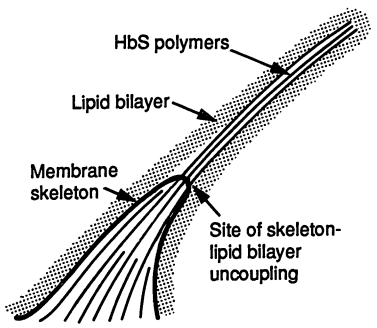

Fig. 4. Model of the long spicule in the deoxygenated sickled cell shows HbS polymers penetrating the membrane skeleton and the lipid bilayer uncoupling from the skeleton.

red cell ghosts that are induced at physiological salt concentrations to undergo transformation into echinocytes, the lipid bilayer becomes uncoupled from the skeleton (*8*), probably because of imbalances between the lipid and skeleton surface areas in the spicules of echinocytic cells. In contrast, the uncoupling of the lipid bilayer in the spicules of deoxygenated sickled cells is passively induced by HbS polymerization through gaps in the skeletal network, not by imbalances in lipid and skeleton surface areas. Lipid bilayers detached from their underlying spectrin skeletons are mechanically unstable and are prone to release from the cell body.

It is possible that the membrane in the spicule region suffers additional damage as a result of the lack of an underlying skeletal network, such as the loss of lipid asymmetry across the lipid bilayer (*9*), which has been demonstrated in RSC spicules (*5, 9*), and an increased leakage of intracellular and extracellular cations across the membrane (*10*). Future studies are needed to determine whether or not the increased cation leakages also occur in the spicules of these deoxygenated RSCs.

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Regulation of Ras-GAP and the Neurofibromatosis-1 Gene Product by Eicosanoids

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Ras-GAP (GTPase activating protein) is a regulatory protein that stimulates the intrinsic guanosine triphosphatase (GTPase) activity of the proto-oncogene product p21^{ras}. A domain of the neurofibromatosis gene product (NF1) that has sequence similarity to the catalytic domain of Ras-GAP and to yeast IRA gene products also has a specific stimulatory activity toward p21^{ras} GTPase. Arachidonic acid and phosphatidic acid inactivate GAP, but no agents have been identified that stimulate GAP and thereby switch p21^{ras} off. With the use of recombinant Ha-c-Ras and Ras-GAP, NF1, and GAP catalytic domains, it was found that prostaglandins $PGF_{2\alpha}$ and PGA_2 stimulated Ras-GAP and that prostacyclin PGI_2 inhibited Ras-GAP. The stimulatory effect of $PGF_{2\alpha}$ was saturable and structure-specific and competed with the inhibitory effect of arachidonic acid. Arachidonic acid also inhibited the catalytic activity of NF1, but prostaglandins were not stimulatory. These results suggest a mechanism for the allosteric control of Ras function through the modulation of arachidonate metabolism.

THE *ras* GENE PRODUCTS ARE 21-KD guanosine triphosphate (GTP)–binding proteins that act as molecular switches to control cell growth (*1*). Guanine nucleotide exchange factors can convert p21^{ras} to the active ("on") state, and a slow, intrinsic GTPase activity returns p21^{ras} to the inactive ("off") state (*2*). GAP accelerates the rate of GTP hydrolysis by p21^{ras} (*3*). The neurofibromatosis gene has sequence similarity to the mammalian *ras*-GAP and yeast IRA genes (*4*), and the catalytic domain of NF1 exhibits GAP-like activity (*5*). Both Ras-GAP and NF1 are commonly expressed (*3, 4*), and the question arises as to whether or not they are differentially controlled. Tyrosine kinases phosphorylate and may indirectly inactivate GAP (*6*). Moreover, certain lipids that are generated in response to mitogenic stimuli, particularly arachidonic acid and phosphatidic acid, also bind to and inactivate GAP (*7*). Additionally, a cytosolic protein has been described that inhibits p21^{ras} GTPase activity (*7*). Either of these inhibitory mechanisms would increase the proportion of p21^{ras} in the on state, but no mechanisms have been identified that stimulate GAP and switch p21^{ras} off.

Cyclooxygenase, which catalyzes the synthesis of prostaglandins from arachidonic acid, is an early-immediate gene product induced by growth factors, cytokines, and certain oncogenes (*8*). Because arachidonic acid inhibits Ras-GAP, we reasoned that other metabolites of arachidonate might also regulate the interaction of Ras and GAP or NF1. Therefore, we studied the effect of prostaglandins on recombinant Ha-c-Ras and recombinant GAP (*9*). The proportion of ^{32}P-labeled GTP hydrolysed over time was estimated either by direct filter binding ([γ-^{32}P]GTP) or by filter binding, extraction, and separation of the nucleotides ([α-^{32}P]GTP) by thin-layer chromatography (*10*) (Fig. 1). In the absence of GAP, none of the prostaglandins tested altered the basal GTPase activity of p21^{ras}. However, both $PGF_{2\alpha}$ and PGE_2 increased the effect of GAP on p21^{ras} GTP hydrolysis, and prosta-

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cyclin (PGI_2) inhibited the effect of GAP. Prostacyclin is highly unstable in aqueous solutions at physiological pH and spontaneously decomposes to 6-oxo-$PGF_{1\alpha}$ (half-life is ~3 min at 37°C, pH 7.4). Therefore, prostacyclin was incubated in tris-HCl, pH 7.5 (0.1 M), for 10 min before use. This treatment reduced the inhibitory effect of PGI_2 on GAP (Fig. 1). Similar data were obtained when rat brain cytosol was used as a source of GAP, instead of bacterially synthesized protein (*11*). To determine structural determinants of GAP activation, we tested a number of other eicosanoids (Table 1). Thromboxane B_2, $PGF_{1\alpha}$, and 9β-$PGF_{2\alpha}$ (a stereoisomer of $PGF_{2\alpha}$) were inactive; $PGF_{2\alpha}$, PGA_2, and PGE_2 stimulated GTPase activity. These and the results in Fig. 1 demonstrate that the allosteric effects of the eicosanoids on *ras*-GAP are structure-specific. In particular, an α-hydroxyl or carbonyl oxygen at C-9 and a double bond between C-5 and C-6 appear to be required for stimulation.

GAP does not increase the rate of guanine nucleotide release from $p21^{ras}$ (*2*). No effects of prostaglandins on the basal rate of guanine nucleotide release from $p21^{ras}$ were observed. In addition, the total amount of ^{32}P associated with $p21^{ras}$ remained constant in the presence or absence of prostaglandins, indicating that these lipids did not increase the rate of release of GTP from $p21^{ras}$ (*11*). These results demonstrate that the prostaglandins are modulating the catalytic effect of GAP on $p21^{ras}$ GTPase activity. The GTPase activity of Ha-v-Ras is not stimulated by GAP in the presence of $PGF_{2\alpha}$ (*11*).

We determined time-course and dose-response curves for activation of GAP by $PGF_{2\alpha}$ (Fig. 2, A and B). $PGF_{2\alpha}$ increased the initial rate of $p21^{ras}$ GTPase activity in the presence of GAP, with no detectable lag and with a half-maximal effect at about 30 μg/ml. This concentration is similar to that for the inhibition of GAP by phosphatidic and arachidonic acids (*7*). To determine whether the stimulatory effect of prostaglandins was caused by an increased affinity of Ras-GAP for $p21^{ras}$, we performed a competitive inhibition assay (*12*) with a low concentration of [γ-^{32}P]GTP–labeled $p21^{ras}$ and GAP (10 nM) in the presence of increasing concentrations of $p21^{ras}$ bound to GTPγS (a nonhydrolyzable analog of GTP). Prostaglandin $F_{2\alpha}$ increased the first-order rate constant for Ras GTPase activity by threefold in the presence of GAP (Fig. 2C) and decreased the affinity of GAP for $p21^{ras}$-GTPγS by about 50%. In the absence of $PGF_{2\alpha}$, the affinity of GAP for $p21^{ras}$-GTPγS was about 3 μM, in agreement with other measurements of this parameter (*5, 12*).

To ensure that the stimulatory effect was not a result of a contaminant in the prostaglandin preparations, we purified $PGF_{2\alpha}$ by thin-layer chromatography, extracted the silica from each section, and assayed the extracts for GAP stimulating activity. A parallel plate was stained for the prostaglandin with iodine vapor. Although a trace of material remained near the origin, the stimulatory activity was extractable only from the section that contained $PGF_{2\alpha}$ (*11*).

Arachidonic acid is an inhibitor of Ras-GAP (*7*). Therefore, we tested whether arachidonic acid could reverse the stimulatory effect of $PGF_{2\alpha}$ on Ras-GAP function. GTPase assays were performed in the presence of arachidonic acid, $PGF_{2\alpha}$, or both agents, with or without GAP (Table 2). In agreement with previous findings (*7*), arachidonic acid (50 μg/ml) almost completely inhibited Ras-GAP function. However, it only partially inhibited the stimulation of Ras-GAP by prostaglandin. A higher concentration of arachidonic acid reduced the GTPase activity to basal level. Therefore, these two agents appear to exert opposite effects on Ras-GAP function in a competitive fashion, possibly by interacting with the same site.

Finally, we tested the responsiveness of the NF1 catalytic domain (*13*) and a 333–amino acid COOH-terminal catalytic domain of GAP (*14*) to allosteric regulation by eicosanoids (Table 3). Although NF1 is inhibited by arachidonic acid, it was not stimulated by $PGF_{2\alpha}$. We reasoned that because the NF1 domain used in these assays represents only a fragment of the whole

Table 1. Effects of various prostaglandins on Ras-GAP function. Complexes between recombinant Ha-c-Ras and [γ-^{32}P]GTP were formed by incubating the protein and labeled nucleotide with EDTA (1 mM), tris-HCl (pH 7.5, 64 mM), and dithiothreitol (1 mM). The GTPase reaction was initiated by addition of $MgCl_2$ (2 mM) and recombinant GAP (10 nM) to a solution that contained 20 mM Hepes (pH 7.5) and $p21^{ras}$–[γ-^{32}P]GTP complexes (70 nM), in the absence or presence of prostaglandins (60 μg/ml), as indicated. Incubation was at 30°C for 20 s. We measured the GTPase activity by filtering the reaction mixture through nitrocellulose filters (0.2 μm pore size) as described (*3*) in order to follow the decrease in the concentration of [γ-^{32}P]GTP bound to $p21^{ras}$. Values are means of two independent experiments ± 2 SEM. TXB_2, thromboxane B_2.

Eicosanoid added	Ras-GAP	[γ-^{32}P]GTP remaining (%)
–	–	99 ± 2
–	+	51 ± 1
$PGF_{2\alpha}$	+	5* ± 1
9β-$PGF_{2\alpha}$	+	59 ± 8
PGA_2	+	11* ± 3
PGE_2	+	27* ± 1
$PGF_{1\alpha}$	+	41 ± 7
TXB_2	+	57 ± 2

*Indicates statistically significant difference from samples treated with GAP alone ($P < 0.05$).

Table 2. Competitive effects of $PGF_{2\alpha}$ and arachidonic acid on Ras-GAP activity. The assay was performed as in Fig. 1 with $p21^{ras}$ (50 nM) in the absence or presence of recombinant GAP (10 nM), $PGF_{2\alpha}$, and arachidonic acid (AA). Incubation time was 10 min at 30°C. Values are means of duplicate samples ± 2 SEM. Each treatment was compared independently to a control (+ GAP alone). In the absence of added Ras-GAP, none of the treatments had any effect on the amount of GTP remaining.

Treatments			GTP remaining (%)
GAP	$PGF_{2\alpha}$ (μg/ml)	AA (μg/ml)	
–	–	–	74 ± 1
+	–	–	30 ± 4
+	50	–	13* ± 2
+	–	50	72* ± 2
+	50	50	29 ± 2
+	50	100	75* ± 4

*Indicates statistically significant difference from samples treated with GAP alone ($P < 0.05$, by unpaired t test).

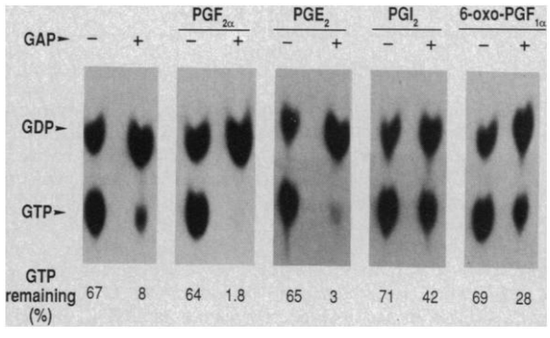

Fig. 1. Effects of various prostaglandins on Ras-GAP function (*9*). Recombinant Ha-c-Ras (50 nM) was incubated for 10 min at 24°C with 50 μCi [α-^{32}P]GTP (3000 Ci/mmol; Du Pont Biotechnology Systems) in buffer A (20 mM MOPS, 200 mM sucrose, 1 mM $MgCl_2$, 5 mM EDTA, 0.2% sodium azide, and 1 mM dithiothreitol, pH 7.4). Insulin (1 mg/ml) was added as a carrier protein. The GTPase reaction was initiated by addition of 10 mM $MgCl_2$, in the absence or presence of recombinant GAP (20 nM), with or without the indicated prostaglandins (60 μg/ml). Total assay volume was 60 μl. Incubation time was 10 min at 30°C. Percent GTP remaining was calculated as [(dpm GTP)/(dpm GTP + dpm GDP)] × 100 and quantitated as described (*9*). Results are representative of more than four similar experiments for each treatment. Samples of the incubated mixtures were separated on thin-layer chromatography plates and detected by autoradiography.

protein, part of the regulatory sequences may have been absent. We therefore compared the effects of NF1 with those of the COOH-terminal catalytic domain of GAP. Although the catalytic activity of full-length GAP is three to five times as great as the activity of this domain (*5*), the catalytic fragment remained responsive to allosteric activation by $PGF_{2\alpha}$. Therefore, it is likely that the regulation of NF1 is significantly different from that of Ras-GAP.

Table 3. Allosteric regulation of the COOH-terminal catalytic domain of GAP (ΔGAP) and the NF1 catalytic domain by eicosanoids. Assay for ΔGAP was performed as in Table 1 with the COOH-terminal catalytic domain of GAP (400 nM) (*14*). Where indicated, the reaction mixture included $PGF_{2\alpha}$ (60 μg/ml) or arachidonic acid (AA) (60 μg/ml), and all mixtures were incubated at 30°C for 30 min. Assay for NF1 was performed as in Fig. 1 with a recombinant fusion protein GST-NF1 (600 nM), which contains the domain with sequence similarity to Ras-GAP (*13*). Incubation time was 10 min at 30°C. Values are means of duplicate samples ± 2 SEM. Each treatment was compared independently.

Protein added	$PGF_{2\alpha}$	AA	[γ-^{32}P]GTP remaining (%)
–	–	–	68 ± 1
ΔGAP	–	–	21 ± 3
ΔGAP	+	–	12* ± 1
ΔGAP	–	+	68* ± 2
–	–	–	74 ± 1
NF1	–	–	55 ± 1
NF1	+	–	55 ± 1
NF1	–	+	70† ± 1

*Indicates statistically significant difference from samples treated with GAP alone ($P < 0.05$, by unpaired t test). †Indicates statistically significant difference from samples treated with GST-NF1 alone ($P < 0.05$).

These allosteric effects of eicosanoids on Ras-GAP and NF1 may be of physiological significance. However, receptors for $PGF_{2\alpha}$ and PGA_2 have been identified, and the concentration necessary to stimulate Ras-GAP (>50 μM) is at least two orders of magnitude higher than the affinity of the prostaglandins for the receptors (*15*). It is not clear that the GTPase assay is being performed under optimal conditions because the recombinant p21^{ras} is not post-translationally modified and is in solution, rather than attached to a plasma membrane. In addition, it is possible that the prostaglandins tested thus far are only weak allosteric activators, as compared to other metabolites of prostaglandins that have not yet been tested. Finally, it is possible that the activation of phospholipase A_2 and the induction of cyclooxygenase leads to high local intracellular concentrations of prostaglandins that bind to and stimulate Ras-GAP.

The likelihood that the stimulation is of physiological significance is heightened by the structural specificity of the effect. Conversion to the β-stereo isomer (9β-$PGF_{2\alpha}$), removal of a double bond in the fatty acid side chain, or introduction of an ether linkage between C-6 and C-9 all extinguished GAP activation. Hence, we suggest that Ras-GAP and NF1 function, and, consequently, the state of p21^{ras} in the cell, may be in part controlled by the relative concentrations of eicosanoids generated by the lipoxygenase and cyclooxygenase pathways. Stimulation of phospholipase A_2 by growth factors would produce an immediate rise in arachidonic acid and lipoxygenase products that could inhibit Ras-GAP and switch p21^{ras} to the on state. The induction of cyclooxygenase, over a period of hours, would lead to the generation of prostaglandins and the consequent stimulation of Ras-GAP and inactivation of p21^{ras}.

In support of this model, certain products of the lipoxygenase pathway are more potent inhibitors of Ras-GAP than is arachidonic acid (*7*). Prostaglandin E_2 inhibits growth of some tumor cells in vitro (*16*). It is intriguing to speculate that prostaglandin analogs might be designed that would not be recognized by the prostaglandin receptors but that would specifically stimulate Ras-GAP function and thereby inhibit cell growth. Such drugs would not be of value in controlling the growth of cells transformed by mutants of p21^{ras} that do not respond to GAP (*3*), but the drugs might inhibit the growth of cells transformed by oncogenes that operate upstream of *ras*, such as *src* or *neu*.

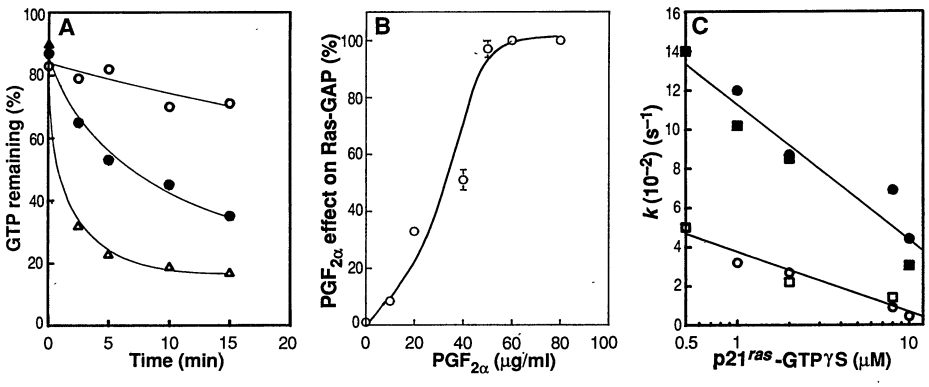

Fig. 2. Stimulatory effect of $PGF_{2\alpha}$ on Ras-GAP function. (**A**) Time-course study of p21^{ras} GTPase activity. Assays were performed as in Fig. 1 in the absence of recombinant GAP (○), with 10 nM recombinant GAP (●), or in the presence of both 10 nM recombinant GAP and $PGF_{2\alpha}$ (60 μg/ml) (△). Data are representative of two independent experiments. (**B**) Dose-response curve for the effect of $PGF_{2\alpha}$ on Ras-GAP function. We used recombinant GAP (10 nM) and the indicated concentrations of $PGF_{2\alpha}$. Assay was performed as in Fig. 1 for 10 min. The effect represents the increased Ras-GAP activity in the presence of $PGF_{2\alpha}$ over basal Ras-GAP activity with GAP alone. The 100% effect corresponds to a ratio of GTP/(GTP + GDP) equal to 0.17. Values are means of duplicate samples ± 2 SEM (where large enough to be indicated). (**C**) Competitive inhibition of Ras-GAP activity by p21^{ras}-GTPγS in the absence or presence of $PGF_{2\alpha}$. Assay was performed as described (*12*) with [γ-^{32}P]GTP–labeled p21^{ras} (70 nM) and recombinant GAP (10 nM) in the absence (○, □) or presence of $PGF_{2\alpha}$ (60 μg/ml) (●, ■) and with increasing concentrations of p21^{ras}-GTPγS as indicated. Complexes between p21^{ras} and GTPγS were formed as described in Table 1. The GTPase reaction was performed at 30°C for 20 s as in Table 1. The first-order rate constant for GTP hydrolysis is k. Squares represent the data from one independent experiment; circles represent the data from a second independent experiment. Lines were fitted to the data by a linear least-square analysis.

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13. A 1104-nucleotide segment of the NF1 gene was cloned into pGEX-1/RCT by R. Weiss (Howard Hughes Medical Institute, Salt Lake City, UT), and the protein was purified from *Escherichia coli* lysates by G. Xu (Utah) and F. Tamanoi (University of Chicago) (*5*).
14. A COOH-terminal fragment of Ras-GAP, consisting of the last 334 amino acid residues, was expressed in *E. coli* by G. Martin and purified by R. Halenbeck (Cetus Corporation) as described by M. S. Marshall *et al.* [*EMBO J.* **8**, 1105 (1989)].
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Spatial Control of Gut-Specific Gene Expression During *Caenorhabditis elegans* Development

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The nematode *Caenorhabditis elegans* was transformed with constructs containing upstream deletions of the gut-specific *ges-1* carboxylesterase gene. With particular deletions, *ges-1* was expressed, not as normally in the gut, but rather in muscle cells of the pharynx (which belong to a sister lineage of the gut) or in body wall muscle and hypodermal cells (which belong to a cousin lineage of the gut). These observations suggest that gut-specific gene expression in *C. elegans* involves not only gut-specific activators but also multiple repressors that are present in particular nongut lineages.

THE *ges-1* GENE OF THE NEMATODE *Caenorhabditis elegans* codes for a carboxylesterase that is expressed only in the intestine (or E) lineage (*1, 2*). *ges-1* expression is due to lineage-autonomous zygotic transcription that occurs when the embryo has 100 to 150 cells and the developing gut has only 4 to 8 cells (*1, 2*). Here we address the spatial control of *ges-1* expression during *C. elegans* development.

We have used DNA-mediated transformation to identify lineage-specific control sequences in *ges-1*. Exogenous transforming DNA can be integrated into the *C. elegans* genome (*3, 4*), but this is a rare event. *Caenorhabditis elegans* can also be "heritably transformed" by means of extra-chromosomal DNA that is passed through the germline with efficiencies of 20 to 90%, depending on the strain (*5–7*). The trans-forming DNA is usually co-injected with a second marker gene that allows transformants to be identified (*8*). We have also "transiently transformed" *C. elegans* by injecting exogenous DNA through the body wall of the mother worm into the oocyte cytoplasm (*9*). The oocyte is then fertilized naturally and stained for *ges-1* activity later in embryogenesis. A wild-type *C. elegans* embryo, stained to show normal *ges-1* activity in the developing gut, is shown in Fig. 1a. An embryo of the *ges-1* null strain JM1041 (*10*), in which the gut does not stain, is shown in Fig. 1b. An example of a *ges-1* null embryo in which gut-localized esterase expression has been accurately reconstituted by transient transformation with the wild-type *ges-1* gene (*11*) is shown in Fig. 1c.

We prepared a unidirectional deletion series beginning from the far upstream 5′ region of *ges-1* and proceeding toward the *ges-1*–coding region. Each deletion construct was introduced into the *ges-1* null worms by the use of both heritable and transient transformation; transformed embryos at roughly the 1½-fold stage of development were then stained for *ges-1* activity.

Six classes of *ges-1* spatial expression patterns were observed, depending on the size of the 5′ flanking *ges-1* DNA in the transforming construct. The top part of Fig. 2 shows examples of individual transformed embryos exhibiting these different *ges-1* expression patterns; the bottom part of Fig. 2 summarizes the pattern frequency (*12*). The different expression patterns were as follows.

I) Gut only. Transformation of *ges-1* null worms with a cosmid containing *ges-1* accurately reproduced the gut-specific pattern of wild-type esterase expression (Fig. 2, a, b, and c).

II) Either gut only or gut + pharynx. When *ges-1* null worms were transformed with deletion −1309, all staining embryos stained in all cells of the gut (Fig. 2, d, e, and f). However, 80 to 90% of the staining embryos also stained, albeit weakly, in the vicinity of the posterior bulb of the pharynx (Fig. 2, d and e). The proportion of embryos showing pharynx staining was reproducible, for example, 89% (n = 37), 89% (n = 19), and 84% (n = 96) in three independently transformed strains.

III) Either pharynx only or gut + pharynx. After transformation with deletion −1220, essentially all (98%) of the staining embryos stained in the gut, but 100% also stained in the pharynx. As more of the 5′ region was deleted, gut expression dropped dramatically, but pharynx expression remained high. With deletion −1140, all of the staining embryos stained in the pharynx, but only half (48%) stained in the gut. The all-or-none nature of these staining patterns was unexpected and striking; the intensity of gut-staining was not graded between individual embryos, but rather the entire pattern shifted (compare g and h in Fig. 2 with i) (*13*).

IV) Pharynx only. Embryos transformed with deletion −1069 expressed *ges-1* in the pharynx, but not in the gut. This pattern persisted down to deletion −1001 and was also found with deletions −521 to −473. In the absence of gut staining, a *ges-1*–expressing cell in the tail now became apparent (arrows in Fig. 2, j, k, and l).

V) Either pharynx or body wall muscle or hypodermis. With deletion −835, *ges-1* was now expressed in three distinct tissues: either the posterior pharynx (Fig. 2m), the posterior body wall muscles (Fig. 2n) or the posterior hypodermis (Fig. 2o) (*14*). As noted earlier, the expression patterns have an unexpected all-or-none character and few embryos stained in more than one of the three tissues. When the 5′ flanking region was deleted a further 10 bp to −825, the same three staining patterns could still be detected, but now the proportion of embryos that stained in the pharynx was increased to 97% and the proportion of embryos that stained in the hypodermis or body wall muscle was decreased to 13%.

VI) Pharynx + random. A predominantly pharynx staining pattern (class IV) was maintained by deletions extending to −473.

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