° (n = 12 parents), 88 at 20° (n = 26) and six at 25° (n = 76). The low brood size of *let-60(ga89)* hermaphrodites is not simply due to abnormal sperm production, since mating *let-60(ga89)* hermaphrodites with wild-type males does not lead to an increase in brood size. *let-60(ga89)* animals have a defect in oocyte maturation or fertilization that resembles that recently described for mutations in *sup-39* (RUN *et al.* 1996). Oocytes accumulate behind the spermathecal opening, and hermaphrodites lay mostly unfertilized oocytes (data not shown). DAPI staining shows that oocyte nuclei appear to have a normal DNA content and are arrested in meiosis I with condensed chromosomes as in wild type. In wild-type animals, a single row of oocytes emerge from the gonadal syncytium at the point where the gonad arm reflexes; in *let-60(ga89)* animals, two rows of oocytes are often seen emerging at this point (data not shown).

At 25°, *let-60(ga89)* animals are larger than wild type in circumference and appear to be filled with fluid, such that the intestine and gonad arms are often floating in an enlarged body cavity (Figure 1D and data not shown). *let-60(ga89)* animals show this "bloated" phenotype at a high penetrance only at 25°.

To score male mating, 30 L4 *let-60(ga89); him-5(e1490)* males grown at 15°, 20° or 25° were mated with five to 10 L4 *unc-24(e138) dpy-4(e1166)* hermaphrodites. The presence of non-Unc non-Dpy F_1 cross progeny indicated successful male mating.

Construction of strains containing *let-60(ga89)*: The strain *let-60(ga89)/let-60(n1876)* was constructed by mating N2 males into *let-60(n1876)/dpy-20(e1362) unc-22(e66)* hermaphrodites. F_1 males from this cross were then mated into *let-60(ga89) unc-30(e191)* hermaphrodites at 15°. Non-Unc-30 F_2 cross progeny were cloned at 15° and animals of genotype *let-60(ga89) unc-30(e191)/let-60(n1876)* were identified since they did not segregate Dpy Unc-22 F_3 progeny. This strain was propagated at 15°, 20° and 25°. Heterozygotes were identified as non-Unc parents that segregated Unc progeny, and their vulval phenotype was scored.

The strain *let-60(ga89)/sDf8* was constructed by mating *dpy-20(e1282); him-5(e1490)* males into *sDf8/nT1* hermaphrodites. F_1 males from this cross were then mated into *let-60(ga89) unc-30(e191)* hermaphrodites at 15°. Non-Unc F_2 cross progeny were cloned and those that did not segregate Dpy or Vul progeny were inferred to be of genotype *sDf8/let-60(ga89) unc-30(e191)*. The vulval phenotype of non-Unc progeny of these animals was scored at 15°, 20° and 25°.

The strain *let-60(ga89)/let-60(n1531dn)* was constructed by mating *dpy-20(e1282); him-5(e1490)* males into *let-60(ga89) unc-30(e191)* hermaphrodites at 15°. Males from this cross were then mated with *let-60(n1531dn)/dpy-20(e1282) unc-30(e191)* hermaphrodites raised at 25° [*let-60(n1531dn)* is also temperature-sensitive (BEITEL *et al.* 1990)]. Non-Dpy non-Unc hermaphrodite progeny were cloned at 25°, and animals that segregated Unc progeny but no Dpy progeny were saved as *let-60(ga89) unc-30(e191)/let-60(n1531dn)*. The vulval phenotype of non-Unc progeny of these animals was scored at 15°, 20° and 25° [*let-60(n1531dn)* homozygotes die as larvae (BEITEL *et al.* 1990; HAN *et al.* 1990)].

The strain *lin-3(n378) let-60(ga89)* was built by crossing N2 males into *lin-3(n378) dpy-20(e1282)* hermaphrodites. F_1 males from this cross were mated with *let-60(ga89)* hermaphrodites. F_2 progeny were cloned, and *lin-3(n378) dpy-20(e1282) +/ + + let-60(ga89)* F_2 cross progeny were identified as those that segregated Vul Dpy F_3 progeny. Vul non-Dpy F_3 recombinants of genotype *lin-3(n378) + let-60(ga89)/lin-3(n378) dpy-20(e1282) +* were cloned at 20°. F_4 self progeny were cloned, and animals that did not segregate Dpy progeny were saved as *lin-3(n378) let-60(ga89)*.

The strain *let-23(sy1); let-60(ga89)* was created by mating *dpy-20(e1282); him-5(e1490)* males with *let-23(sy1)* hermaphrodites. F_1 males were mated with *let-60(ga89)* hermaphrodites. F_2 cross progeny of genotype *let-23(sy1)/+; dpy-20(e1282) +/+ let-60(ga89)* were identified as those that segregated Dpy and Vul progeny. F_3 Vul non-Dpy progeny of genotype *let-23(sy1); dpy-20(e1282) +/+ let-60(ga89)* were shifted to 25° and F_4 Muv progeny were cloned. F_4 animals that did not segregate Dpy animals were saved as *let-23(sy1); let-60(ga89)*.

The strain *let-60(ga89); lin-2(n397)* was constructed by mating *dpy-20(e1282) him-5(e1490)* males with *lin-2(n397)* hermaphrodites. F_1 males from this cross were mated into *let-60(ga89)* hermaphrodites. F_2 progeny form this cross were cloned, and animals of genotype *dpy-20(e1282) +/+ let-60(ga89); lin-2(n397)/+* were identified as those that segregated Dpy and Vul progeny. F_3 Vul non-Dpy animals were shifted to 25° and F_4 Muv progeny were cloned. Animals that did not segregate Dpy animals were saved as *let-60(ga89); lin-2(n397)*.

The *let-23(sy1)* and *lin-2(n397)* mutations enhance the multivulva phenotype of *let-60(n1046)* by a mechanism that is not yet understood (J. SIMSKE, A. HAJNAL, R. HOSKINS and S. K. KIM, unpublished results), but these mutations have no such effect on the multivulva phenotype of *let-60(ga89)* (data not shown).

The strain *mpk-1(n2521); let-60(ga89)* was built by mating *let-60(ga89); him-5(e1490)* males raised at 15° with *unc-79(e1068) mpk-1(n2521)* hermaphrodites. Non-Unc F_1 cross progeny were cloned and allowed to self-fertilize at 25°. Muv non-Unc F_2 hermaphrodites were cloned and allowed to self-fertilize at 20°. The presence of multiple (>10%) F_3 Muv non-Unc animals in the progeny was taken as evidence that the F_2 animals were of genotype *+ +/unc-79(e1068) mpk-1(n2521); let-60(ga89)* (fewer than 0.6% of *let-60(ga89)/+* heterozygotes are Muv at 20°). Unc F_3 hermaphrodites were saved as *unc-79(e1068) mpk-1(n2521); let-60(ga89)*. The Egl phenotype seen in some of these animals at 25° (Table 3) is due to weak effects on vulval induction similar to that described for the *mpk-1(ku1)* mutation (WU and HAN 1994) (data not shown).

The strain *let-60(ga89); gap-1(n1329)* was built by mating N2 males into the strain *lin-7(e1413) unc-52(e250); gap-1(n1329) lon-2(e678)*. Lon males from this cross were mated into *let-60(ga89); dpy-3(e27)* hermaphrodites. Non-Dpy F_2 hermaphrodites were cloned, and those of genotype *let-60(ga89)/+; + gap-1(n1329) lon-2(e678)/dpy-3(e27) + +* were identified because they segregated Muv, Dpy and Lon progeny, but no Vul or Unc progeny. Muv non-Dpy F_3 hermaphrodites were cloned at 20° and those that segregated 100% Muv non-Dpy F_3 progeny at 25° were identified and confirmed to be homozygous for both *let-60(ga89)* and *gap-1(n1329)*. The vulval phenotype of both *let-60(ga89); gap-1(n1329)* and *let-60(ga89); gap-1(n1329) lon-2(e678)* animals was scored; the presence of *lon-2(e678)* did not make a significant difference in the vulval phenotype of the strain. The strain *let-60(n1046); gap-1(n1329)*

was provided by JEFF SIMSKE (J. SIMSKE and S. K. KIM, unpublished results).

Laser ablation of the gonad: The somatic gonad precursor cells Z1 and Z4 were ablated with a laser microbeam during the L1 stage as described (KIMBLE 1981). Animals subjected to laser treatment were then grown at 25°, and their vulval phenotypes were scored as adults. Mock-ablated animals were used as controls. Ablation of the gonad appears to increase the penetrance of the multivulva phenotype of *let-60(ga89)* animals (Table 3), but decreases the level of vulval induction in *let-60(n1046)* animals (SUNDARAM and HAN 1995). The reason for the increase in penetrance of the *let-60(ga89)* multivulva phenotype upon gonad ablation is unknown.

Analysis of the temperature-sensitive period for *let-60(ga89)*: To determine the temperature-sensitive period for the *let-60(ga89)* multivulva phenotype, 20–30 gravid *let-60(ga89)* hermaphrodites raised at 15° were placed on a plate and allowed to lay eggs for 2 hr. The hermaphrodites were then removed and the eggs were either left at 15° or shifted to 25°. At a designated time after the midpoint of the 2-hr laying period, the plate was then either shifted up (15° to 25°) or shifted down (25° to 15°). After the animals had developed to adulthood, the percentage of multivulva and wild-type animals was determined. This experiment also showed that the temperature-sensitive period for the *let-60(ga89)* sterility defect is later than that for the multivulva phenotype, since animals shifted from 15° to 25° during the L4 stage were non-Muv and sterile (data not shown).

Identification of the *let-60(ga89)* mutation: Genomic DNA encoding each of the four exons of *let-60* along with exon/intron junctions was amplified from wild-type (N2) and *let-60(ga89)* worms by PCR using oligonucleotide primers as described (BEITEL *et al.* 1990). Six animals were used per reaction using conditions described for the analysis of single worms by PCR (WILLIAMS *et al.* 1992). PCR products were cloned into pCRII (Invitrogen) and sequenced using Sequenase (United States Biochemical Corp.). For each exon, the sequence of both DNA strands was determined from one wild-type and two independent *let-60(ga89)* DNA clones.

Introduction of mutations into the human H-*ras* gene and purification of H-Ras, H-Ras(L19F) and H-Ras(G12V) proteins: The plasmid pAT-RasH contains a cDNA encoding the first 166 amino acids of wild-type human Harvey Ras driven by the tac promoter (QUILLIAM *et al.* 1995). Single nucleotide substitutions were made in this plasmid using the Unique Site Elimination procedure (Pharmacia) to generate plasmids encoding H-Ras(G12V) and H-Ras(L19F). The mutagenic oligonucleotides were as follows: (G12V) 5′ GGTGGTGGGCGCTGTAGGCGTGGGAAAGAGTGC 3′ and (L19F) 5′ GGACTCCCAGGGCGGTCGACACGCCCACC 3′. Ras proteins expressed from pAT-RAS, pAT-RasG12V and pAT-RasL19F were induced and purified from *Escherichia coli* (DH5α). Briefly, 1 liter of cells grown at 37° to OD_{600} = 0.6 was induced with 0.5 mM IPTG for 2 hr. Cells were pelleted by centrifugation, resuspended in 10 ml TEDP buffer (20 mM Tris pH 7.7/1 mM EDTA/1 mM DTT/0.1 mM PMSF), and lysed by two passes through a French Press. Two hundred fifty milliliters of TEDP/20 μg/ml TLCK/20 μg/ml TPCK/100 μg/ml PMSF was added and the lysate was spun at 14K for 90 min at 4° in a JA-10 rotor. The supernatant was loaded onto a DEAE Sephacryl column and Ras proteins were eluted with a linear gradient from 0 to 500 mM NaCl in TEDP/3 mM $MgCl_2$. Fractions were collected, GDP was added to 10 μM, and fractions were analyzed by SDS-PAGE for Ras protein. Fractions containing Ras protein were pooled, concentrated and then loaded onto an S-100 gel filtration column in TEDP/100 mM NaCl/3 mM $MgCl_2$/0.1 mM GDP. Fractions from this column were collected, analyzed by SDS-PAGE, and those containing Ras protein were pooled. Ras protein samples were at least 90% pure as determined by Coomassie staining.

***In vitro* analysis of Ras GTPase activity:** GTPase assays were performed as described with modifications (MANNE *et al.* 1985). One micromolar [γ-^{32}P]GTP (10 mCi/ml, Dupont NEN) in 50 mM HEPES pH 7.5/1 mM DTT/5 mM $MgCl_2$/1 mM EDTA/100 mM NaCl was incubated at 24°, 30°, 37° and 42°. Assays were begun by the addition of 1 μM Ras protein (H-Ras, H-Ras(L19F), H-Ras(G12V)) or by buffer alone (no Ras control). Fifty microliter aliquots at various time points were taken in duplicate and mixed with 750 μl activated charcoal as described to assess release of radioactive P_i (MANNE *et al.* 1985). GTPase assays were performed at least twice for each of the four temperatures. The calculated rate constant ($k_{observed}$) reflects both the rate of GTP hydrolysis during the assay and the rate of exchange of prebound GDP for GTP at the start of the assay. It is unlikely that the decrease in the rate of GTP hydrolysis at higher temperatures reflects the irreversible unfolding of H-Ras(L19F) since preincubation of the protein at 42° does not alter its activity when measured at 30°. In addition, the percentage active H-Ras(L19F) protein, as measured by GDP binding at saturation concentrations, was similar at different temperatures.

RESULTS

Characterization of *ga89*, a temperature-sensitive mutation in *let-60 ras*: We have isolated a mutation called *ga89* that results in a temperature-sensitive multivulva phenotype. Genetic mapping experiments indicate that *ga89* is located close to *let-60 ras* (within 0.05 map units, see MATERIALS AND METHODS). As described below, complementation test data and DNA sequence analysis indicate that this mutation is a missense mutation in *let-60 ras*.

At 25°, *let-60(ga89)* animals exhibit a multivulva phenotype (Tables 1 and 2 and Figure 1D) and a male mating-defective phenotype (data not shown), which are phenotypes seen at all temperatures in animals containing the gain-of-function mutation *let-60(n1046)* (Figure 1B; FERGUSON and HORVITZ 1985; FERGUSON *et al.* 1987; WOOD 1988). Specifically, 57% of *let-60(ga89)* animals display a multivulva phenotype at 25°, and 18% display a multivulva phenotype at 20°. However, vulval induction and male mating appear virtually normal in *let-60(ga89)* animals at 15° (Table 1; Figure 1C and data not shown). Therefore at 20° and 25°, *let-60(ga89)* animals display phenotypes associated with excess *let-60* activity.

In contrast, *let-60(ga89)* apparently results in lower activity at 15° resulting in a weak vulvaless phenotype in certain genetic strains. At 15°, a single copy of *let-60(+)* is sufficient for normal vulval signaling, whereas a single copy of *let-60(ga89)* is not. Specifically, animals developed a normal vulva when *let-60(+)* was placed in *trans* to a *let-60* reduction-of-function mutation, *let-60(n1876)*, or to a chromosomal deficiency that removes the *let-60* locus, *sDf8* (BEITEL *et al.* 1990; HAN *et al.* 1990). However, animals displayed an egg-laying

TABLE 1

***let-60(ga89)* causes temperature-sensitive defects in vulval induction**

Genotype	15°	20°	25°
+/+	100% wild type (>1000)	100% wild type (>1000)	100% wild type (>1000)
let-60(n1046)/let-60(n1046)	70 ± 3% Muv (1248)	93 ± 1% Muv[a] (1559)	97 ± 2% Muv (336)
let-60(ga89)/let-60(ga89)	1 ± 0.8% Muv (600)	18 ± 2% Muv (1654)	57 ± 3% Muv (925)
+/*let-60(ga89)*	ND	ND	0.6 ± 0.4% Muv (1250)
+/*let-60(n1876)*	100% wild type (150)	100% wild type (150)	100% wild type (150)
let-60(ga89)/let-60(n1876)	77 ± 6% Egl (175)	5 ± 4% Egl (147)	100% wild type (200)
+/*sDf8*	100% wild type (150)	100% wild type (150)	100% wild type (150)
let-60(ga89)/sDf8	93 ± 3% Egl (281)	25 ± 6% Egl (224)	4 ± 3% Egl (148)

Vulval phenotypes of indicated strains at 15°, 20° or 25°. Muv indicates a multivulva phenotype and was scored by the presence of protrusions along the ventral surface of the animal. Egl indicates an egg-laying defective phenotype and was scored by the presence of either excess eggs or living progeny inside the animal. For any given strain, the remaining animals not indicated as Muv or Egl had a wild-type vulval phenotype. The penetrance of the indicated phenotype and the 95% confidence interval [calculated as $1.96(\mathrm{sqr}(x(1-x)/N))$, where x is the penetrance] for each value are shown. Values in parentheses are number of animals. The chromosomal duplication *sDf8* deletes the *let-60* locus (BEITEL *et al.* 1990; HAN *et al.* 1990). The full genotypes of the last two strains are *let-60(ga89) unc-30(e191)/sDf8* and *let-60(ga89) unc-30(e191)/let-60(n1876)*. Note that *let-60(ga89)/sDf8* animals display an egg-laying defective phenotype indicating that one copy of *let-60(ga89)* has less gene activity than wild type at 20°. ND, value not determined.

[a] Data are from BEITEL *et al.* (1990).

defective phenotype when *let-60(ga89)* was placed in *trans* to either *let-60(n1876)* or *sDf8* at 15° (Table 1). To determine whether this Egl phenotype was caused by defects in vulval induction, we examined the number of vulval nuclei present at the L4 stage in 48 *let-60(ga89) unc-30(e191)/sDf8* hermaphrodites raised at 15°. We found 10 animals in which three vulval precursor cells (P5.p, P6.p and P7.p) adopted the 3°, nonvulval cell fate instead of the 1° or 2° vulval cell fates (a strong vulvaless phenotype), 26 animals in which one or two vulval precursor cells expressed the 3° rather than the 1° or 2° cell fate (a weak vulvaless phenotype), and 12 animals in which vulval induction was as in wild type. These results indicate that most animals exhibit defects in vulval induction, suggesting that there is less *let-60 ras* activity in *let-60(ga89)/sDf8* animals than in *let-60(+)/sDf8*. Furthermore, these results indicate that *ga89* is likely to be an allele of *let-60*, since *ga89* fails to complement *let-60(n1876)* for the vulvaless phenotype at 15°. We did not determine whether defects in processes other than vulval induction, such as sex myoblast migration, might also contribute to the egg-laying defective phenotype in *let-60(ga89)/let-60(n1876)* or *let-60(ga89)/sDf8* animals. In summary, the *ga89* mutation causes multivulva and male mating phenotypes at higher temperatures similar to a *let-60* gain-of-function mutation, and can cause a vulvaless phenotype at low temperature similar to a *let-60* reduction-of-function allele.

In addition to temperature, there appear to be other factors that affect the level of signaling activity of *let-60(ga89)*. For example at 20°, 25% of *let-60(ga89)/sDf8* animals displayed an egg-laying defective phenotype (and no animals are multivulva), whereas 100% of +/*sDf8* animals displayed wild-type vulval development (Table 1). This result indicates that one copy of *let-60(ga89)* has less activity than one copy of *let-60(+)* at 20°. In contrast, 18% of *let-60(ga89)/let-60(ga89)* animals displayed a multivulva phenotype at 20°, suggesting that two copies of *let-60(ga89)* are more active than two copies of *let-60(+)*. Although the cause for this difference is unclear, these data suggest that the phenotype caused by *let-60(ga89)* is sensitive to *let-60* copy number. Furthermore, the apparent signaling activity of *let-60(ga89)* can be affected by mutations in other genes in the vulval signaling pathway. In *let-60(ga89); gap-1(n1329)* animals, *let-60(ga89)* appears to be more active than *let-60(+)* at 15° (see Table 4), whereas in *mpk-1(n2521); let-60(ga89)* animals *let-60(ga89)* appears to be less active than *let-60(+)* at 25° (see Table 3).

The results of genetic epistasis and gonad ablation experiments with *let-60(ga89)* are consistent with previ-

TABLE 2

Pn.p cell lineages in *let-60(ga89)* animals grown at 25°

Strain	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
Wild type	3°	3°	2°	1°	2°	3°
let-60(ga89)	**NNNN**	**N**LO	2°	1°	2°	3°
let-60(ga89)	NLO	N**LL**	2°	1°	2°	3°
let-60(ga89)	3°	NO**L**	2°	1°	2°	3°
let-60(ga89)	LLOO	3°	2°	1°	2°	3°

Cell lineage analysis of *let-60(ga89)* animals raised at 25°. T, a transverse axis of division; L, a longitudinal axis; O, an oblique axis; N, the cell did not divide. A letter in boldface type indicates that the cell (N) or its progeny (L) adhered to the ventral cuticle of the animal. The lineages adopted by P3.p–P8.p in wild-type animals are indicated by the designations 1°, 2° and 3° (STERNBERG and HORVITZ 1986). 1°, a vulval precursor cell that generated four progeny that divided in a final pattern of TTTT; 2°, a vulval precursor cell gave four progeny that divided in a final pattern of **LL**TN or NT**LL**; 3°, a vulval precursor cell that divided once. None of the cell lineages adopted by a P3.p or P4.p cell that divided more than once in *let-60(ga89)* animals resembled a strict 1° or 2° lineage.

TABLE 3

Epistasis analysis of *let-60(ga89)*

Genotype	Vulval phenotype at 25°	*n*
+	100% wild type	1000
let-60(ga89)	57 ± 3% Muv	925
let-60(ga89) gonad ablated	83 ± 8% Muv	91
lin-3(n378)	9 ± 1% Egl	200
lin-3(n378) let-60(ga89)	43 ± 5% Muv	306
let-23(sy1)	99 ± 1% Egl	614
let-23(sy1); let-60(ga89)	56 ± 5% Muv	332
lin-2(n397)	87 ± 3% Egl	374
let-60(ga89); lin-2(n397)	52 ± 5% Muv	363
mpk-1(n2521)	100% wild-type	300
mpk-1(n2521); let-60(ga89)	1 ± 1% Muv 16 ± 3% Egl[a]	545

Vulval phenotypes of indicated strains at 25°. Conditions and terms are as described in Table 1; *n* is the number of animals. Although the *mpk-1(n2521)* mutation causes no mutant vulval phenotype on its own, previous genetic analysis indicates it results in a decrease in *mpk-1* activity (LACKNER *et al.* 1994).

[a] The Egl phenotype of these animals is due to a weak effect on vulval induction, similar to that described for the mutation *mpk-1(ku1)* (MU and HAN 1994). In this double mutant, *let-60(ga89)* displays less gene activity than *let-60(+)* at 25°.

ous genetic analyses using the gain-of-function mutation *let-60(n1046)* (BEITEL *et al.* 1990; HAN *et al.* 1990; LACKNER *et al.* 1994; WU and HAN 1994; HOSKINS *et al.* 1996). The penetrance of the multivulva phenotype caused by *let-60(ga89)* was not reduced by laser ablation of the somatic gonad (including the anchor cell) or by reduction-of-function mutations in *lin-3* [encodes the anchor cell signal (HILL and STERNBERG 1992)], *let-23* [encodes the receptor for the anchor cell signal (AROIAN *et al.* 1990)], or *lin-2* [encodes a membrane-associated guanylate kinase that is required for local-

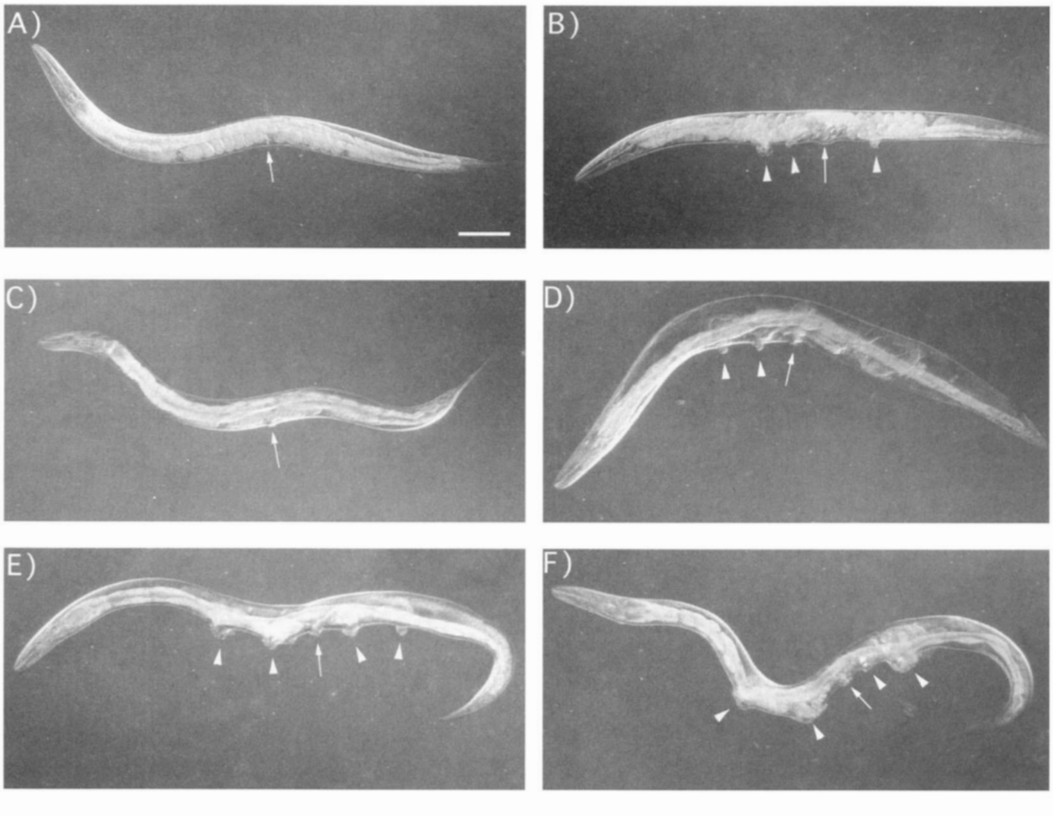


FIGURE 1.—*let-60(ga89)* causes a temperature-sensitive multivulva phenotype. Nomarski photomicrographs of the following: (A) wild type (N2) at 20°, (B) *let-60(n1046)* at 20°, (C) *let-60(ga89)* at 15°, (D) *let-60(ga89)* at 25°, (E) *let-60(ga89); gap-1(n1329) lon-2(e678)* at 15°, (F) *let-60(ga89); gap-1(n1329) lon-2(e678)* at 25°. An arrow indicates the position of the vulva. Arrowheads indicate the position of ectopic vulval inductions resulting in ventral protrusions. The expressivity and penetrance of the multivulva phenotype in *let-60(ga89); gap-1(n1329)* animals is greater than in *let-60(ga89)* animals. Bar, 50 μm.

ization of LET-23 (HOSKINS *et al.* 1996)] (Table 3), suggesting that *let-60* acts downstream of the LET-23 receptor tyrosine kinase. However, the penetrance of the *let-60(ga89)* multivulva phenotype was substantially reduced by a weak reduction-of-function *mpk-1* mutation (Table 3), suggesting that *let-60* acts upstream of the MPK-1/MAP kinase.

The *ga89* mutation causes a phenylalanine for leucine substitution at a conserved residue in LET-60: DNA sequence analysis showed that the *ga89* mutation is a single nucleotide change (CTC to CTT) in *let-60* that is predicted to cause a leucine to phenylalanine substitution at residue 19, L19F. Leucine is found at residue 19 in all Ras proteins identified to date, and in many other small GTPases as well (VALENCIA *et al.* 1991; LOWY and WILLUMSEN 1993). Leucine 19 is located in a small alpha helix (α1) carboxy terminal to the guanine nucleotide phosphate binding loop of Ras (DE VOS *et al.* 1988). Leucine 19 does not directly contact the guanine nucleotide in the active site but rather projects into a hydrophobic pocket in the Ras protein structure as part of a conserved structural core for Ras proteins (DE VOS *et al.* 1988; VALENCIA *et al.* 1991). The effects on Ras activity of a mutation at this residue have not been previously reported.

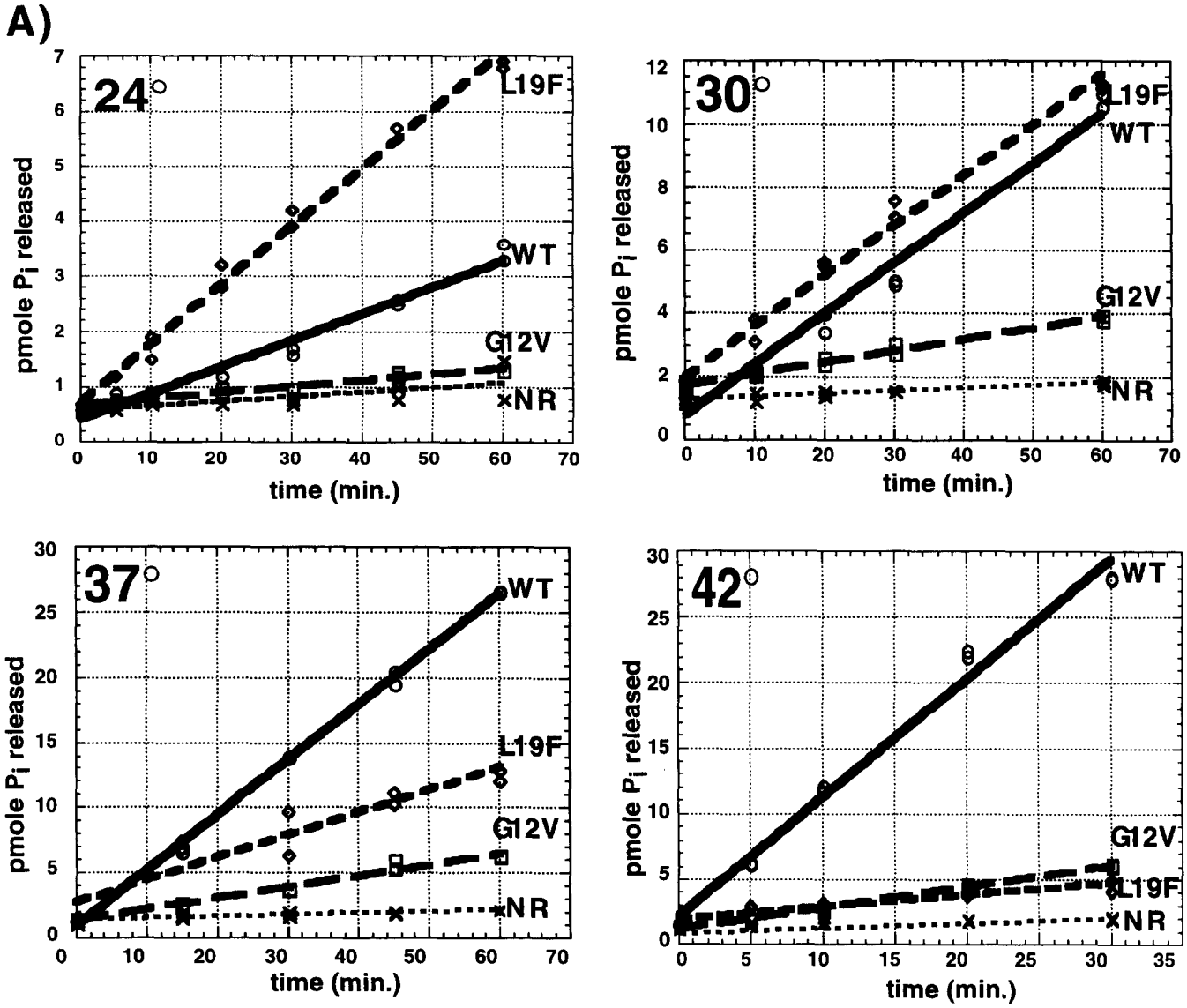
In summary, complementation data and DNA sequence analysis show that the *ga89* mutation is an allele of *let-60*. The *ga89* mutation is a new and unusual allele of *let-60* in two respects. First, it is a mutation that appears to either increase or decrease *let-60* activity depending on the temperature. Second, it affects a residue of LET-60 that is not directly part of the Ras nucleotide binding site or catalytic region, so it would be interesting to determine how this mutation results in increased LET-60 activation at high temperatures. Described below are biochemical and genetic experiments we undertook to try to determine how *let-60(ga89)* might alter the biochemical properties of LET-60 Ras. In particular, we asked whether the L19F substitution alters the intrinsic GTPase activity of Ras *in vitro* or the interactions between LET-60 and GAP or GNEF *in vivo*.

Human H-Ras containing the L19F substitution is a temperature-dependent GTPase *in vitro*: We used an *in vitro* assay to measure the GTPase activity of a Ras protein containing the L19F substitution to determine whether this substitution alters its intrinsic GTPase activity. In this experiment, we used human H-Ras rather than LET-60 since the GTPase activity of LET-60 has not been previously characterized. It seemed likely that the L19F substitution might cause similar defects in human H-Ras as in *C. elegans* LET-60, since the proteins are 89% identical in their first 166 amino acids (HAN and STERNBERG 1990), and all of the residues surrounding the sidechain of leucine 19 in the H-Ras crystal structure [residues 8, 79, 81, 114, 116, 144, 146, 152 and 156 (DE VOS *et al.* 1988)] are conserved in LET-60. We introduced the L19F mutation into a human H-*ras* gene encoding amino acids 1–166, expressed and purified the resulting H-Ras(L19F) protein from *E. coli*, and then measured its GTP hydrolysis activity *in vitro*.

We found that the GTPase activity of H-Ras(L19F) decreased with increasing temperature relative to that of wild-type H-Ras (Figure 2, A and B). Since H-Ras normally acts at 37° whereas LET-60 normally functions at 20° in the laboratory, we assayed H-Ras(L19F) and control proteins at their normal temperature (37°), a higher temperature (42°) and two lower temperatures (24° and 30°). At 42°, H-Ras(L19F) had a reduced rate of GTP hydrolysis (relative to wild-type H-Ras) that is similar to that of H-Ras(G12V), which is a strongly activated Ras mutant protein capable of efficient oncogenic transformation (LOWY and WILLUMSEN 1993). H-Ras(L19F) had a moderate decrease in GTPase activity even at the normal temperature of 37°. Decreasing the rate of GTP hydrolysis should increase the levels of H-Ras(L19F) in the active GTP-bound state, leading to an increase in Ras signaling activity. The overall similarity in protein structure suggests that L19F might have a similar effect on LET-60 as it does on H-Ras, and this effect could account for the multivulva phenotype of *let-60(ga89)* mutants. At a lower temperature than normal (24° *vs.* 37°), the rate of GTP hydrolysis by H-Ras(L19F) is higher than that of wild-type H-Ras. Increasing the rate of GTP hydrolysis should reduce the levels of GTP-bound Ras, leading to a decrease in Ras signaling activity. By analogy, LET-60(L19F) might also have an increased GTPase activity at a temperature lower than normal (15° *vs.* 20°), which could account for the vulvaless phenotype seen in *let-60(ga89)/sDf8* animals. In summary, the L19F substitution causes H-Ras to exhibit a temperature-dependent GTPase activity *in vitro*, and this effect may partially or wholly account for the temperature-sensitive vulval phenotypes observed in *let-60(ga89)* mutants *in vivo*.

***gap-1(n1329)* enhances the multivulva phenotype of *let-60(ga89)*:** We took a genetic approach to determine whether the L19F substitution affects the interaction of LET-60 Ras with GAP-1. *gap-1* encodes a *C. elegans* Ras GAP (A. HAJNAL and S. K. KIM, unpublished results). GAP proteins normally act to inhibit Ras signal transduction by increasing the rate of GTP hydrolysis and thereby decreasing the levels of active, GTP-bound Ras (BOGUSKI and MCCORMICK 1993). In *C. elegans*, *gap-1* inhibits the *let-60 ras* signaling pathway since a reduction-of-function mutation in *gap-1, n1329*, can partially suppress the vulvaless phenotype caused by a reduction-of-function mutation in *let-60* (A. HAJNAL and S. K. KIM, unpublished results). However, *gap-1(n1329)* does not affect vulval induction in an otherwise wild-type background.

We examined whether the *gap-1(n1329)* mutation en-


A)
24°
30°
37°
42°
L19F
WT
G12V
NR
pmole Pi released
time (min.)

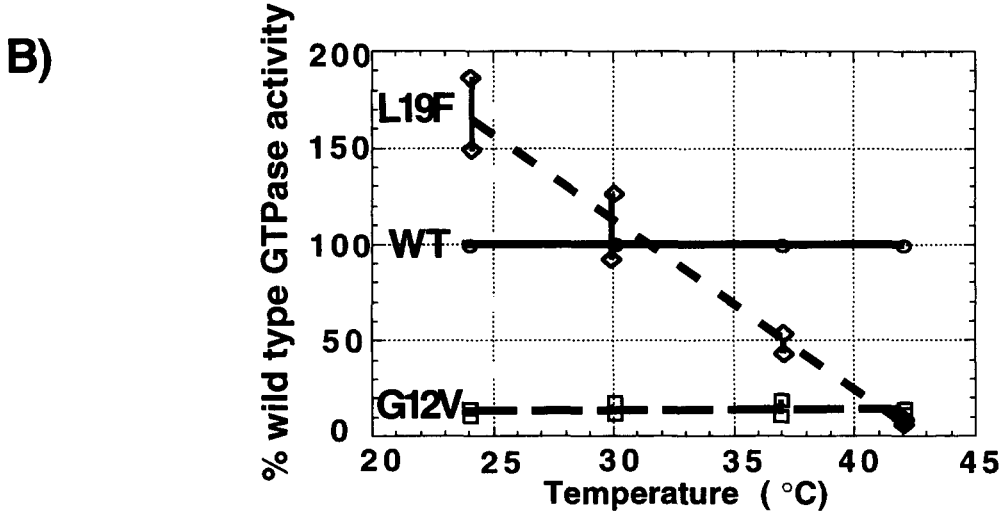

B)
% wild type GTPase activity
L19F
WT
G12V
Temperature (°C)

TABLE 4

A *gap-1* mutation enhances the multivulva phenotype caused by *let-60(ga89)*

Genotype	15°	20°	25°
let-60(ga89)	1 ± 0.8% Muv 1 ± 0.8% Egl (600)	18 ± 2% Muv (1654)	57 ± 3% Muv (925)
let-60(ga89); gap-1(n1329)	41 ± 4% Muv (753)	93 ± 3% Muv (301)	99 ± 1% Muv (200)
let-60(n1046)	70 ± 3% Muv (1248)	93 ± 1% Muv[a] (1559)	97 ± 2% Muv (336)
let-60(n1046); gap-1(n1329)	66 ± 4% Muv (541)	98 ± 2% Muv (220)	99 ± 1% Muv (200)

Vulval phenotypes for the indicated strains at 15°, 20°, and 25°. Conditions and terms are as described in Table 1. Values in parentheses are number of animals. Both the penetrance and expressivity (see Figure 1) of the multivulva phenotype in *let-60(ga89); gap-1(n1329)* animals are greater than in *let-60(ga89)* animals. Note that *let-60(ga89); gap-1(n1329)* animals are Muv at 15°, indicating that *let-60(ga89)* has more gene activity than *let-60(+)* at 15°.

[a] Data are from BEITEL *et al.* (1990).

hanced the multivulva phenotype caused by *let-60(ga89)*. We reasoned that if LET-60(L19F) interacts with GAP-1, then GAP-1 should increase the rate of GTP hydrolysis by LET-60(L19F), which should decrease the amount of LET-60(L19F) in the active, GTP-bound state and decrease the amount of signaling by LET-60(L19F). In this case, removing *gap-1* activity by a loss-of-function mutation should increase the level of vulval induction caused by *let-60(ga89)*. Alternatively, if LET-60(L19F) does not interact with wild-type GAP-1, then a loss-of-function *gap-1* mutation should have no effect on the level of vulval induction caused by *let-60(ga89)*. We found that the penetrance of the multivulva phenotype caused by *let-60(ga89)* was increased by the *gap-1(n1329)* mutation at all three temperatures examined (Table 4; Figure 1, E and F). For example, 42% of *let-60(ga89); gap-1(n1329)* double mutant animals were multivulva at 15°, compared to 1% of *let-60(ga89)* single mutants. This result indicates that LET-60(L19F) interacts with GAP-1, since a mutation predicted to cause a reduction in GAP-1 activity can lead to an increase in signaling by *let-60(ga89)*. However, we cannot determine from this type of *in vivo* experiment whether GAP-1 interacts as well with LET-60(L19F) as it does with wild-type LET-60 protein.

We found that *gap-1(n1329)* did not dramatically increase the penetrance of the multivulva phenotype caused by *let-60(n1046)*, either in *let-60(n1046)* homozygotes (Table 4) or in *let-60(n1046)/+* heterozygotes (data not shown). This result suggests that the GTPase activity of the protein expressed by *let-60(n1046)*, LET-60(G13E), may not be stimulated by GAP-1. This interpretation is consistent with data from *in vitro* experiments showing that H-Ras(G13V) is similarly defective in activation by human GAP (GIDEON *et al.* 1992).

A dominant-negative *let-60* mutation, *let-60(n1531)*, decreases vulval induction caused by *let-60(ga89)*: We also used a genetic approach to determine if the L19F substitution resulted in LET-60 activation that was independent of GNEF. If so, then the phenotype caused by *let-60(ga89)* should not be affected by the loss of GNEF activity. As a GNEF-encoding gene has not yet been genetically identified in the *C. elegans* vulval signaling pathway, it was not possible to directly remove its activity using a loss-of-function mutation. Instead, we took an indirect approach that relies on a dominant-negative form of LET-60 Ras that is believed to inhibit GNEF activity by binding and sequestering it (HAN and STERNBERG 1991). Mutant Ras proteins that act in a dominant-negative manner have been identified in *S. cerevisiae* (POWERS *et al.* 1989), in *C. elegans* (BEITEL *et al.* 1990; HAN *et al.* 1990; HAN and STERNBERG 1991) and by *in vitro* mutagenesis of human H-*ras* (SIGAL *et al.* 1986; FEIG and COOPER 1988). These dominant-negative proteins act to inhibit growth in yeast and mammalian cells, and cause lethality and loss of vulval induction in *C. elegans*. These dominant-negative Ras proteins are believed to sequester guanine nucleotide exchange factor,

FIGURE 2.—H-Ras(L19F) displays a temperature-dependent GTPase activity compared to wild-type H-Ras. (A) pmole Pi released over time at 24°, 30°, 37° and 42° for wild-type H-Ras (WT) (○—○), H-Ras (G12V) (□– – –□), H-Ras (L19F) (◇---◇) and no Ras control (NR) (×· · ·×). The experiments were performed twice at each temperature. Duplicate measurements for each timepoint for a single experiment are shown. (B) Activity of mutant Ras proteins relative to wild type at different temperatures. The data from GTPase assays like those shown in A were combined and averaged to calculate $k_{observed}$ (k_{obs}) values for each protein at the four temperatures. For each temperature, the GTPase activity of wild-type H-Ras is taken as 100% and the percentage activity of G12V and L19F is defined by k_{obs}(mutant)/k_{obs}(WT) × 100%. The k_{obs} values (in pmole Pi released/min/pmol Ras) for wild-type H-Ras are as follows: 0.002 at 24°, 0.006 at 30°, 0.014 at 37° and 0.032 at 42°.

TABLE 5

let-60(n1531dn)* decreases vulval induction caused by *let-60(ga89)

Genotype	Vulval phenotype at 25°	*n*
+	100% wild type	1000
let-60(ga89)/let-60(ga89)	57 ± 3% Muv	925
let-60(ga89)/+	0.6 ± 0.4% Muv	1250
let-60(ga89)/sDf8	4 ± 3% Egl	148
let-60(ga89)/let-60(n1531)	40 ± 5% Egl	336
+/let-60(n1531)	56 ± 7% Egl[a]	>200

Vulval phenotypes of indicated strains at 25°. Conditions and terms are as described in Table 1; *n* is the number of animals.

[a] Data are from BEITEL *et al.* (1990).

since overexpression of GNEFs can overcome the effects of dominant-negative Ras (SIGAL *et al.* 1986; POWERS *et al.* 1989). The mutation *let-60(n1531)* causes a Gly 15 to Asp substitution and 100% of *let-60(n1531)/+* animals are vulvaless at 20°, indicating that LET-60(G15D) inhibits signaling from the wild-type LET-60 protein in a dominant-negative fashion (BEITEL *et al.* 1990; HAN *et al.* 1990; HAN and STERNBERG 1991). We found that *let-60(ga89)/let-60(n1531)* animals exhibited less vulval induction than either *let-60(ga89)/+* or *let-60(ga89)/sDf8* animals (Table 5). Specifically, 40% of *let-60(ga89)/let-60(n1531)* animals had a vulvaless mutant phenotype at 25°, whereas 0% of *let-60(ga89)/+* and 4% of *let-60(ga89)/sDf8* animals had a vulvaless phenotype at 25°. This result suggests that LET-60(L19F) activity is dependent on GNEF activity, since sequestering GNEF activity can decrease signaling by LET-60(L19F).

In contrast, vulval induction caused by *let-60(n1046)* is not strongly dependent on GNEF activity. Previous work has shown that the penetrance of the multivulva phenotype caused by one copy of *let-60(n1046)* is not reduced by a reduction of GNEF activity caused by dominant-negative *let-60(n1531)*. Specifically, it was found that 19% of *let-60(n1046)/let-60(n1531)* animals had a multivulva phenotype, compared with 23% of *let-60(n1046)/let-60(+)* and 7% of *let-60(n1046)/sDf8* animals (BEITEL *et al.* 1990; HAN *et al.* 1990).

In summary, our results indicate that the L19F substitution may primarily affect only one of the biochemical properties of Ras that we tested. This mutation appears to affect the intrinsic GTPase activity of Ras but does not eliminate interactions with GAP or GNEF. These results suggest that the effect of the L19F substitution on the intrinsic GTPase activity of LET-60 may be sufficient to account for the multivulva phenotype observed in *let-60(ga89)* animals.

The temperature-sensitive period for the multivulva phenotype of *let-60(ga89)* is the L3 larval stage: We were able to use the temperature-sensitive nature of the *let-60(ga89)* mutation to determine the time of development during which excess LET-60 signaling can lead to a multivulva phenotype. We shifted *let-60(ga89)* animals from 15° to 25° (upshift) or from 25° to 15° (downshift) at various times during development. These temperature shift experiments show that a period from the late L2 stage to the mid to late L3 stage is the time during which the *let-60(ga89)* mutation can cause a multivulva phenotype (Figure 3). Specifically, the late L2 stage is the earliest time point during which a downshift results in the appearance of a significant percentage of multivulva animals. This result indicates that *let-60(ga89)* only acts after the late L2 stage because *let-60(ga89)* animals must be at 25° after this stage for them to show a multivulva phenotype. The mid to late L3 is the last time period during which an upshift results in an increase in the percentage of multivulva animals. This result indicates that *let-60(ga89)* must act before the late L3 stage because *let-60(ga89)* animals must be at 25° before this stage for them to become multivulva.

The *let-60(ga89)* temperature-sensitive period coincides with the time that the anchor cell signal normally

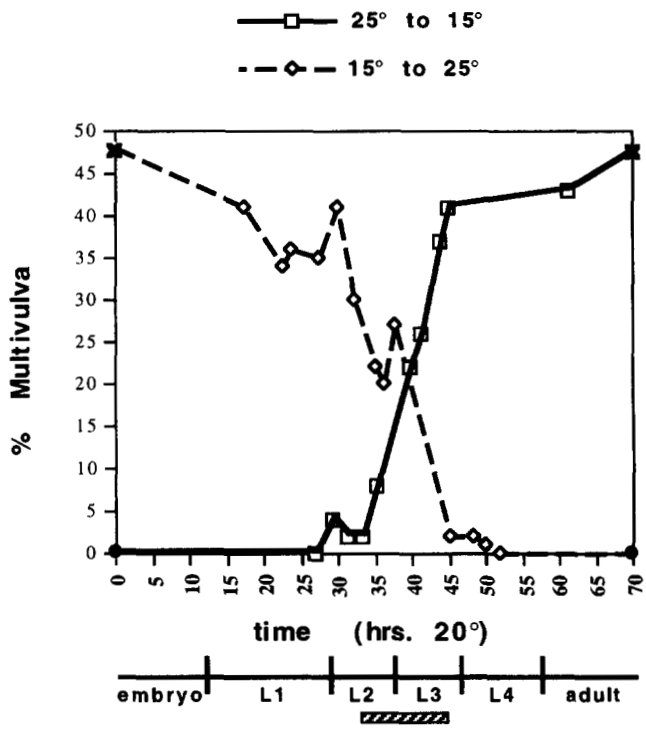


FIGURE 3.—The temperature-sensitive period for the *let-60(ga89)* multivulva phenotype is from the late L2 to late L3 stages. Eggs laid at 15° by *let-60(ga89)* hermaphrodites were incubated at 15° or 25° and then shifted up (15° to 25°, ◇) or shifted down (25° to 15°, □) at the indicated times during development as described in MATERIALS AND METHODS. The animals were allowed to develop into adults and their vulval phenotype was scored. ● and × indicate animals that remained at 15° or 25°, respectively, for the duration of the experiment. Times at 15° and 25° were converted to developmental time at 20° by dividing by 1.61 and 0.77, respectively (LEWIS and FLEMING 1995).

activates the *let-60* signaling pathway. Laser ablation experiments indicate that the anchor cell induces the vulva during the L3 stage (KIMBLE 1981; STERNBERG and HORVITZ 1986). In addition, previous analyses of the temperature-sensitive periods for the genes *let-23* and *lin-10* suggest that cell signaling occurs at this time (FERGUSON *et al.* 1987; KIM and HORVITZ 1990). Since *let-60(ga89)* likely results in constitutive activation of wild-type *let-60* activity, these results suggest that wild-type *let-60* also acts during this time.

DISCUSSION

We have described the isolation and characterization of *let-60(ga89),* a temperature-sensitive mutation in a *ras* homologue that functions in anchor cell signal transduction during *C. elegans* vulval induction. *let-60(ga89)* causes a multivulva phenotype at 20° and 25°, but causes a vulvaless phenotype in hemizygous animals at 15°. The *ga89* mutation is a new and interesting allele of *let-60* in several respects. First, it is a mutation that leads to an increase or decrease in *let-60* activity depending on the temperature. The majority of the *in vivo* data involving *let-60(ga89),* along with the *in vitro* analysis of H-Ras(L19F), support this conclusion. Although temperature-sensitive loss-of-function and dominant-negative mutations in *ras* genes have been described (SIGAL *et al.* 1986; HAN and STERNBERG 1991), this is the first example of a mutation that activates a *ras* homologue in a strongly temperature-dependent manner. Second, the *ga89* mutation may primarily affect one specific LET-60 biochemical activity, since it is likely to alter the GTPase activity of LET-60 based on analogy to the *in vitro* data from H-Ras(L19F), but it does not appear to eliminate the interaction of LET-60 with GAP or GNEF based on genetic experiments in *C. elegans.* In contrast to *ga89,* previously identified activating mutations in vertebrate *ras* genes strongly affect the interaction of Ras with GAP and GNEF in addition to causing a defect in intrinsic GTP hydrolysis. Third, the *ga89* mutation affects a residue of LET-60 that has not previously been found to be altered by Ras mutations isolated from tumors, or by genetic methods in *S. cerevisiae, C. elegans* or *D. melanogaster.* A similar L19F mutation may cause analogous changes in the biochemical properties of all Ras proteins, since a leucine is found in this position in all known Ras proteins (VALENCIA *et al.* 1991; LOWY and WILLUMSEN 1993).

Previously identified activating mutations in *ras* genes appear to have multiple effects on the biochemical functions of Ras. Oncogenic mutations that alter residues 12, 13 or 61 of Ras decrease both the intrinsic rate of GTP hydrolysis and the ability of GAP proteins to stimulate that GTPase activity (LOWY and WILLUMSEN 1993). Both effects are predicted to result in an increase in the levels of GTP-bound, active Ras and therefore both effects are likely involved in cellular transformation by activated Ras. In addition, such mutations allow Ras to be less dependent on the activity of GNEFs (SIGAL *et al.* 1986; FEIG and COOPER 1988; BEITEL *et al.* 1990; HAN *et al.* 1990; STACEY *et al.* 1991; LOWY and WILLUMSEN 1993). It has been difficult to dissect apart the relative contribution of each of these effects on Ras function toward the increase in Ras signaling that results in an activated Ras phenotype. Our analysis of *let-60(ga89)* suggests that this mutation may affect GTPase activity but does not completely eliminate GAP or GNEF interactions, and that the activated Ras phenotype in *let-60(ga89)* animals may be caused primarily by defects in GTPase activity.

However, we cannot rule out that the *ga89* mutation causes quantitative rather than qualitative changes in GAP interaction, or affects other aspects of Ras function. For example, the *ga89* mutation may reduce but not eliminate interactions with GAP-1, and this partial reduction in GAP-1 interaction could contribute to the *let-60(ga89)* multivulva phenotype. In addition, we have not tested the ability of LET-60(L19F) or H-Ras(L19F) to interact with other factors such as Raf or PI3 kinase. Altered binding to the *C. elegans* homologues of these effector molecules could also contribute to the multivulva phenotype caused by the *ga89* mutation. Further genetic and biochemical experiments will be required to determine whether or not the only substantial effect of the *ga89* mutation is on the intrinsic GTPase activity of LET-60.

How might the L19F substitution affect the GTPase activity of Ras? The position of residue 19 in Ras suggests several ways that a substitution of a phenylalanine residue for leucine could affect the structure or function of Ras (DE VOS *et al.* 1988). First, residue 19 is part of an alpha helix that is located immediately carboxy-terminal to the phosphate-binding loop that contains residues G12 and G13. Mutations affecting these residues are the most common substitutions found in *ras* oncogenes isolated from tumors, indicating that their integrity is critical for Ras function. It is possible that a perturbation of LET-60 structure by a substitution at residue 19 could affect the conformation of the loop containing residues 12 and 13 and thereby decrease Ras GTPase activity and increase the fraction of Ras in the active GTP-bound state. Second, it is possible that the L19F mutation alters the interaction of Ras with guanine nucleotides. The sidechain of L19 is found in a hydrophobic pocket where it comes in close contact with residues N116, T144 and A146. Mutations in these residues affect the interaction of Ras with guanine nucleotides such that the intrinsic rate of nucleotide exchange by Ras is increased, leading to an increase in the levels of GTP-bound, active Ras. Such mutations can cause transformation of NIH3T3 cells (FEIG *et al.* 1986; WALTER *et al.* 1986; FEIG and COOPER 1988). It is

possible that the L19F substitution might activate LET-60 or H-Ras in a manner similar to mutations at these residues. Finally, L19 is also in close contact with residues V152 and F156. A recent study has shown that a F156L substitution in Ras can lead to more GTP-bound Ras *in vivo* and to transformation of NIH3T3 cells (QUILLIAM *et al.* 1995). Unlike substitutions at residues 12 and 61 that lead to only small, local changes in Ras structure, the F156L substitution causes larger, global changes in Ras structure including effects on the conformation of residues 17–19. The proximity of L19 to F156 in the Ras structure suggests that L19F could activate Ras in a manner similar to F156L. Further characterization of Ras proteins carrying a L19F substitution should help determine how this change affects Ras structure and function.

Could the L19F substitution result in oncogenic activation of Ras in human tumors? L19 is a conserved amino acid in all known Ras molecules and many steps in the Ras pathway are conserved in worms, flies and mammals. Thus, analogous mutations at residue 19 in human *ras* genes might contribute toward oncogenesis in a manner that is similar to the way that *let-60(ga89)* causes a multivulva phenotype in worms. The L19F substitution causes a significant decrease in the GTPase activity of H-Ras at 37° and 42°. The effect of the L19F substitution on the GTPase activity of H-Ras at 42° is comparable to that caused by the G12V mutation, which is a mutation that strongly activates Ras. Ras(G12V) causes efficient transformation of NIH3T3 cells and contributes to oncogenesis *in vivo*. This result, combined with the fact that *let-60(ga89)* causes a multivulva phenotype in worms, suggests that the L19F mutation might activate H-Ras sufficiently well to cause transformation of NIH3T3 cells. However, we have seen no evidence of transformed foci following the transfection of NIH3T3 cells at 37° or 39° with DNA expressing H-Ras(L19F) (D. EISENMANN and S. K. KIM, unpublished results). One possible explanation for this result is that the vulval precursor cells of *C. elegans* may be more sensitive to LET-60 activation than NIH3T3 cells are to Ras activation. In this case, it may be possible to study small changes in the function or regulation of Ras homologues in *C. elegans*, and this added sensitivity should greatly help us understand how alterations in Ras and Ras signaling pathways contribute to tumorigenesis in humans. Another possibility is that GAP proteins prevent H-Ras(L19F) from transforming NIH3T3 cells by accelerating GTP hydrolysis and thereby decreasing signaling by the mutant protein. We found that removing GAP-1 activity with a *gap-1* loss-of-function mutation in worms dramatically increases the penetrance of the multivulva phenotype caused by *let-60(ga89)*. According to this possibility, transformation of NIH3T3 cells would require a second mutation to eliminate GAP-1 interactions, as well as the L19F mutation to reduce intrinsic GTPase activity. This result would suggest that resistance to GAP activity plays a larger role in the transformation of NIH3T3 cells than in the production of a multivulva phenotype in *C. elegans*.

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