Int. J. Dev. Biol. 39: 653-657 (1995)

Short Contribution

Expression of membrane targeted aequorin in *Xenopus laevis* oocytes

CHRISTIANE DAGUZAN[1], MARIE-THÉRÈSE NICOLAS[2], CHRISTIAN MAZARS[3], CATHERINE LECLERC[1] and MARC MOREAU[1]*

[1]*Centre de Biologie du Développpement, UMR 9925, Université Paul Sabatier, Toulouse,* [2]*Centre de Recherche INSERM, Montpellier and* [3]*Centre de Biologie et Physiologie Végétale, URA-CNRS 1457, Université Paul Sabatier, Toulouse, France*

ABSTRACT We describe here a system for high level of expression of the calcium activated photoprotein aequorin. This protein has been targeted to the plasma membrane of *Xenopus* oocyte by nuclear microinjection of a plasmid containing a construction of a chimeric cDNA encoding a fusion protein composed of the photoprotein aequorin and the 5-HT_{1A} receptor. The expression of this fusion protein is placed under the control of RSV promoter. Functional photoprotein was reconstituted in the oocyte by incubation with coelenterazine. The amount of photoprotein 24 h after nuclear microinjection of the plasmid was sufficient to trigger a detectable light emission following calcium entry. The efficiency of the expression is correlated with the dose of plasmid injected. Intracytoplasmic injection of the plasmid always failed in photoprotein expression. Targeting of the apoprotein was demonstrated by immunolocalization under confocal microscopy. In our experimental conditions, the apoprotein was always localized at the animal pole above the nucleus. We never observed expression and targeting to the plasma membrane of the vegetal pole. We suggest that such expression might be of great interest for the study of numerous problems of developmental biology, in which calcium-dependent pathways are involved.

KEY WORDS: *oocytes, aequorin, calcium, Xenopus, membrane targeting*

Plasma membrane calcium permeability and increase of intracellular free calcium ($[Ca^{2+}]_i$) has been shown to play a key role in specific events involved in mitosis (Helper, 1994), meiosis (Moreau *et al.*, 1985; Guerrier *et al.*, 1993; Deguchi and Osanai, 1994), fertilization (Jaffe, 1985), embryogenesis (Moreau *et al.*, 1994; Leclerc *et al.*, 1995) and differentiation (Holliday *et al.*, 1991).

Photoproteins have several advantages over fluorescent indicators including the need for much simpler recording instrumentation and lack of disturbing factors such as autofluorescence, intracellular compartmentalization, rapid leakage from the cell or buffering of calcium by the probe. Moreover, short wavelength illumination, used with fluorescent probes, affects the development of the embryo (Youn and Malacinski, 1981; Leclerc-David *et al.*, 1985). A relative disadvantage is the need for microinjecting single cells. Aequorin, extracted from the luminous jellyfish *Aequorea aequorea*, is the best photoprotein generally used to measure $[Ca^{2+}]_i$ (for reviews, see Campbell, 1988; Blinks, 1989; Cobbold and Lee, 1991; Shimomura, 1991). It generates a light at 469 nm upon binding Ca^{2+} ions. Aequorin is a 21 kDa photoprotein constituted by apoaequorin, a protein containing three calcium binding sites (Campbell, 1988), and a hydrophobic prosthetic group, coelenterazine, which plays a direct role in the reaction of light emission.

Cloning of the gene of aequorin (Inouye *et al.*, 1985; Prasher *et al.*, 1985) opened the opportunity for *in vivo* expression of the apoaequorin, which could be regenerated in functional aequorin after simple incubation in the presence of coelenterazine (Tsuji *et al.*, 1986). Aequorin used as an indicator for measuring cytosolic $[Ca^{2+}]_i$ has been recently targeted to various cell organelles such as mitochondria (Rizzuto *et al.*, 1992), endoplasmic reticulum (Kendall *et al.*, 1992) or nucleus (Brini *et al.*, 1993; Badminton *et al.*, 1995).

The aim of this work was to develop a method for measuring calcium movements across the plasma membrane during early development of amphibian, using Ca^{2+} activated photoprotein (aequorin) targeted to the plasma membrane of the oocyte. Using the methods described by Gurdon and Wickens (1983) and Swick *et al.* (1992), we directly microinjected into nuclei of intact amphibians oocytes a plasmid, whose expression was under the control of a RSV promoter and which contained the gene of aequorin fused in frame at its 5' end to the $5HT_{1A}$ receptor gene (pRSV-5HTr/Aeq). This plasma membrane protein was employed as a driver to target aequorin to the plasma membrane. We describe the conditions to obtain the functional

Abbreviations used in this paper: Aeq, aequorin; RSV, Roux sarcoma virus; 5HT, serotonin; 5HTr, serotonin receptor.

***Address for reprints:** Centre de Biologie du Développpement, UMR 9925, Université Paul Sabatier, 118 route de Narbonne, F-31062 Toulouse Cedex, France. FAX: 33-61556507.

0214-6282/95/$03.00

© UBC Press
Printed in Spain

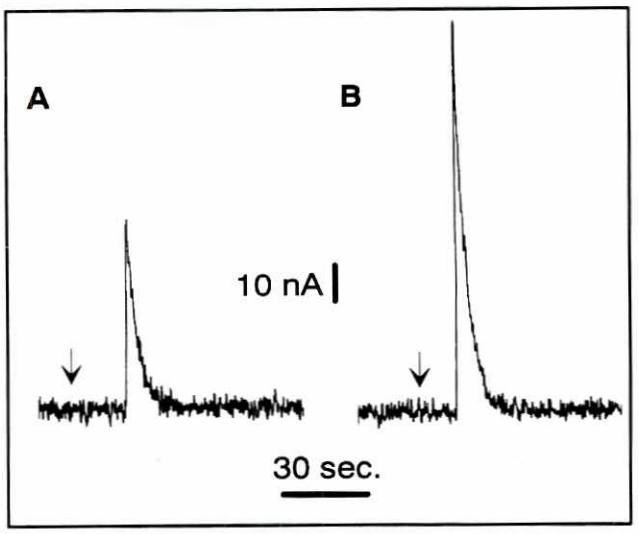

Fig. 1. Typical recording of emitted light after Ca^{2+} entry in *Xenopus laevis* oocyte. *The nucleus of the oocyte was microinjected with 0.25 ng of pRSV-5HTr/Aeq and incubated at 18°C during 48 h.* **(A)** *Stimulation by 10 µM ionomycin.* **(B)** *Emitted light recorded after disruption of the same oocyte following perfusion of hypoosmotic medium. Arrows indicate the addition of ionomycin or the onset of disruption.*

expression of the chimeric aequorin in *Xenopus* oocyte, which is detected by light emission following $[Ca^{2+}]_i$ increase. Expression and targeting of the photoprotein was further assessed by immunolocalization.

Aequorin expression in Xenopus oocytes

Expression of the apoaequorin gene was measured in single isolated oocytes by measuring the extent of light emission observed after increasing cytosolic calcium concentration.

In our experiments, oocytes microinjected with 0.25 ng pRSV-5HTr/Aeq into the nucleus were incubated at 18°C in OR2 modified medium. After respecting a 24 h minimum delay to allow aequorin expression, microinjected oocytes were taken at definite times and transferred for 30 min to a medium containing ß-mercaptoethanol and coelenterazine (see Experimental Procedures). Coelenterazine is highly hydrophobic and readily permeates cell membranes. We have verified that the expression of aequorin in the oocyte and the ß-mercaptoethanol and coelenterazine treatment did not affect meiotic maturation triggered by progesterone (data not shown). Figure 1A illustrates the typical time course of light emission produced by calcium entry triggered by adding 10 µM of ionomycin 48 h after plasmid microinjection into the oocyte nucleus. The rise in $[Ca^{2+}]_i$ provokes a large and rapid increase of aequorin luminescence, the signal returning almost to background level within 30 sec. The

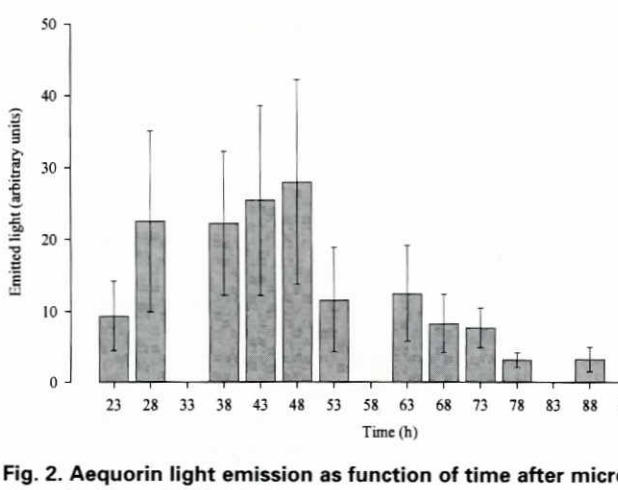

Fig. 2. Aequorin light emission as function of time after microinjection of 0.25 ng pRSV-5HTr/Aeq into the nucleus of *Xenopus* oocyte. *Expression efficiency has been measured by the extent of emitted light observed after disruption of the oocyte. Each bar represents the mean of at least 5 independent experiments. Emitted light was sufficient to be significantly detected only 23 h after microinjection onwards.*

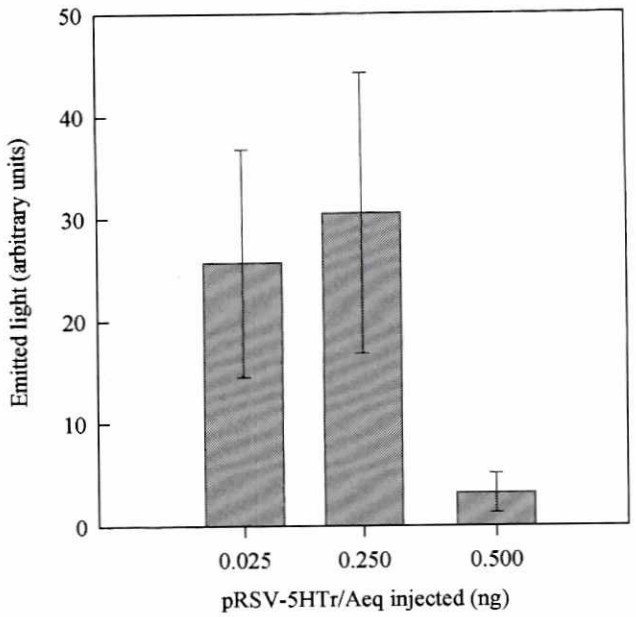

Fig. 3. Dependence of the expression of chimeric protein on the amount of injected pRSV-5HTr/Aeq. *Efficiency was measured following cell disruption. Each bar represents 5 independent experiments.*

experiment (Fig. 1B) is completed by the discharge of all unconsumed aequorin by disrupting the oocyte with an hypoosmotic solution (10 mM calcium in H_20). Oocytes injected with buffer without plasmid never emitted light upon calcium stimulation.

Twenty four hours after pRSV-5HTr/Aeq microinjection, individual oocytes exhibited a detectable level of aequorin. The amount of light emitted and thus probably the amount of the chimeric protein expressed increased up to 50 h and then declined (Fig. 2), but remained detectable up to 96 h. Typically 65% of pRSV-5HTr/Aeq microinjected-oocytes expressed the chimera protein (n= 89). No detectable expression of the photoprotein was obtained when the pRSV-5HTr/Aeq plasmid was injected into the cytoplasm of the oocytes n= 17).

The expression level of chimeric aequorin expressed depends upon the quantity of plasmid microinjected. The activity was evident when 0.025 to 0.5 ng was injected per oocyte. However the highest expression was obtained by injecting 0.25 to 0.3 ng (Fig. 3). The decrease in the light emitted activity of the

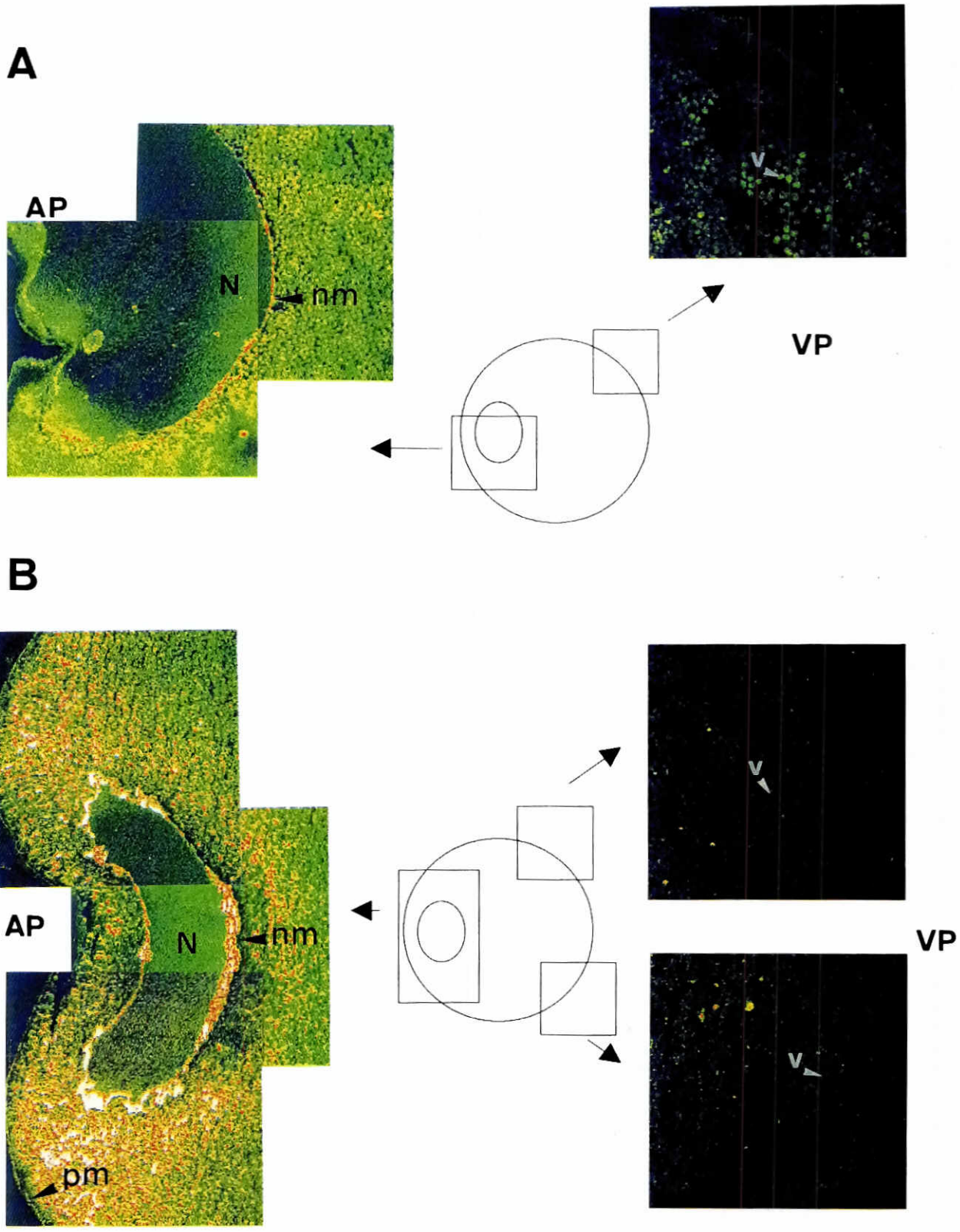

Fig. 4. Immunolocalization of expressed chimeric aequorin, 24 to 72 h after nuclear microinjection of pRSV-5HTr/Aeq, as observed by confocal microscopy. (A) *Expression after 24 h. Labeling is localized on the nuclear membrane (nm) and around the nucleus (N) in intracellular membranes (im).* **(B)** *After 72 h labeling was more intense, always present in intracellular membranes, but also at the level of the plasma membrane (pm). (AP) animal pole, (VP) vegetal pole. In all cases only the animal pole was labeled. The kidney like aspect of the nucleus is due to a fixation artefact.*

expressed chimeric photoprotein after injection of high concentrations (0.5 ng) of pRSV-5HTr/Aeq could be due to promoter competition for pRSV-specific transcription factors, as already suggested by Swick *et al.* (1992) for the pMT_2-cDNA vector.

Immunolocalization of the expressed chimeric apoaequorin

Since we repeatedly observed that the orientation of the oocyte was important to detect light emission, we further tried to localize the expressed aequorin using immunofluorescence techniques under confocal microscopy. First of all, using Western blot analysis and immunolabeling with anti-aequorin antibodies, we found that the chimeric protein immunologically cross-reacted with our anti-aequorin polyclonal antibodies and a major single band was found (data not shown). We thus followed expression of the photoprotein for different times of incubation after pRSV-5HTr/Aeq injection. As illustrated in Figure 4A, after 24 h of incubation, a bright staining is observed in the vicinity of the nucleus and at the level of the nuclear membrane. No fluorescence was detected within the nucleus itself. At this time, fluorescence is only localized at the animal pole. No significant fluorescence was detected at the vegetal pole. After 72 h of incubation the expressed protein migrates towards the plasma membrane, but remains restricted to the animal pole. The vegetal pole was never labeled, even after 96 h of incubation (Fig. 4B).

This localized expression accounts for the importance of orienting the oocyte to measure light emission after calcium entry. This opens the possibility to study the activity of calcium channels localized at the animal pole, especially during fertilization, since the entry site of spermatozoa appears to be localized in this region (Elinson, 1980). The exclusive localization of expressed aequorin at the animal pole may be explained by the distribution of the endoplasmic reticulum in the oocyte. As already described by Campanella *et al.* (1984) endoplasmic reticulum is confined to the immediate vicinity of the nucleus. In addition Oron *et al.* (1988) have demonstrated that acetylcholine receptors (Ach-R) and thyrotropin-releasing hormone receptor (TRH-R) expressed in *Xenopus* oocyte after injection of exogenous corresponding mRNA are expressed mainly in the animal hemisphere.

Experimental Procedures

Plasmid construct

To target aequorin to the plasma membrane, the whole cDNA of 5-HT_{1A} receptor, a plasma membrane protein, was engineered onto the 5' end of aequorin gene. The construction was made following standard procedures (Sambrook *et al.*, 1989). Briefly this new plasmid was constructed in a RSV vector already carrying aequorin cDNA. This starting plasmid (pRSV-Aeq, gift from Dr. Pons, Montpellier, France) was modified by creating a new restriction enzyme site by inserting a polylinker at the 5' end of the aequorin gene. In this new site 5-HT_{1A} receptor cDNA has been inserted and ligated. The correct orientation of the 5-HT_{1A} receptor insert was verified by digestion mapping and PCR analysis. The full plasmid (pRSV-5HTr/Aeq) contained 6262 base pairs.

Solutions

The physiological medium refers to OR_2 medium as described by Wallace *et al.* (1973), 82.5 mM NaCl; 2.5 mM KCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 1 mM Na_2HPO_4; 5 mM Hepes buffer; pH 7.5.

Reconstitution of functional aequorin

Aequorin were reconstituted in the oocyte by adding 2.5 µM coelenterazine (Molecular Probes, Eugene, OR, USA) in a medium containing 5 mM ß-mercaptoethanol according to the procedure proposed by Shimomura (1991).

Oocyte isolation and injection

Adult females of *Xenopus laevis* were anesthetized in 1g/l MS222 solution (Sandoz). Ovarian lobes were surgically removed and freed of follicle cells during gentle agitation (2 h) in a solution of collagenase (1 mg/ml, Type 1A, Boeringher, Manheim). After extensive washing in OR_2 medium, oocytes were maintained in OR_2 at 18°C for 18-24 h. Healthy oocytes were selected under a dissecting microscope. To visualize the nucleus at the animal pole, oocytes were centrifuged 8 to 10 min at 2000g. In these conditions the nucleus went up close to the plasma membrane of the animal pole resulting in the appearance of a transparent spot at this level. This treatment did not affect the biological properties of the cell since progesterone-dependent meiosis reinitiation occurred in these oocytes with the same delay and the same percentage as observed in controls.

Nuclear injection of the plasmid was performed by impaling the nucleus with a pipette of 10 to 12 µm of diameter of aperture. The injection volume (30 nl±15%) was controlled with an automatic pressure generator (Narishige Japan, IM-200). Injected oocytes were incubated in OR_2 medium at 18°C and damaged oocytes were discarded.

Immunolocalization

Oocytes were fixed overnight at 4°C in 3.7% formaldehyde in phosphate buffer (PBS) (0.1 M, pH 7.4). After dehydration they were embedded in paraffin and cut into 7.5 µm serial sections.

Expressed aequorin was visualized using polyclonal antibody raised against aequorin obtained after immunizing a rabbit with 1 ng of pure aequorin extracted and purified from the jellyfish (pure aequorin was a gift from Dr. O. Shimomura). This anti-aequorin antibody was affinity purified and used directly on sections. In paraffin sections of formaldehyde fixed tissue aequorin antigenicity was undetectable but could be fully restored after 2 min treatment with a 0.25% solution of trypsin in PBS (Curran and Gregory, 1977). The indirect immunostaining using primary, secondary antibodies and amplification by biotin-streptavidine was performed according to the classical method adapted for amphibian embryos by Soula *et al.* (1993). After washing, slides were mounted in Mowiol 4-88 (Hoechst) and viewed under epifluorescence confocal microscopy. FITC-stained preparations were excited using an Argon laser (λ= 488 nm). Control slides were processed by omitting or using the preimmune serum in place of primary antibody.

Bioluminescence measurements

Light emission was recorded using a luminometer equipped with a R374 photomultiplier (Hamamatsu, Japan) connected to a chart recorder. The photocurrent was measured directly as an analog signal. Results are expressed in nA or arbitrary units.

Acknowledgments

We are grateful to Dr. Pierre Guerrier for helpful criticism and for correcting the English version and Dr. Anne-Marie Duprat for stimulating discussion. We thank Dr. O. Shimomura for the kind gift of purified aequorin. This work was partly supported by a research grant from AFM (Association Française contre les Myopathies).

References

BADMINTON, M.N., KENDALL, J.M., SALA-NEWBY, G. and CAMPBELL, A.K. (1995). Nucleoplasmin- targeted aequorin provides evidence for nuclear calcium barrier *Exp. Cell Res. 216:* 236-243.

BLINKS, J.R. (1989). Use of calcium-regulated photoproteins as intracellular Ca^{2+} indicators. *Methods Enzymol. 172:* 164-203.

BRINI, M., MURGIA, M., PASTI, L., PICARD, D., POZZAN, T. and RIZZUTO, R. (1993). Nuclear Ca^{2+} concentration measured with specifically targeted recombinant aequorin. *EMBO J. 12:* 4813-4819.

CAMPANELLA, C., ANDREUCCETTI, P., TADDEI, C. and TALEVI, R. (1984). The modifications of cortical endoplasmic reticulum during *in vitro* maturation of *Xenopus laevis* oocytes and its involvement in cortical granule exocytosis. *J. Exp. Zool. 229:* 283-293.

CAMPBELL, A.K. (1988). *Chemoluminescence. Principles and Application in Biology and Medicine.* Horwood/VCH, Weinheim.

COBBOLD, P.H. and LEE, J.A.C. (1991). Aequorin measurements of cytoplasmic free calcium. In *Cellular Calcium: A Practical Approach* (Eds. J.G. McCormack and P.H. Cobbold). IRL Press, Oxford, pp. 55-81.

CURRAN, R.C. and GREGORY, J. (1977). The unmasking of antigens in paraffin sections of tissues by trypsin. *Experientia 33:* 1400-1401.

DEGUCHI, R. and OSANAI, K. (1994). Meiosis reinitiation from the first prophase is dependent on the level of intracellular Ca^{2+} and pH in oocytes of the bivalves *Mactra chinensis* and *Limaria hakodatensis. Dev. Biol. 166:* 587-599.

ELINSON, R.P. (1980). The amphibian egg cortex in fertilization and early development. In *The Cell Surface: Mediator of Developmental Processes* (Eds. S. Subtelny and N. Wessells). Academic Press, New York, pp. 217-234.

GUERRIER, P., LECLERC-DAVID, C and MOREAU, M. (1993). Evidence for the involvement of internal calcium stores during serotonin-induced meiosis reinitiation in oocytes of the bivalve mollusc *Ruditapes philippinarum. Dev. Biol. 159:* 474-484.

GURDON, J.B. and WICKENS, M.P. (1983). The use of *Xenopus* oocytes for the expression of coned genes *Methods Enzymol. 101:* 370-386

HELPER, P.K. (1994). The role of calcium in cell division *Cell Calcium 16:* 322-330.

HOLLIDAY, J., ADAMS, R.J., SEJNOWSKI, T.J. and SPITZER, N.C. (1991). Calcium induced release of calcium regulates differentiation of cultured spinal neurons. *Neuron 7:* 787-796.

INOUYE, S., NOGUCHI, M., SASAKI, Y, TAKAGI, Y., MIYATA, T., IWANAGA, S., MIYATA, T and TSUJI, F.I. (1985). Cloning and sequence analyzing of cDNA for the luminescent protein aequorin. *Proc. Natl. Acad. Sci. USA 82:* 3154-3158.

JAFFE, L.F. (1985). The role of calcium explosions, waves and pulses in activating eggs. In *Biology of Fertilization*, Vol. 3 (Eds. C.B. Metz and A. Monroy). Academic Press, New York, pp. 127-165.

KENDALL, J.M., DORMER, R.L. and CAMPBELL, A.K. (1992). Targeting aequorin to the endoplasmic reticulum living cells. *Biochem. Biophys. Res. Commun. 189:* 1008-1016.

LECLERC, C., DUPRAT, A.M. and MOREAU, M. (1995). *In vivo* labelling of L-type Ca^{2+} channels by fluorescent dihydropyridine: correlation between ontogenesis of the channels and the acquisition of neural competence in ectoderm cells from *Pleurodeles waltl* embryos. *Cell Calcium 17:* 216-224.

LECLERC-DAVID, C, MOREAU, M., VILAIN, J.P. and GUERRIER, P. (1985). UV-irradiation inhibits the electrical block to polyspermy in echinoderms. *Dev. Growth Differ. 27:* 333-348

MOREAU, M. GUERRIER, P. and VILAIN, J.P. (1985). Ionic regulation of oocyte maturation. In *Biology of Fertilization*, Vol. 1 (Eds. C.B. Metz and A. Monroy). Academic Press, New York, pp. 299-345.

MOREAU, M., LECLERC, C., GUALANDRIS-PARISOT, L. and DUPRAT, A.M. (1994). Increased internal Ca^{2+} mediates neural induction in the amphibian embryo. *Proc. Natl. Acad. Sci. USA 91:* 12639- 12643.

ORON, Y., GILLO, B and GERSHENGORN, M.C. (1988). Differences in receptor-evoked membrane electrical responses in native and mRNA-injected *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA 85:* 3820-3824.

PRASHER, D., McCANN, R.O. and CORMIER, M.J. (1985). Cloning and expression of the cDNA coding for aequorin, a bioluminescent calcium binding protein. *Biochem. Biophys. Res. Commun. 126:* 1259-1268.

RIZZUTO, R., SIMPSON, A.W.M., BRINI, M. and POZZAN, T. (1992). Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature 358:* 325-327.

SAMBROOK, J. FRITSCH, E.F. and MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

SHIMOMURA, O. (1991). Preparation and handling of aequorin solutions for the measurement of cellular Ca^{2+}. *Cell Calcium 12:* 635-643.

SOULA, C., FOULQUIER, F., DUPRAT, A.M. and COCHARD, C. (1993). Lineage analysis of early neural plate cells: cells with purely neuronal fate coexist with bipotential neuroglial progenitors. *Dev. Biol. 159:* 196-207.

SWICK, A.G., JANICOT, M., CHENEVAL-KASTELIC, T., McLENITHAN, J.C. and LANE, M.D. (1992). Promoter-cDNA-directed heterologous protein expression in *Xenopus laevis oocytes. Proc. Natl. Acad. Sci. USA 89:* 1812-1816.

TSUJI, F.I. , INOUYE, S., SOTO, T. and SASAKI, Y. (1986). Site-specific mutagenesis of the calcium-binding photoprotein aequorin. *Proc. Natl. Acad. Sci. USA 83:* 8107-8111.

WALLACE, R.A., JARED, D.W., DUMONT, J.N. and SEGA M.W. (1973). Protein incorporation by isolated amphibian oocytes. III. Optimum incubation conditions. *J. Exp. Zool. 184:* 321-334.

YOUN, B.W., and MALACINSKI, G.M. (1981). Axial structure development in ultraviolet irradiated (notochord defective) amphibian embryo. *Dev. Biol. 83:* 339-352.

Accepted for publication: May 1995