general theory for experience-dependent synaptic modification in the mammalian cerebral cortex.

REFERENCES AND NOTES

1. T. V. P. Bliss and T. Lømo, *J. Physiol. (London)* **232**, 331 (1973).
2. B. E. Alger and T. J. Teyler, *Brain Res.* **110**, 463 (1976).
3. S. M. Dudek and M. F. Bear, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4363 (1992).
4. R. M. Mulkey and R. C. Malenka, *Neuron* **9**, 967 (1992).
5. A. Artola and W. Singer, *Nature* **330**, 649 (1987).
6. Y. Komatsu, K. Fujii, J. Maeda, H. Sakaguchi, K. Toyama, *J. Neurophysiol.* **59**, 124 (1988).
7. A. T. Perkins and T. J. Teyler, *Brain Res.* **439**, 222 (1988).
8. F. Kimura, A. Nishigori, T. Shirokawa, T. Tsumoto, *J. Physiol. (London)* **414**, 125 (1989).
9. A. Artola and W. Singer, *Eur. J. Neurosci.* **2**, 254 (1990).
10. N. Kato, A. Artola, W. Singer, *Dev. Brain Res.* **60**, 43 (1991).
11. M. F. Bear, W. A. Press, B. W. Connors, *J. Neurophysiol.* **67**, 1 (1992).
12. R. L. Berry, T. J. Teyler, H. Taizhen, *Brain Res.* **481**, 221 (1989).
13. T. Tsumoto, *Prog. Neurobiol.* **39**, 209 (1992).
14. M. F. Bear and A. Kirkwood, *Curr. Opinion Neurobiol.*, in press.
15. A. Artola, S. Bröcher, W. Singer, *Nature* **347**, 69 (1990).
16. J. C. Hirsch and F. Crepel, *Exp. Brain Res.* **85**, 621 (1991).
17. R. S. Goldman, L. E. Chavez-Noriega, C. F. Stevens, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7165 (1990).
18. Brain slices (400 μm) were prepared and maintained in an interface chamber (Medical Systems, Greenvale, NY) as described (*3, 11*). Microelectrodes were filled with 1 M NaCl (1 to 2 megohms) for extracellular recording or 3 M potassium acetate (80 to 120 megohms) for intracellular recording. Synaptic responses were evoked with 0.02-ms pulses (amplitude, 10 to 200 μA) delivered with a bipolar concentric stimulating electrode (outside diameter, 200 μm). In every experiment, a full input-output curve was generated, and base line responses were obtained at 0.07 to 0.03 Hz with a stimulation intensity that yielded a half-maximal response. To induce LTP, we delivered two to five episodes of TBS at 0.1 Hz. TBS consisted of 10 to 13 stimulus trains delivered at 5 to 7 Hz; each train consisted of four pulses at 100 Hz. To induce LTD, we delivered 900 pulses at 1 to 3 Hz. All rats used were adults (>40 days old).
19. J. Larson, D. Wong, G. Lynch, *Brain Res.* **368**, 347 (1986).
20. R. C. Malenka, *Neuron* **6**, 53 (1991).
21. D. T. Monaghan and C. W. Cotman, *J. Neurosci.* **5**, 2909 (1985).
22. Anatomical experiments indicate that fibers ascending through layer VI terminate densely in layer IV but relatively sparsely in layer III (*30*), and current-source density analysis shows that the prominent current sink in layer III that is evoked by stimulation of the white matter is mostly di- and polysynaptic and always follows activation of layer IV synapses (*6, 31*). Accordingly, the EPSPs evoked by white matter stimulation fail to exhibit a monosynaptic component in half the layer III neurons recorded (*6, 27*). Electrical stimulation of layer IV, besides activating the projection from layer IV neurons, also recruits any direct inputs to layer III that ascend from the white matter.
23. This negative peak was maximal at a depth of ~300 μm from the pia, and current-source density analysis indicates that it reflects a prominent layer III synaptic current sink; therefore, the amplitude of this field potential was used routinely as a measure of synaptic effectiveness.
24. J. Larson and G. Lynch, *Brain Res.* **441**, 111 (1988).
25. We caution, however, that these consequences of layer IV stimulation need not be accounted for solely by activation of the projection of layer IV neurons onto the dendrites in layer III. Clearly, the effects of intracortical stimulation can be quite complex and, in principle, could include the antidromic activation of some layer III neurons and orthodromic activation of intracortical and corticocortical fibers passing through layer IV [cells in the infragranular layers are not part of the essential circuit as these can be cut away and LTP is left intact (131 ± 6%; n = 7)]. However, these complexities are not unique to experiments where layer IV is stimulated. Indeed, stimulation of the cortical white matter may be even more complex considering that in addition to activation of corticipetal fibers and synaptic activation of layer IV neurons, the cells in layers VI, V, and III could all be activated antidromically. The effects of white matter stimulation are further complicated by the requisite use of bicuculline, which, at the concentrations usually used, often promotes widespread epileptiform activation of cortical slices (*32*). Thus, despite some potential complications, layer IV stimulation appears to be an advantageous preparation for the investigation of synaptic plasticity in the superficial layers of the neocortex.
26. One of these residual differences is an early time-course of the responses immediately after the TBS. In the hippocampus, a potentiated response typically is observed immediately after the tetanus and declines to a stable value in about 30 min. The decaying form of potentiation in the hippocampus has been termed short-term potentiation (STP) and may be mechanistically distinct from LTP (*33*). STP typically is not observed in the neocortex.
27. Y. Komatsu, S. Nakajima, K. Toyama, *J. Neurophysiol.* **65**, 20 (1991).
28. V. A. Aroniadou and T. J. Teyler, *Brain Res.* **584**, 169 (1992).
29. L. M. Grover and T. J. Teyler, *Nature* **347**, 477 (1990).
30. A. Burkhalter, *J. Comp. Neurol.* **279**, 171 (1989).
31. K. M. Bode-Greuel, W. Singer, J. B. Aldenhoff, *Exp. Brain Res.* **69**, 213 (1987).
32. Y. Chagnac-Amitai and B. W. Connors, *J. Neurophysiol.* **61**, 747 (1989).
33. C. F. Stevens, *Neuron* **10** (suppl.), 55 (1993).

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Presence of Mitochondrial Large Ribosomal RNA Outside Mitochondria in Germ Plasm of *Drosophila melanogaster*

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Mitochondrial large ribosomal RNA (mtlrRNA) has been identified as a cytoplasmic factor that induces pole cell formation in embryos whose ability to form a germ line has been abolished by treatment with ultraviolet light. In situ hybridization analyses reveal that mtlrRNA is enriched in germ plasm and is tightly associated with polar granules, the distinctive organelles of germ plasm, which supports the idea that mtlrRNA functions in pole cell formation. This suggests that a product from the mitochondrial genome, along with nuclear products, participates in a key event in embryonic development: determination of the germ line.

The segregation of the germ and somatic line in animal embryos represents one of the basic events in cell and developmental biology. In many animal groups, the factor required for germ-line establishment has been postulated to be localized in a histologically remarkable region in egg cytoplasm, the germ plasm (*1*). In *Drosophila*, germ plasm is referred to as polar plasm because it is localized in the posterior pole region of oocytes and cleavage embryos (*2, 3*). The polar plasm contains factors for germ-line and abdomen formation (*4–6*). The factor for abdomen formation has been identified as the product of the *nanos* (*nos*) gene (*7, 8*). The factors for germ-line formation, however, have remained elusive. Recent genetic analyses have identified seven maternally acting genes called posterior group genes [*cappuccino* (*capu*), *spire* (*spir*), *staufen* (*stau*), *oskar* (*osk*), *vasa* (*vas*), *valois* (*vls*), and *tudor* (*tud*)] whose functions are required for the localization of factors for the germ line as well as of the *nos* product in the polar plasm (*4, 9, 10*). Embryos from these mutants fail to form a germ line and abdomen. In addition, they lack polar granules, the distinctive organelles of polar plasm, which suggests that polar granules are essential for both germ-line and abdomen formation (*9*).

After fertilization, nine nuclear divisions take place without cytokinesis in the central region of embryos (the cleavage stage). The nuclei then migrate to the periphery (the syncytial blastoderm stage). As the nuclei enter the posterior polar plasm, each of them is included in a cytoplasmic protrusion that contains polar

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plasm. These protrusions later become pole cells, the germ-line precursor in this animal (*11–13*).

Previously, we have proposed that the mitochondrial large ribosomal RNA (mtlrRNA) is a component of functional polar plasm on the basis of the fact that injection of mtlrRNA rescues embryos from the failure to form pole cells that is caused by treatment with ultraviolet (UV) light (*14*). However, there is the possibility that this result does not represent the normal pathway to pole cell formation. To exclude this possibility, we carried out experiments to locate mtlrRNA in normal embryos at early stages. Here, we report that mtlrRNA is enriched in the polar plasm of cleavage embryos but not in pole cells. In situ hybridization for electron microscopy revealed that the mtlrRNA signal is restricted to polar granules in the polar plasm of early cleavage embryos, although we cannot exclude the fact that some mtlrRNA is present in mitochondria (*15*). These results, along with our previous ones, lead to the conclusion that mtlrRNA is a component of polar granules and probably cooperates with the other components for polar plasm function. Our findings here may reveal a role for a mitochondrially encoded product in the function of the polar plasm in germline formation.

The temporal and spatial distribution of mtlrRNA in early embryos was determined by in situ hybridization (*16*). In cleavage embryos, the mtlrRNA signal was strongest in the polar plasm and very weak in the other regions (Fig. 1A). During and after pole cell formation, the mtlrRNA signal was hardly detectable in pole buds and pole cells but was prominent in the periplasm beneath pole cells (Fig. 1B). At the late syncytial blastoderm and cellular blastoderm stages, a localized mtlrRNA signal was no longer discernible (Fig. 1, C and D). For a control, we used a sense DNA probe (*17*) and found no localized signal in cleavage embryos (Fig. 2D, inset). Furthermore, there was no localization of the transcripts of two other genes, the mitochondrial small ribosomal RNA and *ND-1*, which are located adjacent to the *lrRNA* gene in the mitochondrial genome (Fig. 1, E and F).

We previously reported the results of Northern (RNA) blot analyses, which suggested that the mtlrRNA is present outside mitochondria in cleavage embryos and that the amount of this extramitochondrial mtlrRNA decreases during development from the cleavage to the blastoderm stages. No significant change was evident in the amount of intramitochondrial mtlrRNA (*14*). This decrease of the mtlrRNA outside mitochondria correlates well with the developmental changes in the mtlrRNA signal in polar plasm observed here by in situ hybridization. Our observations suggest that the mtlrRNA is transported out of mitochondria into the cytosol only in the polar plasm, from which it is subsequently removed. This idea is directly supported by our in situ hybridization data at an ultrastructural level (*18*). In early cleavage embryos, the mtlrRNA signal is enriched in the polar plasm, and 73% of the total signal in the polar plasm is present on the surface of polar granules (*19, 20*) (Fig. 2, A, B, and F). In contrast, a signal is occasionally observed on mitochondria either in the polar plasm or in lateral regions and is sometimes observed unassociated with any organelles. This signal probably represents nonspecific hybridization (background) (*19*).

Several lines of evidence from control experiments definitively support our conclusion that mtlrRNA is present on polar granules in early cleavage embryos. First, the presence of excess amounts of unlabeled antisense RNA results in the disappearance of signal on polar granules in early cleavage embryos (*21, 22*). Second, when we used a sense probe for mtlrRNA (*17*), we did not observe a signal on polar granules (Fig. 2D). Third, when *ND-1* or the mitochondrial small ribosomal RNA gene was used as a probe, no signal was discernible on the polar granules (*22*). Finally, when the mtlrRNA probe was applied to blastodermal embryos, the signal was no longer detected on polar granules in pole cells (Fig. 2G). This developmental change of the mtlrRNA signal on polar granules correlates well with the disappearance of the signal in pole cells.

Polar granules have been suggested to function in pole cell formation and differentiation (*2, 9*). Mutation of any one of the seven posterior-group genes involved in pole cell and abdominal pattern formation (*4, 9, 10*) is known to affect polar granules, pole cells, and abdomen formation. Thus, polar granules are regarded as being associated with both pole cell factors and the abdominal factor. The factor for abdomen formation is encoded by the posterior group gene *nos*, mutation of which results in the failure of embryos to form abdomens but affects neither polar granules nor pole cells (*7, 8*). However, none of the posterior group genes so far cloned has been shown to encode a specific factor alone sufficient to cause pole cell formation.

The pole cell–forming factor is expected to have the following properties: (i) to be crucial for pole cell formation; (ii) to depend on the seven posterior group genes for its localization in the polar plasm; and (iii) to have no effect on abdomen formation. We propose that extramitochondrial mtlrRNA is a candidate for such a factor on the basis of the following evidence. First, the extramito-

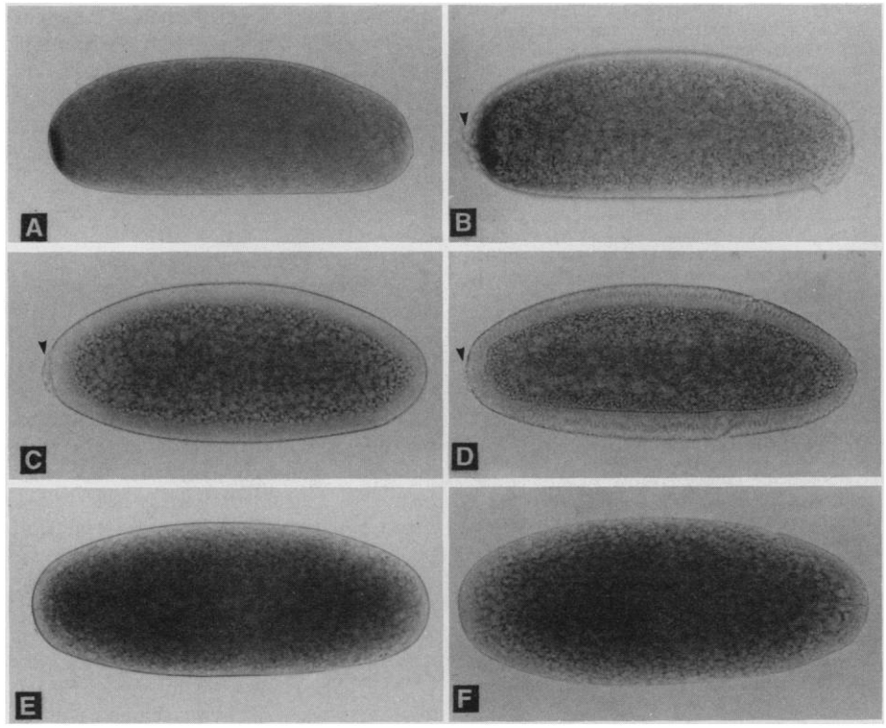

Fig. 1. Distribution of mtlrRNA in early embryos. In situ hybridization of the mtlrRNA cDNA (*28*) to embryos at the cleavage stage (**A**), the pole-cell formation stage (**B**), the syncytial blastoderm stage (**C**), and the cellular blastoderm stage (**D**). In situ hybridization of the *ND-1* gene probe (*14*) (**E**) and pdyHC DNA (*29*) containing the small ribosomal RNA gene (**F**) to cleavage embryos. In all panels, anterior is to the right. Arrowheads point to pole cells.

chondrial mtlrRNA is localized in the polar plasm of cleavage embryos (Figs. 1A and 2, A and B). Second, the seven posterior group mutations disrupt localization of the extramitochondrial mtlrRNA, but a mutation in *nos* never affects the localization of mtlrRNA (*23*). This suggests that mtlrRNA, like *nos* RNA, depends on the function of posterior group genes for its localization in polar plasm. Third, mtlrRNA is present on the surface of polar granules in early cleavage embryos (Fig. 2, A, B, and F). Fourth, in embryos from *Bic-D* females, the mtlrRNA is enriched only in the posterior pole (Fig. 3D). *Bic-D* embryos develop an ectopic abdomen with reverse polarity at the expense of the head and thorax but form pole cells only at the posterior pole (*24*). This suggests that mtlrRNA is dispensable for abdomen formation but is required for pole cell formation. Fifth, UV irradiation of the polar plasm at a dose sufficient to prevent pole cell formation but not enough for affecting abdomen formation reduces the amount of mtlrRNA localized in the polar plasm (Fig. 3, B and C). Finally, as we previously reported, when injected mtlrRNA is able to rescue embryos from UV-caused inability to form pole cells (*14*).

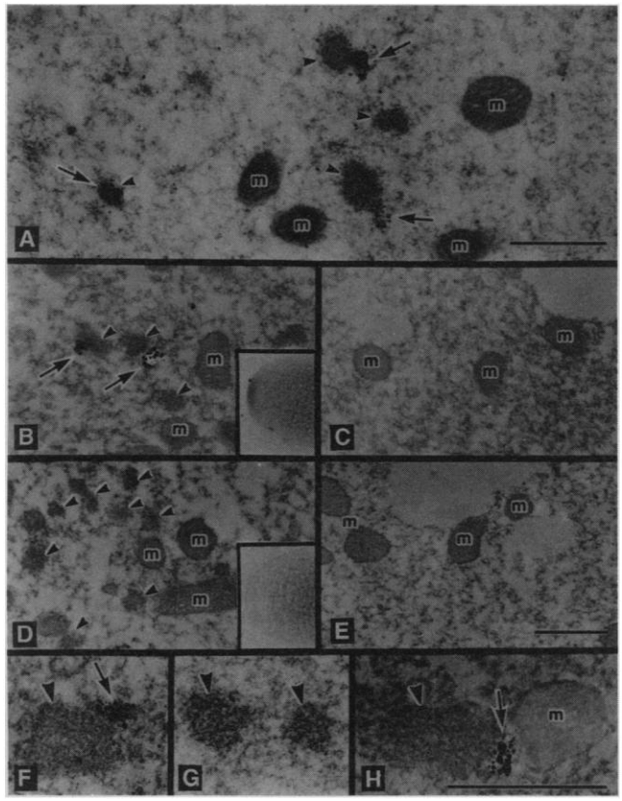

Fig. 2. Distribution of mtlrRNA in the polar plasm and lateral region of early embryos. (**A**) Electron micrograph of a section through the polar plasm of a cleavage embryo hybridized with a double-stranded DNA probe for mtlrRNA. Electron micrographs of polar plasm (**B** and **D**) and lateral regions (**C** and **E**) of early cleavage embryos hybridized with an antisense (B and C) or a sense (D and E) mtlrRNA probe (*17*). Micrographs at a higher magnification of polar granules in an early cleavage embryo (**F**), in a very early cleavage embryo (*20*) (**H**), and in pole cells of a blastodermal embryo (**G**), which have been hybridized with an antisense mtlrRNA probe. The mtlrRNA signal (arrows) could be detected on the surface of polar granules (F) and at the boundary between the polar granule and the mitochondrion (H). The mtlrRNA in mitochondria cannot be detected by this method (*15*). (**Insets**) Light micrographs of posterior poles of cleavage embryos hybridized with an antisense (B) and a sense probe (D) and processed for immunogold labeling and silver enhancement (*18*). Arrowheads show polar granules; m, mitochondria. Bars = 0.5 μm.

We have previously reported that mtlrRNA can induce pole cells at the anterior pole if it is co-injected with UV-irradiated polar plasm. However, the pole cells induced by the RNA in UV-irradiated posterior poles never develop into germ cells (*14*). This may suggest a requirement for additional factors that are localized in the polar plasm and required for pole cell formation and germ cell determination. Recently, it has been shown that mislocalization of *osk* RNA to the anterior pole leads to induction of an abdomen and functional pole cells at the ectopic site. Furthermore, of the seven posterior group genes only two, *vas* and *tud*, are required for the induction of an abdomen and pole cells at the ectopic site (*25*). The possibility that a polar plasm component or components encoded by one or both of these posterior group genes represents the additional factor or factors required for germ-line determination remains to be tested. Moreover, transport of mtlrRNA from mitochondria to polar granules could be mediated by *tud* (*26*). Investigation of pathways involving *osk*, *vas*, *tud*, and mtlrRNA will give a better understanding of the problem of germ-line determination.

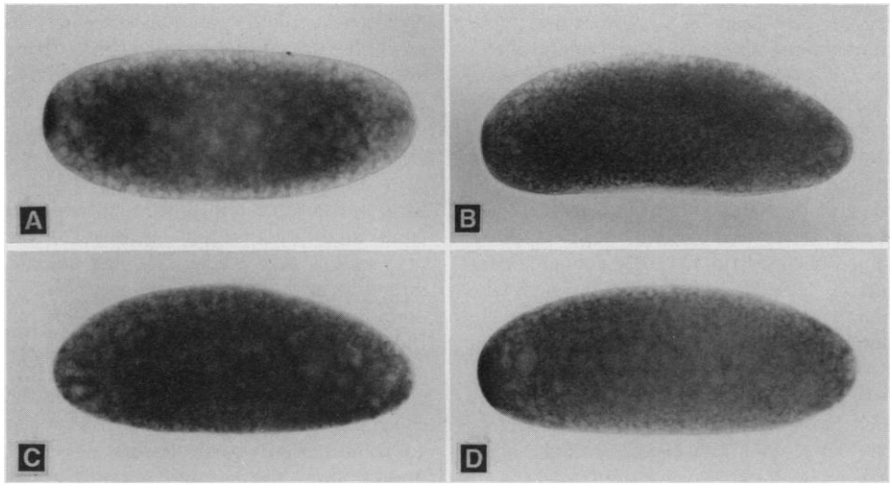

Fig. 3. Effects of UV irradiation and *Bic-D* mutation on the distribution of the mtlrRNA in cleavage embryos (*30*). In situ hybridization of the mtlrRNA cDNA probe to an intact embryo (**A**), embryos UV-irradiated posteriorly (**B** and **C**), and an embryo derived from a *Bic-D*/+ female (**D**). Note that the mtlrRNA signal is detected only in the posterior pole of the embryo. This monopolar distribution was also observed in embryos from *Bic-D*/*Bic-D* females. When embryos are UV-irradiated posteriorly at an early cleavage stage, the mtlrRNA signal in the polar plasm is diminished (B) or completely disappears (C). In all panels, anterior is to the right.

REFERENCES AND NOTES

1. E. M. Eddy, *Int. Rev. Cytol.* **43**, 229 (1975).
2. A. P. Mahowald, *J. Exp. Zool.* **167**, 237 (1968).
3. ———, *ibid.* **176**, 329 (1971).
4. R. Lehmann and C. Nüsslein-Volhard, *Cell* **47**, 141 (1986).
5. K. Illmensee and A. P. Mahowald, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1016 (1974).
6. M. Okada, I. A. Kleinman, H. A. Schneiderman, *Dev. Biol.* **37**, 43 (1974).
7. C. Wang and R. Lehmann, *Cell* **66**, 637 (1991).
8. R. Lehmann and C. Nüsslein-Volhard, *Development* **112**, 679 (1991).
9. A. P. Mahowald, *Science* **255**, 1216 (1992).
10. T. Schüpbach and E. F. Wieschaus, *Dev. Biol.* **113**, 443 (1986); R. E. Boswell and A. P. Mahowald, *Cell* **43**, 97 (1985); L. J. Manseau and T. Schüpbach, *Genes Dev.* **3**, 1437 (1989).

11. E. M. Underwood, J. H. Caulton, C. D. Allis, A. P. Mahowald, *Dev. Biol.* **77**, 303 (1980).
12. G. M. Technau and J. A. Campos-Ortega, *Wilhelm Roux's Arch. Dev. Biol.* **195**, 489 (1986).
13. B. Hay *et al.*, *Development* **103**, 625 (1988).
14. S. Kobayashi and M. Okada, *ibid.* **107**, 733 (1989).
15. Although mtlrRNA is present in mitochondria [M. Binder *et al.*, *J. Cell Biol.* **102**, 1646 (1986)], we could not detect an mtlrRNA signal in mitochondria by our in situ hybridization technique because we sectioned embryos for electron microscopy after in situ hybridization (*18*). This is probably because the mtlrRNA probe hardly permeates mitochondrial membrane under the conditions used.
16. In situ hybridization to whole-mount Oregon-R embryos was carried out principally according to Tautz and Pfeifle (*27*). We used a double-stranded DNA probe for in situ hybridization.
17. Sense and antisense DNA probes were obtained by asymmetric polymerase chain reaction with a full-length complementary DNA (cDNA) of mtlrRNA (*28*) as a template.
18. In situ hybridization of the mtlrRNA cDNA probe to whole-mount Oregon-R embryos was carried out principally according to Tautz and Pfeifle (*27*), but we used antibody to digoxigenin (anti-digoxigenin) (Bio Cell Research Laboratories, Cardiff, U.K.) that was conjugated to 1-nm gold particles instead of alkaline-phosphatase–conjugated anti-digoxigenin. After the antibody reaction, the immunogold-labeled embryos were processed for silver enhancement with a silver enhancing kit (Bio Cell). It is well known that gold catalyzes the reduction of silver ions to metallic silver. Thus, in preparations processed for silver enhancement, gold particles are surrounded by a growing shell of metallic silver [G. Danscher, *Histochemistry* **71**, 81 (1981)]. In embryos hybridized with an antisense mtlrRNA probe and processed as above, a signal localized in the polar plasm was easily observed under a light microscope (Fig. 2B, inset). The embryos were embedded in epoxy resin for transmission electron microscopy. Thin sections were cut with an LKB Nova Ultramicrotome (LKB, Bromma, Sweden) and observed under a JEM 100CXII electron microscope (JEOL, Tokyo, Japan). The detailed method for in situ hybridization for electron microscopy can be found elsewhere (R. Amikura *et al.*, in preparation).
19. We counted the number of signals in areas that were a total of 500 μm^2 in the polar plasm and in the same areas in the lateral region of early cleavage embryos (within 60 min after egg laying). In embryos hybridized with an antisense probe for mtlrRNA, we observed 167 signals in the polar plasm and 63 in the lateral region. One hundred twenty-two signals (73% of the total signals) in the polar plasm were present on the surface of polar granules. The remaining signals in the polar plasm and all signals in the lateral region were scattered all over the cytoplasm, including the mitochondria. These observations show that enrichment of the signal in the polar plasm is not a result of an intramitochondrial signal but a result of an extramitochondrial signal on polar granules. In the control embryos hybridized with a sense probe, we observed 59 signals in the polar plasm and 32 in the lateral region. No signal was present on the polar granules, and all signals were on mitochondria or in residual cytoplasm. These observations suggest that almost all signals on mitochondria and in the residual cytoplasm represent background.
20. As shown in Fig. 2, the mtlrRNA signal can be observed on one side of the polar granules. The side with the signal may be the side previously associated with mitochondria because the mtlrRNA signal is frequently found at the boundaries between mitochondria and polar granules in very early cleavage embryos (within 30 min after egg laying). In 250-μm^2 areas in the polar plasm of a very early cleavage embryo, we found 33 signals on polar granules associated with mitochondria and 32 on free polar granules. Of the 33 signals on polar granules associated with mitochondria, 30 signals were observed at the boundaries between polar granules and mitochondria (Fig. 2H).
21. In situ hybridization was carried out in the presence of 300 times excess (relative to the labeled antisense mtlrRNA probe) of an unlabeled antisense RNA that was transcribed from a full-length cDNA of mtlrRNA in vitro.
22. S. Kobayashi, R. Amikura, M. Okada, data not shown.
23. D. Ding, K. Whittaker, H. D. Lipshitz, personal communication.
24. J. Mohler and E. H. Wieschaus, *Genetics* **112**, 803 (1986).
25. A. Ephrussi and R. Lehmann, *Nature* **358**, 387 (1992).
26. The protein encoded by *tud* is detected in mitochondria and in polar granules during the cleavage stage (A. Bardsley, K. McDonald, R. E. Boswell, personal communication). The *tud* gene is the only posterior gene product known to localize in mitochondria. The causal relation between the protein encoded by *tud* and the transport of mtlrRNA remains to be tested.
27. D. Tautz and C. Pfeifle, *Chromosoma* **98**, 81 (1989).
28. S. Kobayashi and M. Okada, *Nucleic Acids Res.* **18**, 4592 (1990).
29. D. O. Clary and D. R. Wolstenholme, *ibid.* **13**, 4029 (1985).
30. In situ hybridization was carried out as described (*27*). Embryos (*mwh* e^{11}) were UV-irradiated as described [S. Togashi, S. Kobayashi, M. Okada, *Dev. Biol.* **118**, 352 (1986)]. The genotype of the females that produced *Bic-D* embryos was *b dp Bic-D*$^{71\ 34}$*/CyO*.
31. We thank R. E. Boswell, A. Bardsley, K. McDonald, D. Ding, K. Whittaker, and H. D. Lipshitz for allowing us to cite their unpublished data, D. R. Wolstenholme for his gift of pdyHC plasmid, S. Sugiyama for valuable comments on this work, and A. Ephrussi, A. P. Mahowald, and C. S. Goodman for critical reading of the manuscript. Supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture, Japan, a grant from the Naito Foundation, and a Research Project grant from the University of Tsukuba.

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Predisposition to Neoplastic Transformation Caused by Gene Replacement of H-*ras*1

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Homologous recombination was used to introduce a nominally transforming mutation into an endogenous H-*ras*1 gene in Rat1 fibroblasts. Although both the mutant and the remaining normal allele were expressed equally, the heterozygous cells were not neoplastically transformed. Instead, spontaneously transformed cells arose from the heterozygotes at a low frequency, and the majority of these cells had amplified the mutant allele. Thus, the activated H-*ras*1 allele was not by itself dominant over the normal allele but predisposed cells to transformation by independent events, such as amplification of the mutant allele.

Certain point mutations within coding sequences of *ras* proto-oncogenes (H-*ras*, K-*ras*, and N-*ras*) generate oncogenes that, when ectopically expressed, can transform rodent cell lines (*1*). Furthermore, the transforming ability of these mutant alleles prevails even though the normal alleles are also expressed (*1*, *2*). The mutant alleles have therefore been referred to as dominant. Consistent with this assessment, the mutant genes are gain-of-function alleles; in general, their protein products have lost the ability to hydrolyze guanosine triphosphate efficiently and, thus, become constitutively activated (*1*, *3*). Although the mutant *ras* alleles are strongly implicated in human tumorigenesis (*1*, *4*, *5*), their proposed dominance has been questioned (*6*).

To address the issue of *ras* dominance, we used homologous recombination to replace one copy of normal H-*ras*1 in Rat1 fibroblasts [subclone B1a(+/+) (*7*)] with the mutant allele NMU-H-*ras*1 and then assessed whether the heterozygous cells were neoplastically transformed. The NMU-H-*ras*1 gene (isolated from rat mammary tumors after mutagenesis with nitrosomethylurea) contains a single missense mutation that converts Gly^{12} to Glu (*8*) and can transform Rat1 fibroblasts when expressed in abundance after DNA transfection (*8*, *9*).

We replaced H-*ras*1 with NMU-H-*ras*1 by a two-step method that resembles strategies used previously in yeast (*10*) and mammalian cells (*11*). The first step required integration of the vector DNA that contained a truncated mutant *ras* allele into the chromosomal H-*ras*1 locus by homologous recombination (Fig. 1). The resultant cells were neoplastically transformed because the recombination event reconstituted a full-length NMU-H-*ras*1 allele expressed from the strong promoter of the murine leukemia virus–long terminal repeat (MLV-LTR). These transformed cells, referred to as Me12/+, also possessed a truncated normal H-*ras*1 allele and a copy of the *gpt* gene driven by the SV40 promoter-enhancer (SV-*gpt*) that rendered them resistant to mycophenolic acid.

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