and Marler's final two criteria appear to be met by the white-lipped frog's seismic signals—they are short-lived relative to the animal's life-cycle dynamics, they reflect internal states of the sender, and they are capable of altering internal states of the receiver. With respect to the evolutionary criteria, the frog saccule seems to be a specialized sensor of seismic signals (*1, 2*); one can argue for selective advantage of seismic communication—for example, the difference in velocities between the airborne chirp and the substrate-borne (Rayleigh-wave) thump provides a temporal clue (approximately 7 msec/m) to the distance from the source (as in the lightning-thunder phenomenon), and thus could help male frogs establish and maintain closely spaced territories.

Acute seismic sensitivity has been reported in reptiles, but has not been implicated in intraspecific communication (*7*). Several mammalian species exhibit ground-thumping behavior, but the intraspecific communication channel in such cases has been assumed to be auditory (airborne) rather than vibratory (substrate-borne) (*8*). Ground thumping has also been observed in arthropods, and in some cases may be involved in intraspecific communication (*9*). Direct vibrotactile communication has been convincingly demonstrated in some amphibian species (*10*). As far as we know, however, the evidence we have presented here provides the first strong implication of the use of substrate-borne seismic signals in intraspecific communication in vertebrates.

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References and Notes

1. D. Ross, *J. Physiol. (London)* **86**, 117 (1936); H. Koyama, E. R. Lewis, E. L. Leverenz, R. A. Baird, *Brain Res.* **50**, 168 (1982).
2. P. M. Narins and E. R. Lewis, *Assoc. Res. Otolaryngol. Abstr.* **6**, 88 (1983); *J. Acoust. Soc. Am.*, in press.
3. Field recording equipment comprised: Realistic 343-992B cardioid dynamic microphone, hermetically sealed AMF Geo-Space 10395 vertical geophone (calibrated with a Bruel & Kjaer 4810 exciter and 4370 accelerometer), laboratory-built geophone preamplifier, Sony WM-D6 stereo tape cassette recorder, and TEAC R61 cassette data recorder.
4. G. E. Drewry, in *Puerto Rico Nuclear Center Rain Forest Project Annual Report 1970* (Puerto Rico Nuclear Center, San Juan, 1970), pp. 16–63.
5. D. B. Lewis and D. M. Gower, *Biology of Communication* (Wiley, New York, 1980).
6. S. Green and P. Marler, in *Handbook of Behavioral Neurobiology*, P. Marler and J. G. Vandenbergh, Eds. (Plenum, New York, 1979), vol. 3, pp. 73–158.
7. P. H. Hartline, *J. Exp. Biol.* **54**, 349 and 373 (1971); M. L. Lenhardt, *Assoc. Res. Otolaryngol. Abstr.* **7**, 92 (1984).
8. H. H. Swanson, *Anim. Behav.* **22**, 638 (1974); G. J. Kenagy, *J. Mammalogy* **57**, 781 (1976); J. H. Kaufman, *Anim. Behav.* **22**, 281 (1974).
9. M. D. Burkenroad, *Ecology* **28**, 458 (1947); M. Salmon, *Zoologica* **47**, 15 (1962); F. G. Barth, in *Spider Communication*, P. N. Witt and J. S. Rovner, Eds. (Princeton Univ. Press, Princeton, N.J. 1982), pp. 67–122.
10. L. E. Brown and M. J. Littlejohn, in *Evolution in the Genus* Bufo, W. F. Blair, Ed. (Univ. of Texas Press, Austin, 1972), pp. 310–323.

11. This work was supported by NSF grants BNS-8005834 and BNS-8317639, NIH grant NS19725, and grant UR-3501 from the UCLA Academic Senate. We are grateful to T. M. Chin, K. R. Krieg, E. M. Lewis, P. T. Lopez, and S. W. Moore for technical assistance and to P. H. Hartline for thoughtful reviews of the manuscript.

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Detection of Two Viral Genomes in Single Cells by Double-Label Hybridization in Situ and Color Microradioautography

Abstract. *Double labeling and color microradioautography were used in a new method of hybridization in situ to identify different genes in individual cells. The method is based on the unequal penetration of ^{3}H and ^{35}S into two layers of nuclear track emulsion separated by a thin barrier film. Hybridization of a ^{35}S-labeled probe specific for one kind of gene results in silver grains over cells in both layers of emulsion; a ^{3}H-labeled probe for a second gene provides grains only in the first layer of emulsion. Silver grains are converted to magenta-colored grains in the first layer and to cyan-colored grains in the second to facilitate enumeration of grains in each layer. This technique should be widely applicable in analyses of differential gene expression in single cells or in discrete populations of cells.*

With recent advances in hybridization in situ, single copies of viral genomes can be detected in cells or single genes in chromosomes (*1–6*). These sensitivities (in the range of 10^{-18} g of specific nucleotide sequences per cell) have been achieved with improvements in the hybridization methodology itself and the high specific radioactivities of probes labeled with ^{3}H or ^{125}I precursors (*7*). The recently introduced ^{35}S-labeled precursors (*8*) afford even greater sensitivities on light microscopy, in part because of the increased efficiency of formation of silver grains in radioautographs (about 0.5 grain per disintegration for ^{35}S versus about 0.1 grain per disintegration for ^{3}H (*9*). The higher energy of ^{35}S responsible

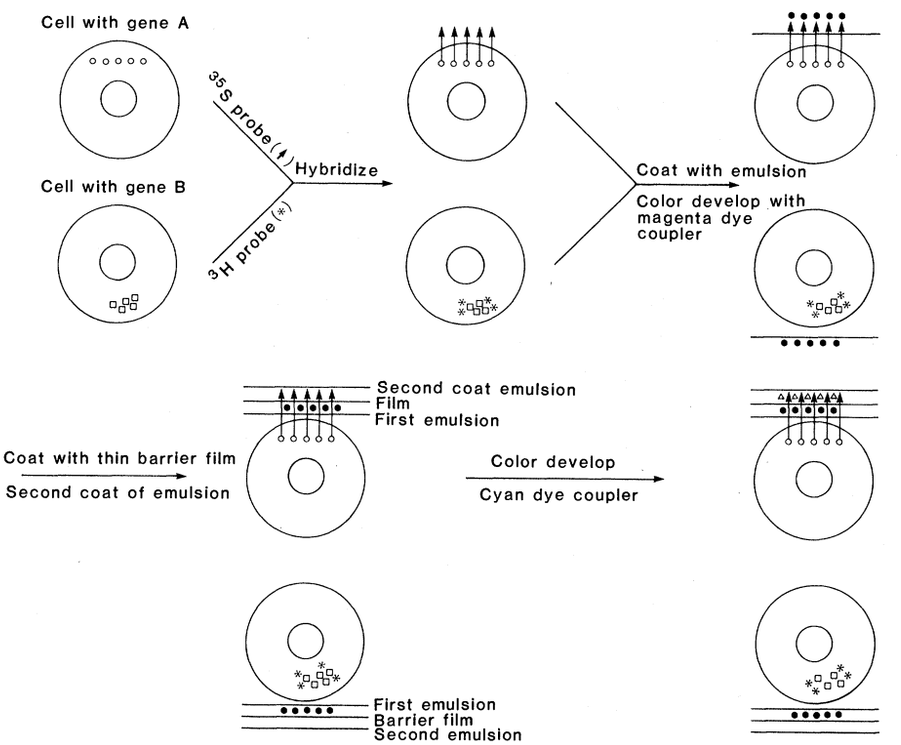

Fig. 1. Principles and major steps of double-label hybridization in situ and color radioautography. Cells with gene A (○) are hybridized to a probe labeled with ^{35}S (↑); cells with gene B (□) are hybridized to a probe labeled with ^{3}H (*). After slides are coated with nuclear track emulsion, the grains in the first layer are color-developed with a magenta dye coupler (●). A thin barrier film and a second coat of emulsion are applied and color developed with a cyan dye coupler (△).

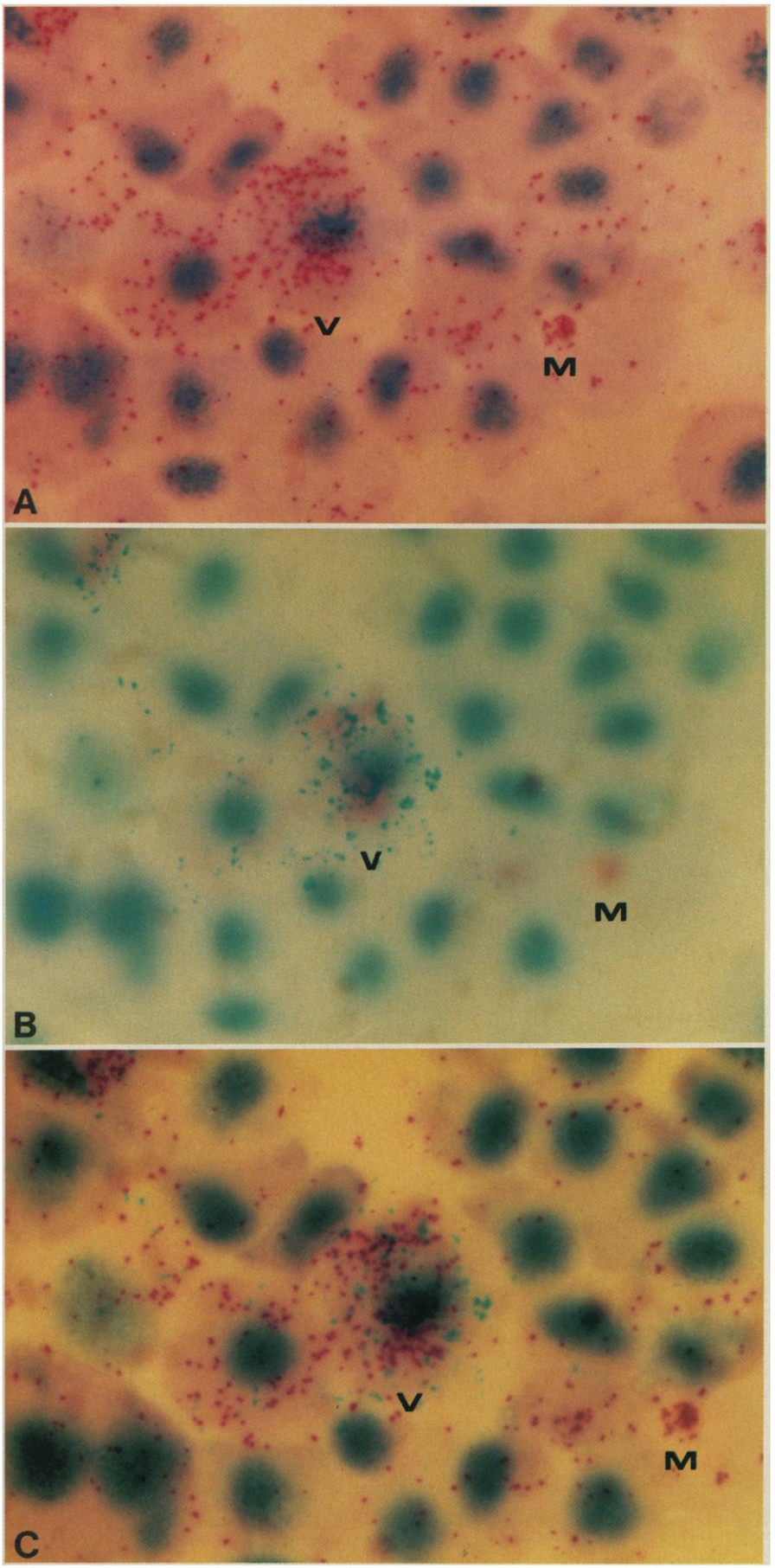

Fig. 2. Field containing several cells with visna virus RNA (*V*) and one cell with measles virus RNA (*M*). (A) First layer, showing magenta-colored grains. (B) Second layer, showing cyan-colored grains. (C) Color transparencies (A and B) superimposed.

for this increased efficiency suggested to us that it would be possible to devise a method of sensitive double-label hybridization in situ based on differential penetration of the β electrons of ^{3}H and ^{35}S into two layers of emulsion (*9–12*). We now describe such a method and a technique for microscopic color radioautography to readily distinguish the two isotopes.

The principles and major steps involved in the method are presented in Fig. 1. Cells containing gene A or gene B are hybridized in situ with a ^{35}S-labeled probe specific for gene A and a ^{3}H probe specific for gene B. After hybridization, the cells are coated with the first emulsion and then developed after a suitable exposure. The silver grains are converted to magenta grains, and the first emulsion is covered with a thin clear film that blocks penetration of ^{3}H into a second emulsion. The second emulsion is then developed, and the silver grains are converted to cyan grains. Cells with gene B that bound the ^{3}H-labeled probe are easily recognized, as they contain magenta grains in the first emulsion but lack cyan grains in the second. Cells with gene A that bound ^{35}S probe have magenta grains in the first layer and cyan grains in the second. If a cell has both genes, the relative amounts are determined by enumerating the number of magenta and cyan grains in their respective layers. The number of cyan grains is converted to the number of copies of gene A, and its contribution to the number of grains in the first layer is subtracted from the total number of magenta grains to determine the number of copies of gene B.

We developed and tested our method of double-label hybridization in situ by introducing into two kinds of cells small numbers of RNA genomes from two different viruses—measles virus and visna virus. Visna virus is a retrovirus with a positive-strand RNA genome; we detected this RNA by hybridizing in situ with a virus-specific probe labeled with ^{35}S. This RNA is equivalent to gene A in Fig. 1. Measles virus is a paromyxovirus with a negative-strand RNA genome; this RNA was detected with a ^{3}H probe as was gene B in Fig. 1.

The detailed experimental protocols were as follows:

1) Introduction of viral RNA's into two cell types. Sheep choroid plexus cells were infected with 3 plaque-forming units (PFU) of visna virus per cell. One hour after infection, the cells were removed by trypsinization, washed in buffered saline, and mixed with Vero monkey kidney cells that had been infected for 1 hour with 5 PFU of measles virus,

and collected in the same manner (*4, 13*). Cells were deposited by cytocentrifugation on acetylated and coated glass slides, and fixed in a mixture of ethanol and acetic acid and then in ethanol.

2) Hybridization in situ. The cells were first treated to increase the diffusion of the probe and then hybridized with a mixture of ^{35}S-labeled visna virus–specific probe and a ^{3}H-labeled measles virus–specific probe in buffered 50 percent formamide containing 10 percent dextran sulfate (*7*).

3) Probes. Both probes were prepared by reverse transcription of purified viral RNA's with random primers (*4, 13*). The measles-specific probe was labeled to a specific activity of 4×10^8 dpm/μg with [^{3}H]dATP (50 Ci/mmol), [^{3}H]dCTP (50 Ci/mmol) and [^{3}H]dTTP (100 Ci/mmol) (dATP, dCTP, and dTTP are the 5′-triphosphates of deoxyadenosine, deoxycytosine, and deoxyribosylthymine, respectively). The ^{35}S visna-specific probe was labeled to a fivefold lower specific activity because the efficiency of latent image formation was greater for ^{35}S than for ^{3}H. The specific activity was reduced to 10^8 dpm/μg by diluting ^{35}S-labeled dATP (1200 Ci/mmol) with unlabeled thionucleotide precursor.

4) We devised a method of color development of nuclear track emulsions that is based on principles of development of color films (*14–16*). After the slides were washed, they were coated with NTB-2 emulsion, and after 3 days they were developed with Kodak D19 developer. The metallic silver grains were converted to magenta-colored grains as follows. Slides were immersed for 3 minutes in a solution of 0.37 percent Formalin (Fisher) and 0.6 percent Na_2CO_3, washed for 2 minutes in tap water at room temperature, bleached for 1 minute in 10 percent $K_3Fe(CN)_6$ and 5 percent KBr, washed again for 2 minutes in tap water, and developed for 1 minute in freshly prepared color developer with magenta dye coupler (*17*). The slides were then washed, bleached, and washed again as above, fixed for 5 minutes in a solution containing 24 g of $Na_2S_2O_3$ and 15 g of Na_2SO_3 per 100 ml of water, washed again for 5 minutes as above, stained in 0.5 percent $CuSO_4$ for 5 minutes, dipped in water, stained in 1 percent methyl green for 5 minutes, and washed briefly in water to remove excess dye.

5) Barrier film. Krylon (Borden) from a spray can was collected in a glass vessel. The liquid Krylon (0.4 ml) was added dropwise to water contained within a circle of polyethylene tubing (circumference, 40 cm) floating on water. As the solvent evaporates, a thin film of uniform thickness forms in the center. The polyethylene tubing was removed, and the film was deposited on the slide by picking the film up from underneath with the slide. The slide and film were drained and dried in a semivertical position. Most of the dried excess Krylon film was gently scraped from the back of the slide. The slide was then dipped in a filtered aqueous solution containing 0.5 percent gelatin and 0.05 percent $CrK(SO_4)_2 \cdot 12\ H_2O$ for 5 minutes, then drained and dried.

6) Second color development. The slide was coated with NTB-2 emulsion again and after suitable exposure was developed as described in (*4*) but with a cyan dye coupler C-16 (*N-o*-acetamide phenethyl-1-hydroxy-2-naphthamide; Eastman Kodak).

To illustrate how easily the two labels can be distinguished by color radioautography, we selected a field with several cells with visna virus RNA and one cell with measles virus RNA. In the first layer (Fig. 2A) there are several prominently labeled cells with visna virus RNA diffusely distributed in the cytoplasm and one cell with measles virus RNA in a vacuolar structure. In the second layer of emulsion (Fig. 2B) there are cyan grains over the cells with visna virus RNA, but none over the cells with measles virus RNA. This distinction is clearer in Fig. 2C where the two-color transparencies have been superimposed and printed in the same plane of focus.

We evaluated the efficiency of energy transfer to the second layer of emulsion, and the efficiency of conversion of silver grains to colored grains in experiments with cells labeled either only with ^{3}H or with ^{35}S, with one coat of NTB-2, or with the barrier film and a second coat. We found that the barrier film blocked all ^{3}H penetration into the second layer and attenuated the ^{35}S signal by a factor of about 0.2. The conversion ratio of developed silver grain to dyed grain was 0.72. The overall efficiency of colored grain development in the second layer is therefore about 0.6. This factor, and the number of cyan grains, can be used to determine the number of magenta grains contributed by ^{35}S to the first layer of emulsion. The ^{3}H contribution is the difference between the total number of magenta grains and the ^{35}S contribution.

To estimate the sensitivity of the method, we converted grain counts to copy numbers by comparing the average number of grains per cell in a population of cells infected with visna virus to the average copy number in the population, as assessed by standard hybridization techniques with RNA extracted from the cells (*7*). In the experiments described in this report, the cells infected with visna virus contained about 30 copies of the 10-kilobase viral genome (*4*) or the equivalent of 150 copies of an average sized messenger RNA (mRNA) (*18*). With an exposure time of three days, 30 copies of mRNA would have produced about 10 grains per cell over background, so that mRNA's representing about 0.1 percent of the mass of the cellular species (*18*) could be detected.

Double-label hybridization in situ is therefore a sensitive quantitative method that offers new opportunities to examine gene expression at the single cell level. The direct evaluation of virus gene expression in tissues should provide fresh insights into pathogenesis and the molecular ecology of virus infections; for example, whether the same or different cells harbor herpes simplex and herpes zoster in the latently infected ganglion; (*19–22*) or whether cytomegalovirus and hepatitis B virus cohabit cells in Kaposi's sarcoma (*23, 24*). More generally in cellular and developmental biology, this technique should provide valuable information about relative levels of gene expression in cells undergoing differentiation.

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References and Notes

1. H. E. Blum *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6685 (1983).
2. D. S. Gerhard *et al.*, *ibid.* **78**, 3755 (1981).
3. A. T. Haase *et al.*, *Virology* **119**, 399 (1982).
4. M. E. Harper, A. Ullrich, G. F. Saunders, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4458 (1979).
5. A. Tereba, M. M. C. Lai, K. G. Murti, *ibid.* **76**, 6486 (1979).
6. J. M. Trent, S. Olson, R. M. Lawn, *ibid.* **79**, 7809 (1982).
7. A. T. Haase, M. Brahic, L. Stowring, H. Blum, in *Methods in Virology*, K. Maramorosch and H. Koprowski, Eds. (Academic Press, New York, 1984), vol. 7, pp. 189–226.
8. M. J. Vincent, W. R. Beltz, S. H. Ashton, *Miami Winter Symp.*, Abstr. 107 (1982).
9. A. W. Roger, *Techniques of Autoradiography*, (Elsevier/North-Holland, Amsterdam, ed. 3, 1979).
10. M. Krause and W. Plaut, *Nature (London)* **188**, 511 (1960).
11. R. Baserga and D. Malamud, *Autoradiography, Techniques and Application* (Harper & Row, New York, 1969).
12. N. Van Rooijen, *Int. J. Appl. Radiat. Isot.* **27**, 547 (1976).
13. A. T. Haase *et al.*, *J. Infect. Dis.* **144**, 154 (1981).

14. W. Thomas, Jr., in *SPSE* [Society of Photographic Scientists and Engineers] *Handbook of Photographic Science and Engineering* (Wiley, New York, 1973), pp. 549–565.
15. E. O. Field *et al.*, *Stain Technol.* **40**, 295 (1979).
16. L. H. Kronenberg, *Anal. Biochem.* **93**, 189 (1979).
17. The magenta dye coupler was prepared as follows. Immediately before use, a 5-ml solution containing 100 mg of M-38 (1-2,4,6-trichlorophenyl-3-nitroanilino-2-pyrazolin-5-one; Eastman Kodak) dye coupler in ethanol containing 0.2*N* NaOH was mixed with 45 ml of a solution of 100 mg of Na_2SO_3, 100 mg of color developer CD2 (4-*N*, *N*-diethyl-2-methylphenylenediamine monohydrochloride; Eastman Kodak), 50 mg of KBr, and 1 g of Na_2CO_3 dissolved in water.
18. R. P. Perry, E. Bard, B. D. Hames, D. E. Kelley, U. Schibler, *Prog. Nucleic Acid Res. Mol. Biol.* **19**, 275 (1976).
19. R. W. Hyman, J. R. Ecker, R. B. Tenser, *Lancet* **1983-II**, 814 (1983).
20. D. H. Gilden *et al.*, *Nature (London)* **306**, 478 (1983).
21. A. Puga, J. D. Rosenthal, H. Openshaw, A. L. Notkins, *Virology* **89**, 102 (1978).
22. D. A. Galloway, C. M. Fenoglio, J. K. McDougall, *J. Virol.* **41**, 686 (1982).
23. G. Giraldo, E. Beth, E.-S. Huang, *Int. J. Cancer* **26**, 23 (1980).
24. A. Siddiqui, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4861 (1983).
25. We thank M. Shell, R. Cordella, and Y. Guptill for manuscript preparation; J. Gibbs, E. Hahn, and H. Hunter for discussion and samples of Eastman Kodak color developers and dye couplers; and the Veterans Administration, American Cancer Society, National Multiple Sclerosis Society, National Institutes of Health for support. A.T.H. was a medical investigator at the Veterans Administration in the course of this work; H.B. is a Heisenberg Fellow of the Deutsches Forschuengsgruneinschaft.
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Dinoflagellate with Blue-Green Chloroplasts Derived from an Endosymbiotic Eukaryote

Abstract. *The dinoflagellate,* Amphidinium wigrense, *contains triple membrane-bound bodies we have termed "blue-green chloroplasts." We believe these chloroplasts were derived from a cryptomonad endosymbiont similar to that present in another blue-green dinoflagellate,* Gymnodinium acidotum. *These dinoflagellates provide evidence that a chloroplast has evolved from an endosymbiotic eukaryote.*

The chloroplasts of red and green algae (and higher plants) evolved from photosynthetic, prokaryotic endosymbionts (*1, 2*). These chloroplasts are surrounded by an envelope consisting of two membranes. With few exceptions (*2, 3*), however, chloroplasts of other photosynthetic eukaryotes are bound by either three membranes (euglenoids and dinoflagellates) or four membranes (chlorophyll c–containing organisms other than dinoflagellates). It has been proposed that these chloroplasts resulted from the acquisition and degeneration of endosymbiotic eukaryotes (*1, 2, 4, 5*).

Evidence that a chloroplast arose from a eukaryotic endosymbiont has come from studies on the Cryptophyceae, a small group of flagellates whose photosynthetic members contain phycobilin accessory pigments in addition to chlorophylls a and c_2. The nucleomorph, a double membrane–bound body characteristic of Cryptophyceae, is found in the space called the "periplastidal compartment" between the two pairs of membranes surrounding the cryptomonad chloroplast. The periplastidal compartment also contains starch and eukaryotic-sized ribosomes (*6–8*). The nucleomorph may represent the degenerate nucleus of a red alga–like cell, to which the ribosomes presumably once belonged (*5, 7, 8*). This view has been strengthened by the demonstration of DNA and RNA in the nucleomorph (*9*). The red alga–like cell is thought to have taken in a prokaryote, which eventually became its chloroplast, and, in turn, to have been acquired by some ancestral cryptomonad and subsequently reduced to its present state (*5, 7, 8*).

The blue-green dinoflagellate *Gymnodinium acidotum* contains an endosymbiotic cryptomonad (*10*). Like the ciliate *Mesodinium rubrum*, which also harbors a cryptomonad (*11, 12*), and the dinoflagellates *Peridinium balticum* and *Kryptoperidinium foliaceum*, which contain chrysophycean endosymbionts (*13*), *G. acidotum* and its endosymbiont may resemble a relatively early stage in the evolution of a eukaryotically derived chloroplast.

In contrast to their free-living counterparts, the cryptomonad endosymbionts of *G. acidotum* and *M. rubrum* each lack a periplast, ejectosomes, and a flagellar apparatus (*10, 12*). Cells of one *M. rubrum* strain have numerous discrete compartments of endosymbiont cytoplasm, each containing a chloroplast, microbody, and mitochondrion. These "chloroplast-mitochondrial complexes" are apparently not connected to the portion of cytoplasm containing the endosymbiont nucleus (*11*). Moreover, a cell of *G. acidotum* was found which lacked an endosymbiont nucleus (*10*). Similar changes would be expected to occur during the transformation of a whole cell endosymbiont into a chloroplast.

The blue-green dinoflagellate, *Amphidinium wigrense* (*14*), contains bodies we have chosen to call "blue-green chloroplasts" on the basis of their consistent

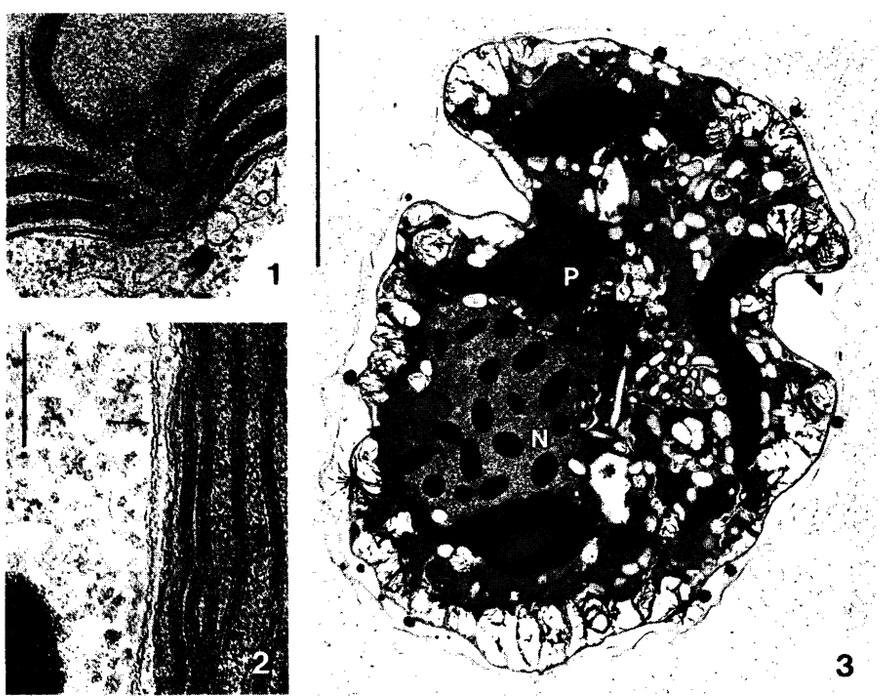

Fig. 1. The three bounding membranes of a blue-green chloroplast are indicated by arrows. The electron-opaque contents of the paired thylakoids are visible. Scale bar, 0.25 μm. Fig. 2. Chloroplast adjacent to nucleus showing the nuclear envelope (arrow) and the three membranes of the chloroplast envelope lying next to it. Scale bar, 0.25 μm. Fig. 3. Longitudinal section of an *A. wigrense* cell showing its nucleus and profiles of several of this cell's seven chloroplasts. The fibrillar material external to the cell is a mucilaginous substance released from peripheral vacuoles upon fixation. *N*, nucleus; *P*, pyrenoid; scale bar, 5 μm.