Mediation of anion transport in oocytes of *Xenopus laevis* by biosynthetically inserted band 3 protein from mouse spleen erythroid cells

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mRNA from the spleens of anemic mice was purified by oligo(dT)-cellulose chromatography and fractionated by density gradient centrifugation. After injection into oocytes of *Xenopus laevis*, two of the four fractions obtained led, after 16 h of incubation at 20°C, to the expression of mouse band 3 protein, as demonstrated by immunoprecipitation with polyclonal antibodies against mouse band 3. Flux measurements showed an ~2- to 4-fold increment of $^{36}Cl^-$ uptake, which could be abolished by two different stilbene disulfonates, specific inhibitors of band 3-mediated anion transport in red blood cells.

Key words: anion transport/band 3 protein/mouse spleen mRNA/*Xenopus* oocytes

Introduction

The use of cell-free translation systems is essential for the study of the biosynthesis of transport proteins. However, the functional state of the synthesized product can only be assessed after its insertion into bilayers where the transport process can be monitored. Recently, the biosynthetic insertion of foreign transport proteins into the plasma membrane of oocytes of *Xenopus laevis* has been reported, such as the acetylcholine receptor (Barnard *et al.*, 1982; Sumikawa *et al.*, 1984; Mishina *et al.*, 1984), the Na channels of rat and human brain (Gundersen *et al.*, 1983a, 1984a), the chloride channel of the electrical organ of Torpedo (Sumikawa *et al.*, 1984) and other drug-controlled channels (Gundersen *et al.*, 1983b, 1984b, 1984c).

The basis for the expression of the transport systems in the oocyte plasma membrane is the following. The oocytes mature within the follicles of the ovaries until, at the beginning of the first meiotic division, their further development becomes arrested. When a hormonal stimulus takes place, maturation continues. The meiotic division is completed and the now mature oocytes are ready to be shed. During the so-called prophase arrest preceding hormonal stimulation, protein biosynthesis is reduced to a very low level. mRNA injected into these oocytes will successfully compete with the endogenous mRNA and translation products of the foreign mRNA will be produced. Since the oocytes contain the necessary machinery for post-translational processing and intracellular transport, the newly synthesized membrane transport protein will eventually appear in the plasma membrane of the cell where its functional state can be studied.

We were interested to explore whether or not the anion transport protein of the red blood cell (the so-called band 3 protein) can be expressed and become inserted in a functionally active state into the oocyte's plasma membrane. As opposed to the channel-forming transport proteins, whose expression has been studied previously, the band 3 protein mediates an ion exchange that does not contribute to the electrical conductance of the membrane (for reviews, see Cabantchik *et al.*, 1978; Macara and Cantley, 1983; Knauf, 1985; Jennings, 1985; Passow, 1985). Hence, in contrast to the work published so far, sensitive electrophysiological methods could not be applied to monitor the incorporation of the transport protein into the oocyte membrane. Instead, flux measurements with radioactive anions had to be made to demonstrate the occurrence of a band 3 protein-mediated component of anion flux in the oocyte. This requires the insertion of a considerable number of band 3 molecules into the oocyte plasma membrane. The present paper describes the successful expression of functional band 3 protein from mouse by the oocytes of *X. laevis*.

Results

For the isolation of mRNA use was made of the previous work of Braell and Lodish (1981, 1982) and Sabban *et al.* (1981). The spleens of anemic mice were homogenized and the RNA was extracted by a standard procedure based on the work of Chirgwin *et al.* (1979) as applied by Braell and Lodish (1982). When the bound RNA from the oligo(dT) column was subjected to density gradient centrifugation on a sucrose gradient, the sedimentation profile presented in Figure 1 was obtained. The contents of the gradient were subdivided into four fractions of decreasing mol. wt., designated A–D. The functionality of these fractions was tested in cell-free reticulocyte lysates and the appearance of [^{35}S]-methionine-labelled band 3 was shown by immunoprecipitation of products of unfractionated mRNA and a high mol. wt. fraction with polyclonal anti-mouse band 3 antibodies (whose specificity is demonstrated in Figure 2) and subsequent SDS-
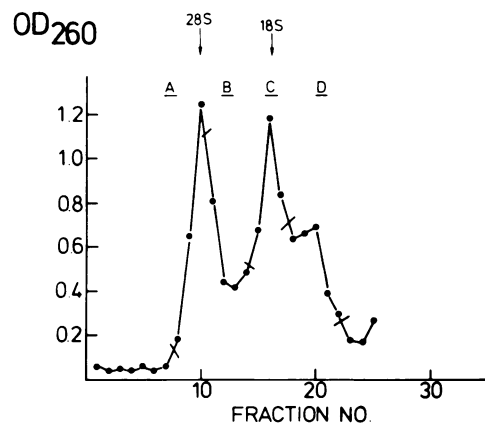

Fig. 1. Sucrose density gradient centrifugation profile of mouse anemic spleen poly(A)$^+$ RNA. Positions of 18S and 28S rRNA run in a parallel gradient are indicated. Fractions from regions A–D were pooled separately, ethanol precipitated, and used for translation in reticulocyte lysate and microinjection into oocytes.
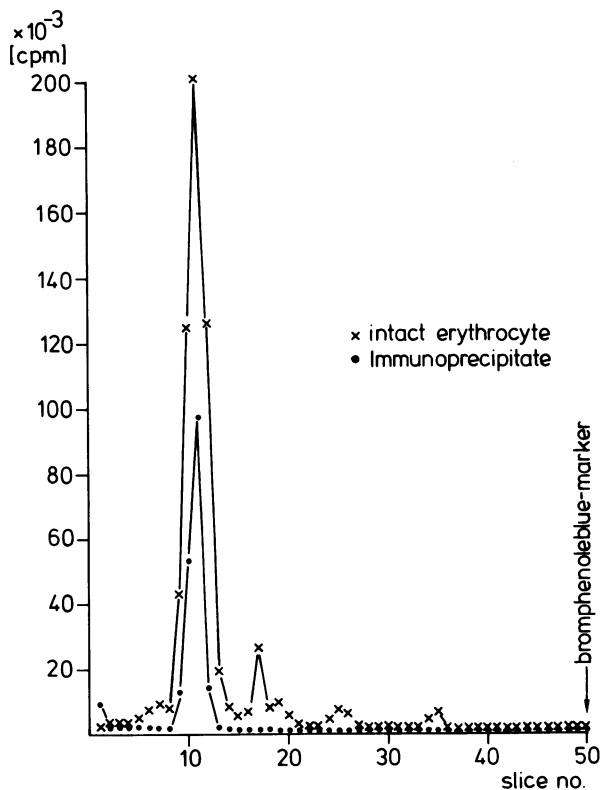
Fig. 2. Immunoprecipitation of $^3H_2DIDS$-labeled mouse red cell membranes with the anti-mouse band 3 antibodies used in this paper. Labeling of the cell membrane with $^3H_2DIDS$ according to Passow *et al.* (1982). The major peak at slice no. 11 represents the location on SDS-PAGE of the $^3H_2DIDS$-labeled band 3 protein in the untreated red cell membrane and in the immunoprecipitate.

PAGE. In accordance with what one would expect for a protein of the size of the band 3 protein (mol. wt. 96 000) fractions B and C contained the mRNA for the immunoprecipitable material at the 96 000-dalton location, and these fractions were chosen for microinjection. For translation, the injected oocytes were incubated for ~ 16 h at 20°C.

When the incubation of the injected oocytes was performed in the presence of [^{35}S]methionine, and the oocytes subsequently dissolved in SDS and subjected to polyacrylamide gel electrophoresis, many labelled bands were found that essentially coincided with the band pattern observed in uninjected control oocytes. However, after specific immunoprecipitation the synthesis of band 3 protein could be demonstrated in the mRNA-injected oocytes, but not in the controls that had received no mRNA or in micro-injected oocytes immunoprecipitated with pre-immune serum (Figure 3). Only fractions B and C yielded the immunoprecipitates, not fractions A and D.

After demonstration of the occurrence of biosynthesis of band 3 in the oocytes, anion transport was studied.

After the 16 h incubation period mentioned above, both control oocytes and microinjected oocytes were incubated in Barth's medium in the presence of $^{36}Cl^-$. At suitable time intervals (usually 2 or 3 h) ~ 10 − 20 oocytes were collected, washed and their radioactive contents were individually counted by placing each one into a separate counting vial. Uninjected oocytes show some Cl^- uptake during the flux period of 2 or 3 h. This uptake is little if at all affected by the addition of stilbene disulfonates, which are known to be specific inhibitors of anion transport (Knauf and Rothstein, 1971; Cabantchik and Roths-
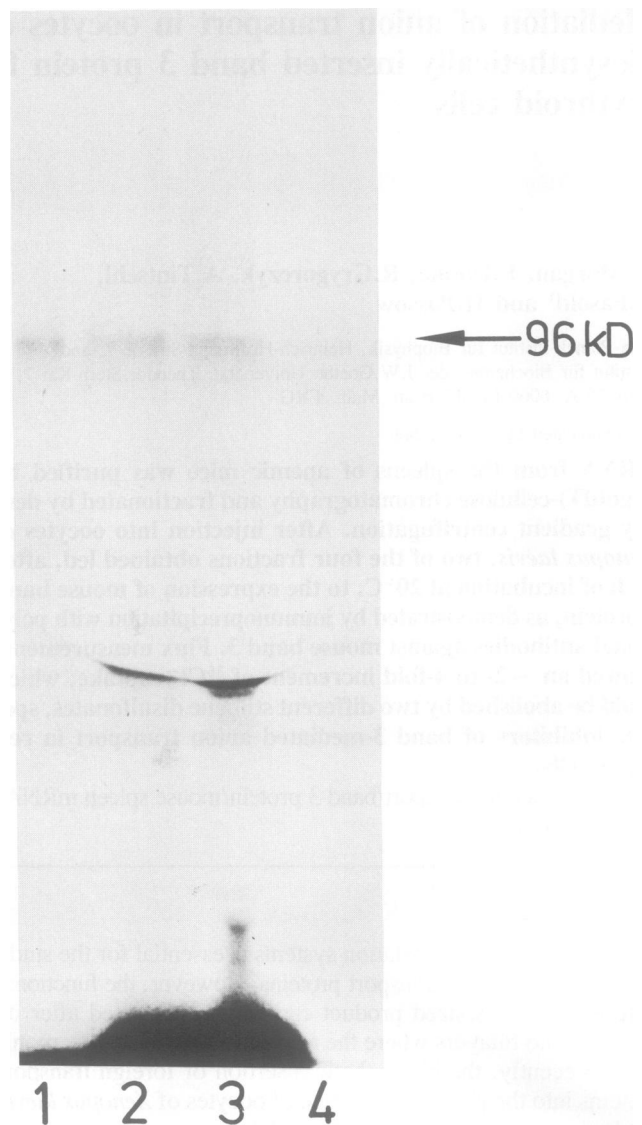

Fig. 3. Typical autoradiogram of SDS-PAGE of immunoprecipitates of extracts of oocytes microinjected with mouse spleen mRNA using anti-band 3 antibodies (**lane 1**) and two different preparations of antibodies against solubilized mouse erythrocyte ghosts (**lanes 2** and **3**). **Lane 4** shows the control with pre-immune serum. The 96-kD marker indicates the band 3 location. The dark zones in **lanes 2 − 3** appear in some but not all autoradiograms of similar experiments with antibodies against erythrocyte ghost membranes. They seem to be associated with protein A transferred to the gels together with the immunoprecipitate. For unknown reasons they never appear (as in **lane 1**) in immunoprecipitates with anti-mouse band 3 antibodies.

tein, 1974; for review, see Passow *et al.*, 1982). The non-covalently binding 4,4'-dinitro stilbene-2,2'-disulfonate (DNDS) produced consistently a slight inhibition while the covalently binding diisothiocyanate 4,4'-diisothiocyano stilbene-2,2'-disulfonate (H_2DIDS) exerted either no significant effect or a slight increase in the rate of $^{36}Cl^-$ uptake. In the mRNA-injected oocytes, the rate of $^{36}Cl^-$ uptake was increased as compared with the control and the increment was abolished by the presence of DNDS (Figure 4) or H_2DIDS (Figure 5). These results are based on experiments with the oocytes from seven different females. In five of these experiments, including two experiments on the action of H_2DIDS, the specific activity of the $^{36}Cl^-$ was not sufficiently well known to permit a conversion of the radioactivity uptake into the uptake per oocyte of chloride. In four experiments,
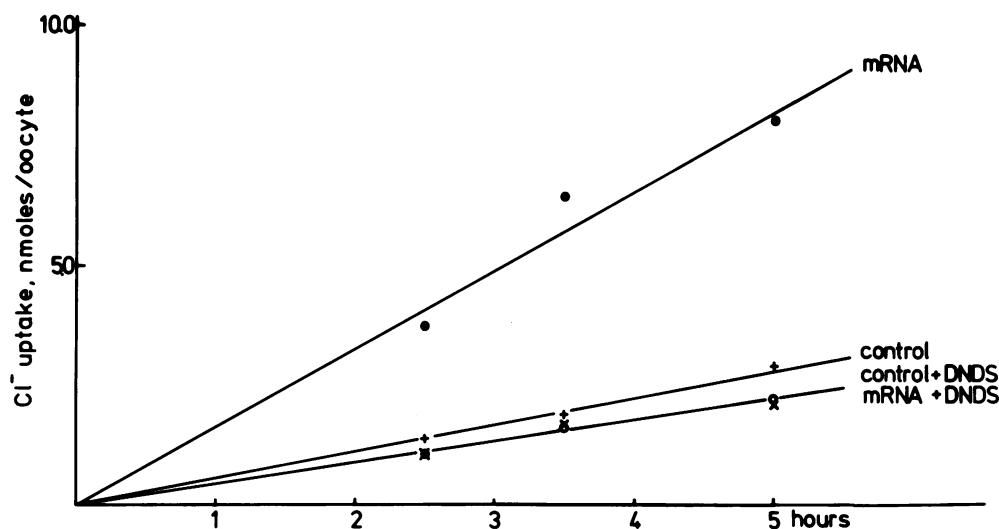
Fig. 4. Effect of microinjected mRNA from mouse spleen on Cl^- uptake by prophase-arrested, fully grown oocytes from *X. laevis*. Control and control + DNDS: controls, in Barth's medium without and with 0.4 mM DNDS, respectively. mRNA and mRNA + DNDS: oocytes containing mRNA, in Barth's medium without and with 0.4 mM DNDS, respectively. 20°C.
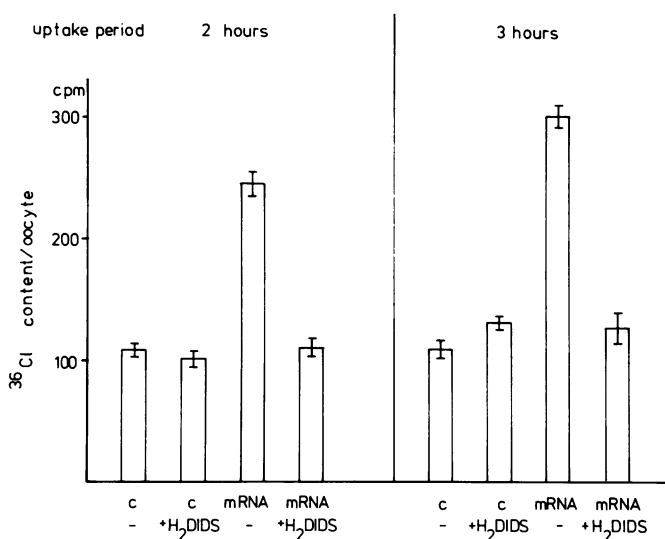

Fig. 5. Effects of H_2DIDS on Cl^- uptake of control oocytes (c) and oocytes containing microinjected mRNA from mouse spleen. Same experimental arrangement as in Figure 4. H_2DIDS concentrations: 20 μM. Error bars indicate standard error of the mean values.

the specific activity could be determined and absolute values of the uptake were calculated (Table I).

From the stilbene disulfonate inhibitable increment of $^{36}Cl^-$ uptake appearing after the injection of spleen mRNA, it is possible to make a rough estimate of the number of band 3 molecules incorporated per oocyte. If one assumes that the turnover number in the oocyte is similar to the turnover number in the intact red cell (i.e., $\sim 10^4$ ions/band 3 molecule/s; Brahm, 1977), one arrives at a value of $\sim 10 - 20 \times 10^6$ band 3 molecules/oocyte. This would be equivalent to the incorporation of the band 3 molecules of $\sim 10 - 20$ red blood cells.

It should be noted that the calculation is based on the assumption that the (unknown) turnover number of mouse band 3 is the same as that of human band 3 and that this turnover number remains unaffected by incorporation into the lipid environment of the oocyte plasma membrane, which may differ from that in the human or mouse red cell. Differences of lipid composition are

known to influence the turnover number of the band 3 protein (Deuticke *et al.*, 1980). In mammalian red cells these differences account for an ~ 5-fold change of transport activity of band 3. It seems reasonable to assume, therefore, that the lower limit of incorporation into the oocytes corresponds to the band 3 molecules in about two red cells, the upper limit to the band 3 molecules in ~ 100 red cells.

In the membrane of the human red cell, the density of band 3 molecules is $\sim 7000/\mu m^2$. Assuming that the density in the mouse red cell membrane is about the same, from this number one can calculate that in a spherical oocyte (diameter ~ 1.2 mm), after incorporation of the band 3 molecules of $10 - 20$ red cells, the surface density of band 3 molecules is $\sim 4 - 5/\mu m^2$. This value represents an overestimate since the surface of the oocytes carries microvilli which increase the surface area by at best a factor of 2. Thus the surface density of band 3 molecules in the mRNA-injected oocytes is at least $2 - 3/\mu m^2$ or $\sim 0.03 - 0.04\%$ of the density in the red blood cell.

The experiments demonstrate that it is feasible to express band 3 protein of mouse red cells in the plasma membrane of *X. laevis*. The protein molecules are functional and the amount incorporated into the membrane suffices to induce a stilbene disulfonate-sensitive increment of anion flux that can be measured in single oocytes. Therefore it may now be possible to express band 3 molecules from different species in the same lipid bilayer and thus to study the effects of species differences on transport in the same environment. Moreover, it should become possible to measure the voltage dependence of 'electrically silent' anion fluxes in single oocytes. Such measurements would be desirable since, even without a contribution of the transported anion to the electrical conductance of the membrane, the conformation of the transport protein could be voltage dependent and hence membrane potential could affect chloride transport.

Materials and methods

Table I. Cl⁻ uptake by *Xenopus* oocytes with or without injected mouse spleen mRNA, in media with or without DNDS (400 μM)

Exp. no.	Uptake period h	Cl⁻ uptake, nanoequivalents/oocyte				DNDS inhibitable uptake nanoequivalents/oocyte	
		Control	Control + DNDS	mRNA	mRNA + DNDS	Control	mRNA injected
1	2.5	2.70 ±0.06 n=23	2.25 ± 0.045 n=23	9.22 ± 0.85 n=9	2.67 ± 0.071 n=11	0.45	6.55[a]
2	2.5	3.68 ± 0.079 n=16	3.29 ± 0.056 n=16	8.68 ± 0.15 n=13	3.95 ± 0.065 n=14	0.39	4.73[a]
3	2.5	3.75 ± 0.071 n=10	3.40 ± 0.067 n=9	5.03 ± 0.11 n=11	3.55 ± 0.075 n=14	0.35	1.48
	5.0	5.69 ± 0.084 n=12	5.20 ± 0.058 n=11	7.42 ± 0.092 n=12	5.67 ± 0.083 n=9	0.49	1.75
4	2.5	1.43 ± 0.05 n=8	1.11 ± 0.019 n=12	3.79 ± 0.45 n=8	1.07 ± 0.017 n=11	0.32	2.72
	3.5	1.93 ± 0.036 n=12	1.66 ± 0.054 n=12	6.50 ± 0.38 n=11	1.75 ± 0.033 n=13	0.27	4.75
	5.0	2.96 ± 0.049 n=14	2.20 ± 0.046 n=12	8.11 ± 0.33 n=13	2.14 ± 0.11 n=9	0.76	5.97

[a]In contrast to all other experiments described in this paper, control oocytes and mRNA injected oocytes had been incubated for 40 h (rather than the usual 16 h) prior to the initiation of the ^{36}Cl⁻ uptake measurements. Oocytes in experiments 1 and 3 were made with oocytes from the same female. Experiments 2 and 4 were made with oocytes from another female.

stream of nitrogen, resuspended in 4 ml of 0.1% SDS to which was added 4 ml of RNA extraction buffer [0.5% SDS, 0.1 M NaCl, 50 mM sodium acetate (pH 5.2) and 5 mM sodium EDTA], and finally extracted with phenol:chloroform: isoamylalcohol (25:24:1). After removing the aqueous phase, the organic layer was re-extracted with 3 ml of 0.1% SDS solution plus 3 ml RNA extraction buffer. The aqueous phases were combined, re-extracted with an equal volume of phenol: chloroform:isoamylalcohol, made 0.2 M in sodium acetate (pH 5.2) and precipitated with two volumes of ethanol overnight at $-20°$C. The RNA was recovered by centrifugation and the pellet washed once with 10 ml of 70% ethanol. Chromatography on oligo(dT)-cellulose (Bethesda Research Laboratories) was performed according to Braell and Lodish (1982).

Poly(A)-containing RNA was recovered by centrifugation, washed once with 70% ethanol, dried with nitrogen, dissolved in a small volume of autoclaved H_2O to give a final concentration of ~ 1 mg/ml and an aliquot taken to determine optical density of 260 and 280 nm. Approximately 250 μg in water was made 1 mM EDTA and 0.5% SDS, heated at 60°C for 10 min, cooled briefly on ice, and run on 10−30% (w/w) sucrose gradients, in 10 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% SDS, in a SW 40 rotor at 25 000 r.p.m., 17 h, 16°C. Alternatively, 150 μg of denatured RNA was run in 5−20% sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM sodium EDTA, 0.2% SDS, on the SW 55 rotor, 45 000 r.p.m., 4 h, 16°C. Poly(A)⁻ RNA was run in a parallel gradient as a size marker. 0.4 ml fractions were collected, and the optical density determined at 260 nm. The fractions were either pooled or individually ethanol precipitated.

Cell-free translation of mRNA

Unfractionated poly(A)⁺ RNA or gradient fractions were added at a final concentration of 40 μg/ml to nuclease-treated reticulocyte lysate (Bethesda Research Laboratories, Inc.) containing 10 μCi of [^{35}S]methionine (Amersham-Buchler, sp. act. 1000 Ci/mmol) per 20 μl sample. Incubation was at 30°C for 2 h. 4 μl aliquots were taken into gel sample buffer (Laemmli, 1970).

Antibodies

Mouse erythrocyte band 3 protein was purified from mouse erythrocyte membrane by preparative SDS-PAGE according to Koziarz *et al.* (1978), using the Laemmli buffer system. The column used (7900 uniphor column electrophoresis system, LKB) was 2.5 cm in diameter and contained 40 ml 6% separating gel and 10 ml 3% stacking gel.

Mouse anti-band 3 antibodies and antibodies against solubilized mouse erythrocyte ghosts were raised in rabbits. A total of 1 mg antigen/rabbit in 2% Brij 58 was administered in three injections at 2-week intervals followed by a last injection after another 4 weeks. The rabbits were bled 1 week after the last injection. An IgG fraction was prepared by ammonium sulfate precipitation of the immune serum, followed by chromatography on protein A-Sepharose Cl 4B (Sigma) (Walker and Mayer, 1977; Williams and Chase, 1967). The antibodies were characterized by Ouchterlony immuno-diffusion (Ouchterlony, 1967) and Western blotting (Burnette, 1981) and immunoprecipitation of ^{3}H$_2$DIDS-labeled mouse band 3 protein (Figure 2).

Immunoprecipitation

Each microinjected oocyte (see below) was incubated overnight at 19°C in 10 μl Barth's medium containing 0.02 g/l penicillin, 0.025 g/l streptomycin and 3 μCi [^{35}S]methionine (Amersham). Homogenization and immunoprecipitation were carried out by combining the methods of Colman (1984a) and Anderson and Blobel (1983). Oocytes were homogenized in 10 μl/oocyte of homogenization buffer containing 50 mM Tris, 50 mM NaCl, 1% SDS, 0.1 mM phenyl methyl sulfonylfluoride (PMSF), pH 7.2 at 0°C. After centrifugation (2 min, Eppendorf centrifuge) 50 μl aliquots of the extract of five oocytes were mixed with 2 μl 25% SDS and heated at 100°C for 4 min. Then the samples were diluted with 200 μl dilution buffer containing 1.25% Triton X-100, 190 mM NaCl, 60 mM Tris, 6 mM EDTA, 0.1 mM PMSF, pH 7.4, followed by an overnight incubation at 4°C with antibody (60−80 μg/sample). Control samples included microinjected oocytes reacted with pre-immune serum, or non-injected oocytes reacted with specific antibodies. The resulting immunocomplex was bound to protein A-Sepharose (Sigma) (30 μl of a 1:1 suspension of protein A-Sepharose in dilution buffer). After end-over-end mixing for 4 h, the beads were washed several times. The bound protein was eluted with SDS-PAGE sample buffer containing 1% SDS and 0.5% β-mercaptoethanol. After heating at 100°C for 4 min, the samples were applied to a SDS-PAGE (Laemmli, 1970) using a 3% stacking gel and a 8−20% gradient separating gel.

Microinjection and measurements of Cl⁻-flux in the Xenopus oocytes

Pieces of the ovaries were removed under anesthesia (for details, see Colman, 1984b), suspended in Barth's solution (in mM/l: 88 NaCl, 1.0 KCl, 2.4 Na HCO$_3$, 0.82 MgSO$_4$, 0.33 Ca(NO$_3$)$_2$, 0.41 CaCl$_2$, 5.0 Hepes, pH 7.6) containing 0.5 units/ml collagenase and incubated for 8−16 h at ~18−20°C. After subsequent washings in Ca^{2+}-free Barth's medium, prophase arrested, full grown (stage VI) oocytes were collected in normal (i.e., Ca^{2+}-containing) Barth's medium. One half of the oocytes received fractions B or C (25 ng in 50 nl) of the RNA preparation by microinjection using a motor driven syringe (micropump, Bachofer). Controls were either mock-injected without RNA or not injected. After ~16 h of incubation at 18−20°C in Barth's medium containing penicillin and streptomycin at the concentrations indicated above, the oocytes were washed and resuspended in Barth's medium containing ^{36}Cl⁻ (Amersham). After 2 h or more of incubation at 20°C, 15−20 oocytes were removed, washed free of extracellular radioactivity and placed individually into separate counting vials. Cl⁻ uptake was calculated from the radioactivity per oocyte and the known specific activity of the medium.

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