be formed between Cys^{99} and Cys^{115} in the mTNF-tip peptide. Similarly, a disulfide bridge between Cys^{100} and Cys^{116} was introduced in the tip peptide of hTNF. We synthesized the tip subpeptides with NH_2- and COOH-terminal cysteines in order to investigate the effect of ring closure on peptide activity. In contrast to the mTNF and hTNF tip peptides, these short peptides were very difficult to close by air oxidation, and no closure could be demonstrated within the time span of these experiments. The oxidized (closed) forms of these subpeptides were inactive (*10*). Furthermore, the peptide TPEGAE was synthesized and found to have an activity indistinguishable from that of tip subpeptide in its reduced (open) form (*10*).

25. For expression of mature mTNF in *Escherichia coli*, mature mTNF cDNA was fused exactly to an ATG codon in the expression vector pIG2 (Innogenetics, Ghent, Belgium). The T104A, P105A, and del(T^{104}–E^{109}) mutations were introduced directly into this vector by a variation on the cassette-mutagenesis method [J. A. Wells *et al.*, *Gene* **34**, 315 (1985)] with the use of synthetic oligonucleotides. The oligonucleotides used for the construction of mTNF(T104A) were 5′-CAAGGACGCTCCGGAGGGGGCTGAGCT-3′ and 5′-CAGCCCCCTCCGGAGCGTC-3′; for the construction of mTNF(P105A), 5′-CAAGGACACCGCTGAGGGCGCCGAGCT-3′ and 5′-CGGCGCCCTCAGCGGTGTC-3′; and for the construction of mTNF[del(T^{104}–E^{109})], 5′-CAAGGACCTCAAACCATGGTATGAGCCCATATAC-3′ and 5′-ATGGGCTCATACCATGGTTTGAGGTC-3′. Expression was carried out in *E. coli* strain MC1061(pcI857) [K. F. Wertman *et al.*, *Gene* **49**, 253 (1986); E. Remaut *et al.*, *ibid.* **22**, 103 (1983)].

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Mesodermal Patterning by a Gradient of the Vertebrate Homeobox Gene *goosecoid*

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Amphibian mesoderm arises from the marginal zone of the early gastrula and generates various tissues such as notochord, muscle, kidney, and blood. Small changes (twofold) in the amount of microinjected messenger RNA encoding the *goosecoid* (*gsc*) homeodomain protein resulted in marked changes in the differentiation of mesoderm in *Xenopus laevis*. At least three thresholds were observed, which were sufficient to specify four mesodermal cell states. Endogenous *gsc* messenger RNA was expressed in the marginal zone in a graded fashion that is congruent with a role for this gene in dorso-ventral patterning of mesoderm at the early gastrula stage.

Concentration gradients of molecules that elicit multiple discontinuous cellular responses at different threshold concentrations have been shown to play important roles during *Drosophila* embryogenesis (*1*). The view that concentration gradients of molecules might pattern mesodermal tissues in vertebrates has recently gained support from the discovery that uncommitted embryonal animal cap cells from *Xenopus* can respond to the peptide growth factor activin, giving rise to various mesodermal cell types at distinct threshold concentrations (*2, 3*). However, it is not known whether activin molecules are present in a graded manner at the right time and place during mesoderm specification. The homeobox gene *goosecoid* (*gsc*) was cloned from a dorsal lip complementary DNA (cDNA) library and was shown to encode a DNA-binding protein with a DNA-binding specificity similar (*4*), but not identical (*5*), to that of the *Drosophila* anterior morphogen *bicoid*. The *gsc* gene is expressed in the Spemann organizer of *Xenopus* embryos and is a primary response gene inducible by activin in the absence of protein synthesis (*6*). Its misexpression in ventral mesoderm is sufficient to induce morphogenetic movements and secondary embryonic axes (*6, 7*). Here we show that *gsc*, which is expressed at the right time and place for mesoderm specification, can pattern mesodermal differentiation through a concentration gradient.

Xenopus laevis embryos were injected with serial twofold dilutions of synthetic *gsc* mRNA; for each experiment, all eight vegetal blastomeres of a 16-cell stage embryo were injected. Embryos were cultured until the early gastrula stage, at which time ventral marginal zone (VMZ) explants were prepared. Explants were then cultured for 2.5 days until sibling embryos reached the tadpole stage (*8*). Increasing doses of *gsc* mRNA led to the inhibition of blood differentiation [the normal self-differentiation of VMZs (*9*)] and to the appearance of successive peaks of pronephros, muscle, notochord, and neural tissue (Fig. 1). These results showed that *gsc* is able to dorsalize the VMZ in a highly dose-dependent manner and suggested that discontinuous responses might underlie these tissue transitions.

To reveal the existence of concentration thresholds in the action of *gsc* mRNA, we analyzed the expression level of various marker genes in injected VMZ explants by quantitative polymerase chain reaction (PCR) (*10*). Explants cultured until sibblings were neurulae (Fig. 2A) show the following: (i) The expression of α-globin, typical of ventral cells (*11*), is turned off by *gsc*. (ii) The *Xenopus* homolog of the *brachyury* gene (*Xbra*), expressed normally in both ventral and dorsal cells (*12*), is induced over basal levels by small amounts of *gsc*. (iii) A *Xenopus* homolog of the *Hox* gene *labial*, *Xlab* (*4*), is induced by *gsc*. (iv) Cardiac actin, expressed in myotomes (*13*), is induced by moderate amounts of *gsc* mRNA. Sufficient *Xbra* mRNA can lead to muscle differentiation in animal caps (*14*), which raises the possibility that *gsc* might induce muscle by acting on *Xbra*. (v) UVS.2, a metalloprotease expressed dorsoanteriorly in hatching gland tissue (*15*), is induced with intermediate amounts and repressed with larger amounts of *gsc* mRNA, as is actin. (vi) Endogenous *gsc*, which at the neurula stage is expressed in head mesoderm (*16*) [the most dorso-anterior mesoderm (*3*)], is induced exclusively at the highest doses of *gsc* mRNA. Endogenous *gsc*, and not microinjected *gsc* mRNA, is detected in these PCR assays because the 5′ PCR primer we used hybridizes to a sequence in the 5′ untranslated region that is deleted in the injected *gsc* RNA (*10*). Autoactivation, such as that observed here, is a property expected of genes that generate stable cell states (*1*). (vii) Histone H4 (*17*) remains unaffected by *gsc* injection and was used as a reference. All PCR assays were carried out at cycle numbers below amplification-saturation levels, which were determined empirically for each primer pair (*10*).

We conclude that as little as a twofold difference in *gsc* concentration can affect cell fate after microinjection of *gsc* mRNA. Thus, sharp thresholds appear for *Xbra*, cardiac actin, and endogenous *gsc* at 2.5, 5, and 10 pg per blastomere, respectively (Fig. 2A). This defines at least four states of cell differentiation within an eightfold concentration range (1.25 to 10 pg per blastomere). The results do not, however, distinguish whether the genes analyzed are directly affected by the *gsc* DNA-binding

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protein or are indirectly activated through intermediate gene regulators.

The effect on cell differentiation is specific for *gsc*, because microinjection of two control RNAs—preprolactin and Δ*gsc*, a deletion construct lacking the homeodomain (*10*)—fail to dorsalize (Fig. 2B). At very high doses (300 pg), however, Δ*gsc* can induce *Xbra* and cardiac actin. This dorsalization is not sufficient to turn off α-globin, and the dose needed is 60 times higher than the dose of wild-type *gsc* mRNA required to evoke a similar effect. The weak dorsalizing effect of Δ*gsc* might be explained by a mechanism similar to that observed in mutants of the SP1 transcription factor that lack a DNA-binding domain. They are still able to oligomerize with wild-type molecules when overexpressed and therefore activate transcription (*18*).

To determine whether the levels of injected *gsc* mRNA were comparable to those normally found in the early gastrula, we measured injected transcripts and compared them to a standard curve of synthetic mRNA by quantitative reverse transcription PCR (RT-PCR). It was found that at the highest concentration used in Fig. 2, the amount of injected *gsc* mRNA that remained in VMZ explant equivalents at the early gastrula stage was about the same (1.1-fold) as the amount of *gsc* mRNA present in the organizer (*19*). Thus, the injected amounts of mRNA are in the physiological range, although the steady-state level of protein made from it is unknown because of lack of a suitable antibody.

Dorsalization was also observed in VMZ explants microinjected with a DNA construct in which *gsc* cDNA is under the control of the cytomegalovirus promoter (pCMV*gsc*) and is expressed only after the midblastula transition (*7*). Although injection of DNA was less effective than injection of mRNA (*20*), the results exclude the possibility that the dorsalizing activity of *gsc* is due to premature expression before Spemann's organizer is formed.

How do these threshold responses to *gsc* relate to normal development? The specification map of the early *Xenopus* gastrula, determined by culturing explants from different regions of the marginal zone (9), shows that they differentiate largely as blood and that only the dorsolateral and dorsal marginal zones give rise to pronephros, muscle, and notochord tissue (Fig. 3A). Thus, the specification map parallels the effects of injecting increasing doses of *gsc* mRNA.

We analyzed the expression of endoge-

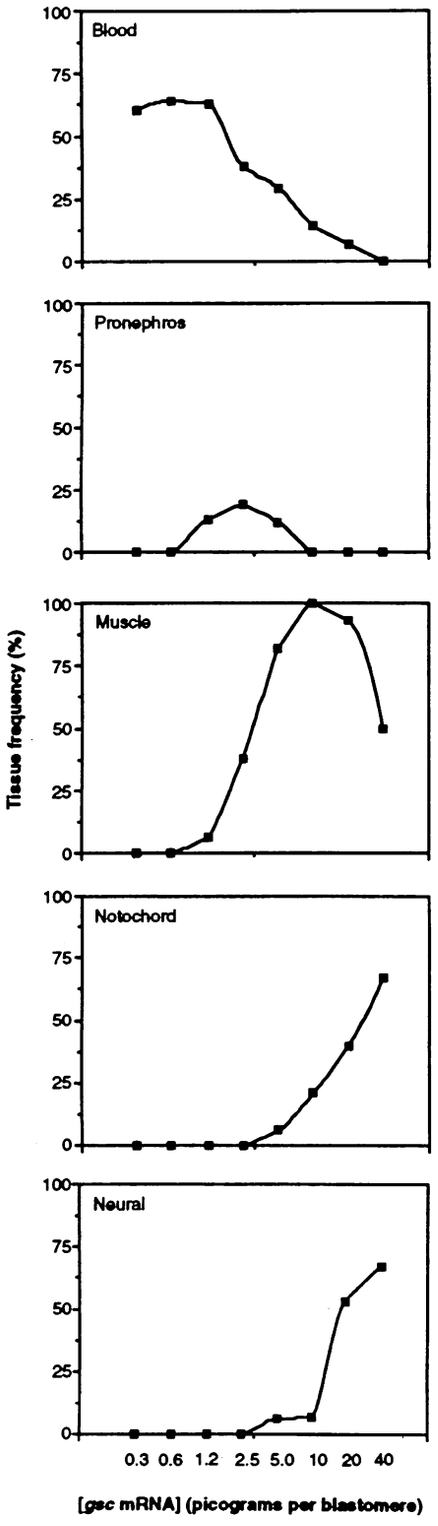

Fig. 1. *goosecoid* mRNA leads to the formation of dorsal tissues in a dose-dependent manner. Embryos were injected with synthetic *gsc* mRNA at the indicated dose per blastomere (shown in a semilogarithmic scale). Ventral marginal zone explants were prepared at the early gastrula stage and cultured for 2.5 days, then fixed and processed histologically (*8*). For each injected *gsc* dose, 13 to 18 independent explants were analyzed for the presence of the indicated marker tissues, the frequency of which is given as a percent and plotted against the injected *gsc* dose. The complete experiment was performed twice, yielding similar results. The frequency of pronephric tubules reached 70% (*19*). The total number of scored explants is represented by 100%, and each individual explant may contain multiple tissues. Note that successive peaks of tissue differentiation are observed.

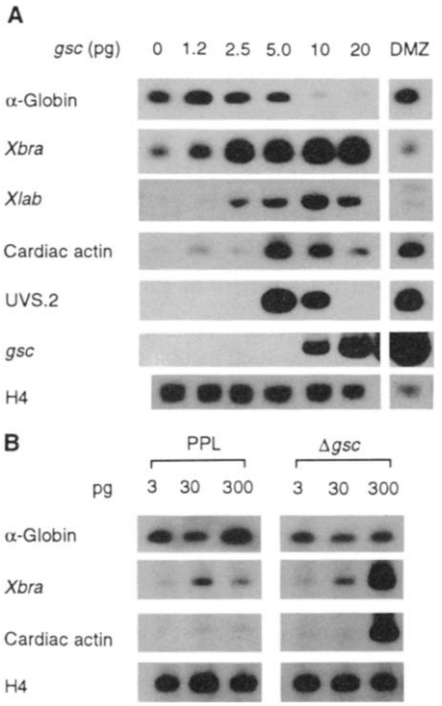

Fig. 2. *goosecoid* mRNA elicits multiple threshold responses. Embryos were injected with mRNA at the indicated dose per blastomere. Ventral marginal zone explants were cut at the early gastrula stage, cultured overnight until they reached the late neurula stage (stage 19), and processed for RNA quantitation by RT-PCR assays (*10*). (**A**) The RT-PCR of RNA from explants injected with *gsc* mRNA. The 0-pg dose corresponds to injection of buffer without RNA. Note the sharp changes in response with twofold concentration differences of injected *gsc* mRNA. The dorsal marginal zone (DMZ) lane corresponds to RNA from dorsal marginal zones of uninjected control embryos. The entire experiment was performed twice, with the same results. Separate PCR assays were carried out for all probes (to avoid overloading the PCR reactions), with the exception of α-globin and cardiac actin, which were assayed together. Histone H4 primers were included in all reactions as controls, and the amount of amplified product was not found to vary significantly between samples; three independent RT reactions were performed. The ventral marker α-globin is expressed in the DMZ lane; these explants form head-like embryoids that presumably include blood islands. (**B**) The RT-PCR of RNA from explants injected with control RNAs. PPL, preprolactin mRNA (*27*); Δ*gsc*, a *gsc* mRNA lacking the homeobox. Note that Δ*gsc* has a dorsalizing effect at very high doses.

nous *gsc* in the early gastrula by whole mount in situ hybridization and histological sections (Fig. 3) (*21*). The *gsc* mRNA is not distributed uniformly but is present at a high concentration in the dorsal side of the marginal zone and in smaller concentrations dorsolaterally. This graded distribution of *gsc* RNA is congruent with the specification map of the early gastrula marginal zone (Fig. 3A). A dorso-ventral gradient of *gsc* mRNA is also prominently seen in the zebrafish late blastula (*22*).

Densitometric analysis of the RNA gradient (Fig. 3E) reveals that the range of concentration detected is sevenfold (Fig. 3). This is consistent with the eightfold concentration range over which *gsc* mRNA injection effects the activation of *Xbra*, actin, and *gsc* itself.

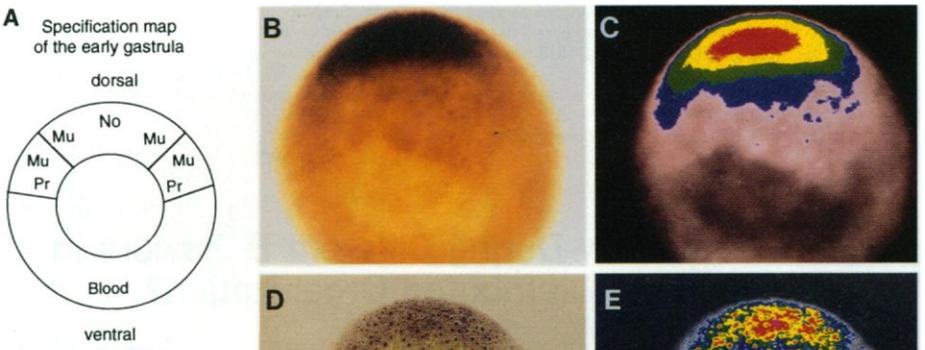

Fig. 3. The specification map of the early gastrula is congruent with the expression pattern of *goosecoid*. (**A**) Specification map of the early gastrula *Xenopus* embryo, vegetal view. Abbreviations: Mu, muscle; No, notochord; and Pr, pronephros. [This diagram is based on data from the classical study by Dale and Slack (*9*).] (**B**) A *gsc* whole mount in situ hybridization of an early gastrula, vegetal view. (**C**) Image analysis of the *gsc* expression seen in (B). (**D**) Ten-micrometer section through the marginal zone of a *gsc* whole mount in situ hybridization of an early gastrula. (**E**) Computer densitometric analysis of the *gsc* expression seen in (D); different gray levels were assigned to false colors and threshold ranges for gray levels in relative units were set as 0 to 10 blue, 10 to 20 green, 20 to 30 yellow, and 40 to 70 red, after subtraction of background.

Thus, the endogenous distribution of *gsc* RNA and the results of the mRNA injections suggest that this DNA-binding protein may pattern the differentiation of the marginal zone of the early *Xenopus* gastrula, as summarized in the simplified model shown in Fig. 4.

The effect of *gsc* mRNA on cell fate presumably requires cofactors that are present in the marginal zone, as *gsc* mRNA injection has no mesoderm-inducing effect on explanted animal cap cells (*7*). Indeed, other transcription factors such as *Xlim-1* and *XFKH-1* (*23*) spatially overlap the expression of *gsc* and may cooperate with it. Our experiments address only the specification of the marginal zone in the early gastrula (the stage at which the explants were performed) and not the function of an additional dorsalizing signal, also called the horizontal signal, which emanates from the organizer at later stages (*1*).

In *Drosophila*, three morphogens that act through a concentration gradient have been described; *bicoid*, encoding a DNA-binding protein present in a gradient through the syncytial embryo (*24*); *decapentaplegic*, which encodes a signaling growth factor (*25*); and *dorsal*, encoding a DNA-binding protein found in a nuclear gradient established by extracellular signals transduced by the *toll* receptor (*24*). Here *gsc* is shown to pattern cell fate through a concentration gradient. The gene is expressed in a gradient at the right time and place to pattern mesoderm in the early gastrula (Fig. 3). As is the case for the *Drosophila* protein encoded by *dorsal*, the gradient of *gsc* must be established by extracellular signals. Although it is not known which molecule or combination of molecules controls expression of *gsc* in the embryo, candidate signals include peptide growth factors of the transforming growth factor–β, fibroblast growth factor, *noggin*, and *Wnt* families (*1–3*, *26–28*). Although our overexpression studies show that *gsc* is sufficient to pattern cell fate, only loss-of-function studies will show whether *gsc* is also necessary.

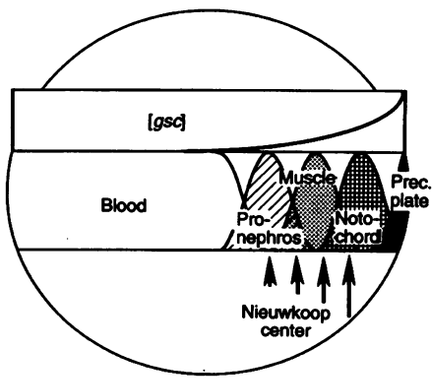

Fig. 4. *goosecoid*-gradient model of mesoderm specification in the early gastrula. A dorso-ventral gradient of *gsc* acts as a patterning system where the local concentration of *gsc* determines the position of cells in the marginal zone. Mesodermal cells, destined by default to become blood and mesenchyme, can respond at differing concentrations by differentiating into pronephros, muscle, notochord, and prechordal (prec.) plate (head mesoderm). The *gsc* pathway is probably only one out of several that lead to mesoderm specification in the embryo, as suggested by observations that *gsc* alone is unable to cause mesoderm differentiation in animal caps (*7*); that *gsc*-induced secondary axes usually lack complete head structures (*6*, *7*); and that FGF can induce muscle in animal cap cells without inducing *gsc* (*6*). The expression of *gsc* is thought to be controlled by signals emanating from the Nieuwkoop center, indicated by arrows. The Nieuwkoop center signal may consist of a concentration gradient of a single inducer or arise by the combinatorial action of several inducers. The *gsc* transcription factor is proposed to be an intermediate agent in this pathway, translating the positional information released by the Nieuwkoop center into positional specification in the overlying marginal zone. Two important components in mesoderm specification, which are not indicated in this simplified model, are the secreted proteins Xwnt-8 and noggin. *Xwnt-8* mRNA is expressed in the VMZ and acts as a repressor of dorsal cell differentiation (*28*). *Xwnt-8* expression is repressed by *gsc* (*28*). Interaction between these two genes presumably further sharpens the tissue responses that pattern mesoderm. The *noggin* gene, which is coexpressed with *gsc*, is a soluble factor that is able to dorsalize early gastrula VMZ and might correspond to the horizontal signal known to emanate from organizer cells (*26*).

REFERENCES AND NOTES

1. J. M. W. Slack, *Mech. Dev.* 41, 91 (1993).
2. J. B. A. Green and J. C. Smith, *Nature* **347**, 391 (1990).
3. J. B. A. Green, H. V. New, J. C. Smith, *Cell* 71, 731 (1992).
4. B. Blumberg, C. V. E. Wright, E. M. De Robertis, K. W. Y. Cho, *Science* **253**, 194 (1991).
5. D. Wilson *et al.*, *Genes Dev.* 7, 2120 (1993).
6. K. W. Y. Cho, B. Blumberg, H. Steinbeisser, E. M. De Robertis, *Cell* **67**, 1111 (1991).
7. C. Niehrs, R. Keller, K. W. Y. Cho, E. M. De Robertis, *ibid.* **72**, 491 (1993).
8. Embryos at the 16-cell stage were injected radially in the equatorial region of all eight vegetal blastomeres with 1 nl of solution during a 1-s interval. The diameter of the injection needles did not exceed 5 μm. Larger injection volumes may lead to unspecific dorsalization and explant elongation, probably by some mechanical activation as noted (*9*). VMZ explants were dissected, cultured in 0.3× modified Barth's saline, and ana-

lyzed as described (7). We constructed the pSP*gsc* expression vector by creating an Nco I site at the initial methionine and a Sal I site after the termination codon of the *gsc* cDNA type B form [nucleotides 115 to 847 of the sequence reported in (6)] by means of PCR add-on mutagenesis. This fragment was then used to replace a bovine preprolactin fragment in pSP35T (27) after excision with Nco I and Sal I. The parent vector of pSP35T is a modified form of pSP64 [P. A. Krieg and D. A. Melton, *Nucleic Acids Res.* **12**, 7057 (1984)]. The resulting pSP*gsc* construct contains 5′ and 3′ untranslated sequences of β-globin, as well as a polyadenylate tail. Capped synthetic mRNA was transcribed from pSP*gsc* in vitro with SP6 RNA polymerase (6). This mRNA could be diluted 20 times to yield results similar to those obtained in our previous studies with the vector p*gsc* (6), which lacks β-globin sequences.

9. L. Dale and J. M. W. Slack, *Development* **100**, 279 (1987).
10. Preparation of synthetic mRNA, microinjection, culture of embryos, and preparation of explants were as described (Fig. 1) (7). Preprolactin mRNA was synthesized with the use of the pSP35T vector (27) as a template. We created pSPΔ*gsc* by excising the homeodomain of pSP*gsc* with Pst I followed by re-ligation. Isolation of RNA, reverse transcription, and PCR were carried out essentially as described [R. Rupp and H. Weintraub, *Cell* **65**, 927 (1991)], but with the following modifications: RNA was prepared with the RNA STAT-60 kit (Tel-Test) according to the manufacturer's instructions. Polymerase chain reaction was carried out in 25-μl reactions with 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; NEN) in a PTC-100 (MJ Research) temperature cycler at 95°, 55°, and 72°C for 1 min each cycle and terminating with 15 min at 65°C. Ten microliters of the reaction product was analyzed on 5% polyacrylamide gels and visualized by autoradiography. Experiments always included non–reverse-transcribed RNA samples as negative controls (19). Polymerase chain reactions were carried out in the linear range of the amplification cycle number, which was determined empirically in each case, as well as of template input. The forward (F) and reverse (R) primer sequences (developed empirically), the amplified product sizes, and the optimal number of amplification cycles were as follows: α-globin (F, 5′-TTGCTGTCTCACACCATCCAGG; R, 5′-TCTGTACTTGGAGGTGAGGACG; 126 bp; 30 cycles), *Xbra* (F, 5′-GCTGGAAGTATGTGAATGGAGA; R, 5′-TTTAAGTGCTGTAATCTCTTCA; 318 bp; 30 cycles), cardiac actin (F, 5′-TCCCTGTACGCTTCTGGTCGTA; R, 5′-TCTCAAAGTCCAAAGCCACATA; 252 bp; 20 cycles), UVS.2 (F, 5′-CTCATGAACAGAACAGAAGCGA; R, 5′-GAAGCGTAGTCATATTCAATCC; 130 bp; 32 cycles), *Xlab* (F, 5′-CCGCGTCTAACTTCTGC; R, 5′-ACATACATGGC- A TGTGT; 379 bp; 31 cycles), *gsc* (F, 5′-GGAGAGGACTGCAGTCTGCATG; R, 5′-[TCTAGAGTCGACTCAACT]CTGCAGCTCAGTCCTGGA, the sequence in brackets does not prime in *gsc*; 590 bp; 33 cycles), and histone H4 (F, 5′-CGGGATAACATTCAGGGTATCACT; R, 5′-ATCCATGGCGGTAACTGTCTTCCT; 188 bp; 19 cycles).
11. R. K. Patient *et al.*, *Nucleic Acids Res.* **10**, 7935 (1982).
12. J. C. Smith, B. M. Price, J. B. A. Green, D. Weigel, B. G. Herrmann, *Cell* **67**, 79 (1991).
13. T. J. Mohun, S. Brennan, N. Dathan, S. Fairman, J. B. Gurdon, *Nature* **311**, 716 (1984).
14. V. Cunliffe and J. C. Smith, *ibid.* **358**, 427 (1992).
15. S. M. Sato and T. D. Sargent, *Dev. Biol.* **137**, 135 (1990).
16. H. Steinbeisser and E. M. De Robertis, *C. R. Acad. Sci. Ser. III Sci. Vie* **316**, 966 (1993).
17. M. Perry, G. Thomsen, R. G. Roeder, *J. Mol. Biol.* **185**, 479 (1985).
18. A. Courey, D. A. Holtzman, S. P. Jackson, R. Tjian, *Cell* **59**, 827 (1989).
19. C. Niehrs, H. Steinbeisser, E. M. De Robertis, unpublished results.
20. The pCMV*gsc* construct does not contain β-globin flanking sequences and when microinjected is only expressed after the midblastula stage (7). Radial injections were performed as described (8), with 4, 13, 40, 80, and 160 pg of pCMV*gsc* DNA per blastomere. At least nine explants were injected with each concentration (total, 69). Explants were analyzed histologically. At the highest injected concentration, 15% of the explants contained notochord. In addition, RT-PCR revealed a sharp threshold for cardiac actin at 80 pg of DNA per blastomere (19).
21. Whole mount in situ hybridizations were carried out as described, with a full-length *gsc* clone as probe (6). Embryos were developed in the staining solution for 6 hours to avoid reaching reaction saturation. Slides of intact whole mounts or histological sections were scanned and analyzed digitally at the computing facility of the CNRS Strasbourg, directed by J.-L. Vonesch.
22. S. Schulte-Merker *et al.*, *Development*, in press.
23. M. Taira, M. Jamrich, P. J. Good, I. B. Dawid, *Genes Dev.* **6**, 356 (1992); M. L. Dirksen and M. Jamrich, *ibid.*, p. 599.
24. C. Nusslein-Volhard, *Development* (suppl.) **1**, 1 (1991).
25. E. L. Ferguson and K. V. Anderson, *Cell* **71**, 451 (1992).
26. W. C. Smith, A. K. Knecht, M. Wu, R. M. Harland, *Nature* **361**, 547 (1993).
27. E. Amaya, T. J. Musci, M. W. Kirschner, *Cell* **66**, 257 (1991).
28. J. L. Christian and R. T. Moon, *Genes Dev.* **7**, 13 (1993).
29. We thank J.-L. Vonesch and P. Dolle for image analysis carried out in Strasbourg; R. Rupp for advice on PCR; E. Amaya and M. Kirschner for the pSP35T plasmid; and U. Banerjee, J. Lengyel, and Y. Sasai for improving the manuscript. C.N. is a recipient of an American Cancer Society senior postdoctoral fellowship, and H. S. is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. Supported by a grant from NIH (HD21502-08).

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Neuronal Activity During Different Behaviors in *Aplysia*: A Distributed Organization?

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The active neuronal populations in the *Aplysia* abdominal ganglion during spontaneous and evoked behaviors were compared with the use of multineuronal optical measurements. In some preparations, more than 90 percent of the neurons activated during the reflex withdrawal of the gill also were activated during respiratory pumping and during small spontaneous gill contractions. Although the same neurons made action potentials in all three behaviors, the activity patterns were different. There was a substantial interaction between the neural substrates underlying evoked and spontaneous behaviors when they were made to occur together. If a gill withdrawal reflex was elicited a few seconds after a respiratory pumping episode, the evoked neuronal activity in most neurons was clearly altered. These results suggest that a distributed organization involving a large number of neurons may be responsible for generating the two behaviors. Different behaviors appear to be generated by altered activities of a single, large distributed network rather than by small dedicated circuits.

Behaviors can be generated either by dedicated neuronal circuits or by a distributed neuronal network (Fig. 1) that can be reconfigured to generate several behaviors. It may be difficult to distinguish a distributed neuronal organization from dedicated circuits by direct observations. For example, earlier voltage-sensitive dye recordings show that between 200 and 300 neurons in the abdominal ganglion of *Aplysia* are activated during the gill withdrawal reflex (*1*, *2*). However, as shown in Fig. 1 both kinds of organization can be constructed with similar numbers of neurons and both may share sensory and motor neuron pools among several behaviors. A straightforward way to distinguish the two organizations is to examine all the possible synaptic connections in the system. Unfortunately, this is often impractical because the number of possible connections can be very large (increasing approximately as the square of the number of neurons).

Some researchers have suggested that dedicated circuits may generate some simple behaviors (*3*, *4*). On the other hand, there is evidence of distributed neuronal organizations in both invertebrate and vertebrate systems (*5*). However, in these studies only a small fraction of the active neurons was examined. Thus, it was impossible to determine how the whole nervous system functioned during different behaviors.

We attempted to distinguish the two possible organizations by comparing neuronal activity in *Aplysia* during spontaneous and evoked gill withdrawal behaviors. In the dedicated model (Fig. 1), sensory neurons activate many distinct circuits and interneurons. However, during spontaneous behaviors sensory neurons are not involved (6). This dedicated model would predict that only one circuit should be activated by the intrinsic generator, and thus we would expect a much smaller number of active inter-

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