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Topical Review

Use of *Xenopus* Oocytes for the Functional Expression of Plasma Membrane Proteins

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Introduction

Membrane Transport and Signalling

The phospholipid bilayer of the plasma membrane of the eucaryotic cell acts as a permeability barrier to many metabolic substrates and ions, whose transmembrane movement is catalyzed by selective membrane transport proteins. Control of cytosolic ion concentrations is achieved by the concerted action of numerous proteins working in the plasma membrane and the membranes of intracellular organelles: active pumps driven by ATP, carriers catalyzing co- or countertransport and ion channels.

A second important function of plasma membrane proteins is the reception of signals from the extracellular milieu, and the transmission of these signals either along the membrane, or across it, to the cytoplasmic side. The extracellular signals include such diverse entities as fast neurotransmitters, modulatory peptides, hormones, substrate-cell and cell-cell contacts.

Many methods are now available for the study of mechanisms underlying membrane transport and signalling. Biochemical isolation and purification to homogeneity, reconstitution into artificial bilayer membranes, functional studies in these model systems, chemical modification, immunochemical and electrophysiological methods have helped to elucidate many features of membrane protein function. But, it should be emphasized here, that to date no protein catalyzed transmembrane transport mechanism is fully understood at the molecular level. The powerful tools recently developed by molecular biologists have raised new hopes for such an understanding. The availability of a quickly growing number of cDNA sequences coding for membrane transport proteins and receptors has provided a wealth of structural information, and insight into the evolutionary relationship between these proteins. In order to understand the functional implications of these structures, the proteins encoded must be functionally expressed and characterized.

Key Words *Xenopus laevis* · *Xenopus oocyte* · membrane protein · expression · mRNA · cDNA

Functional Expression of Membrane Proteins

Biosynthesis of membrane proteins requires at least partial translocation of the nascent polypeptide chain(s) into the endoplasmic reticulum, proper folding in the membrane, often assembly of multiple subunits, post-translational modification in the Golgi apparatus, and, in some cases, sorting of the newly formed protein to defined surface membrane domains. Due to this complexity, it is difficult to achieve synthesis of functional eucaryotic membrane proteins in vitro or in procaryotic expression systems, such as *Escherichia coli*. Thus, functional expression of eucaryotic membrane proteins usually requires use of a eucaryotic host cell.

cDNA may be expressed in eucaryotic cells, either by using transient or stable transfection, or by using microinjection into the nucleus. Alternatively, the cDNA may be transcribed in vitro, capped, polyadenylated and the resulting mRNA transferred into the cytoplasm. Functional expression helps to confirm the identity of cloned DNA sequences and to investigate the functional properties of the encoded proteins. As the cDNA sequences can be altered, e.g., by site-directed mutagenesis, the perturbation of function in the expressed mutant protein contributes to the understanding of the functional architecture of these membrane proteins.

Functional expression of proteins can also be achieved by introduction of crude tissue mRNA into a foreign cell. This allows:

—characterization and partial purification of mRNA(s) coding for these functions;

—a direct approach to the cDNA solely based on function (functional expression cloning);
—observation of a membrane protein in a foreign host cell, where it may be more accessible than *in situ* to studies of its biosynthesis and modulation by second messengers or drugs; and
—comparison of membrane proteins originating from different tissues and stages of ontogeny of the same species or from different species in a standardized environment.

For reasons discussed in this review, the most frequently used cell for the functional expression of plasma membrane proteins from mRNA is the *Xenopus* oocyte, but similar experiments have been performed with oocytes from other species and with somatic cells. For example, the oocytes of the newt *Cynops pyrrhogaster* have been shown to express eel electroplax acetylcholine receptor [6, 108], and Dahl et al. [37, 38] demonstrated functional expression of gap junctions in a tumorigenic mouse cell line after fusion of the cells with liposomes containing total rat myometrium mRNA.

The aim of this review is to provide a survey of the use of the oocyte of the South African frog *Xenopus laevis* as a functional expression system for plasma membrane proteins. Limitations of each application will also be discussed.

Experimental Approaches

Historical Aspects

Expression of foreign proteins in the *Xenopus* oocyte, either following microinjection with mRNA into the cytoplasm [74] or of cDNA into the nucleus [144] has been pioneered by Gordon and collaborators. The oocyte efficiently transcribes and translates injected genetic information, performs assembly of the protein products, correctly processes the nascent polypeptides, and targets them to the proper subcellular compartment. It quickly became a standard in vivo expression system [for reviews *see* 35, 73, 75, 112, 120, 209]. But only in 1981, the *Xenopus* oocyte was "discovered" for the expression of plasma membrane proteins. Sumikawa et al. [216] demonstrated the presence of specific ^{125}I-α-bungarotoxin binding sites newly expressed in the oocyte surface membrane, following microinjection of the cells with crude mRNA, isolated from the electric organ of *Torpedo marmorata*. One year later, the same authors showed, in collaboration with Miledi [16], that this protein truly represented a functional ion channel gated by acetylcholine. Thus, it was recognized that the oocyte is also able to properly assemble foreign multi-subunit proteins and to incorporate them in a functional form into its surface membrane. Following this discovery, the technique was rapidly applied to other ion channels [69, 88, 151, 152] and later to many other membrane proteins. Tables 1 and 2 summarize plasma membrane proteins that have so far been functionally expressed in the *Xenopus* oocyte, either using crude mRNA isolated from various tissues (Table 1), or starting from cDNA (Table 2). Both tables list selected references only, for each protein. The literature review was completed at the end of 1989.

Comparison of the Oocyte Expression System with Functional Expression in Somatic Cells

The major advantage of the oocyte is the experimental ease with which expression of mRNA can be achieved. Due to the difficulties of efficiently transferring mRNA into small cells, handling and recognizing individual cells, and performing experiments, somatic cells have largely been abandoned as mRNA expression sytems. A valuable alternative exists for the expression of cDNA, namely transfection of cDNA into a suitable cell line. Advantages for the expression of cDNA in the oocyte *versus* transfection in somatic cells, especially for the purpose of electrophysiological experiments, are:
—simple handling of single identified cells after transfer of genetic information, using a pasteur pipette;
—the high proportion (>98%) of cells that express the genetic information after the transfer;
—the possibility of controlling the environment of the oocytes by simple bath perfusion;
—simple control of the membrane potential.

Drawbacks of the oocyte include:
—the seasonal variation of the oocyte quality, that is observed in some laboratories;
—the short expression period, which lasts usually not longer than fourteen days, compared to stable transfection;
—the relatively small number of cells that can be handled in a single experiment; and
—cDNA that has to be transcribed, capped and polyadenylated in vitro, unless it is directly injected into the nucleus of the oocytes.

Thus, the choice of an expression system will depend on the type of experiments to be done.

Oocytes Are Easily Handled in Culture

The morphology and development of this polarized cell have been described [47]. The fully grown oocyte from *X. laevis* is an extraordinarily large cell

Table 1. Plasma membrane proteins that have been expressed in the *Xenopus* oocyte after injection with crude mRNA

Protein function Agonist or substrate (type)	References
Ion channels	
Na^+	[63, 67, 69, 86, 111, 132, 145, 176, 198, 199, 202, 221, 223, 224, 233, 238]
Ca^{2+}	[43, 52, 72, 100, 121, 131, 202, 206, 240]
K^+	[24, 25, 53, 68, 132, 188, 221, 223, 230, 259]
Cl^-	[62, 220]
K^+ (ATP-regulated)	[10]
Na^+ (amiloride-sensitive)	[60, 83, 113]
GABA receptor channel	[9, 30, 88, 152, 174, 175, 200, 201, 202, 207, 221, 223, 243, 255]
Glycine receptor channel	[1, 2, 9, 30, 71, 88, 177]
Acetylcholine receptor channel	[16, 26, 145, 151, 153, 176, 216, 220]
Glutamate receptor channel (kainate or quisqualate)	[51, 69, 70, 85, 87, 88, 124, 173, 177, 186, 189, 215, 221, 241, 244, 246]
Glutamate receptor channel (NMDA)	[51, 59, 70, 107, 118, 123, 124, 141, 244–246]
Carriers	
Na^+-Ca^{2+}	[130, 204]
Na^+-glucose	[7, 82]
Na^+-iodide	[247]
Na^+-neurotransmitters	[22, 193]
Na^+-amino acid	[8]
Amino acid (Na^+-independent)	[8, 232]
Anion-exchanger (band 3)	[64, 76, 158]
Receptors acting via IP_3	
Serotonin ($5HT_{1c}$, $5HT_2$)	[5, 42, 66, 69, 134, 167, 190, 228, 237]
Angiotensin (type II)	[36, 142, 147, 192, 254]
Neurotensin	[84, 178]
Substance P	[5, 78, 178, 258]
Substance K	[139]
Neurokinin (type A)	[78]
Quisqualate	[51, 70, 85, 214, 215]
Vasopressin (V1, V2)	[142, 147, 159, 254]
Acetylcholine (M1, M3)	[5, 42, 84, 101, 167, 169]
Cholecystokinin	[159, 161, 254]
Bombesin	[146, 161, 254]
Gastrin-releasing peptide	[146]
Gonadotrophin-releasing hormone	[50]
Thyrotropin-releasing hormone	[142, 147, 169–171]
Noradrenaline	[222]
Dopamine	[222]
Other proteins	
Light (rhodopsin)	[105]
Odorant mixture	[61]
Adrenergic receptors ($\beta1$, $\beta2$)	[13]
Fc receptors	[185]
Interferon (γ)	[117]
IgE receptor	[127]
G-proteins (G_i, G_o)	[99]
Cyclase (cAMP)	[208]
Gap junction	[251]
Semliki Forest virus envelope protein	[89]

with a diameter of 1–1.2 mm which is surrounded by the noncellular, fibrous vitellin layer and by several layers of follicular cells [46]. The latter may be removed, either mechanically or by enzymatic procedures, to give "naked," "denuded" or defolliculated oocytes. Most experiments are carried out using naked oocytes that are still surrounded by the vitellin layer, which provides mechanical stability to the oocyte. For the recording of single ion channel currents using the patch-clamp technique, it is also necessary to remove the vitellin layer [145], or to rupture it locally [198], in order to expose the plasma

Table 2. Cloned plasma membrane proteins that have been expressed in the *Xenopus* oocyte

Protein function Ligand or substrate (type)	References
Channels	
Na^+ channel	[11, 145, 163, 210, 211, 225, 239]
K^+ channel	[32, 53, 162, 212, 213, 230]
K^+ channel (A-type)	[94, 136, 234, 235, 261, 262]
Ca^{2+} channel (L-type)	[148]
Acetylcholine receptor channel	[91, 92, 116, 122, 140, 145, 154–156, 182, 191, 217–219, 227, 236,
(skeletal muscle, electroplax)	252, 253, 260]
(brain)	[14, 23, 48, 172, 248]
GABA receptor channel	[21, 106, 125, 126, 128, 129, 137, 138, 184, 195, 203, 256, 257]
Glycine receptor channel	[194]
Kainate receptor channel	[87]
Connexins (gap junctions)	[39, 49, 226, 250]
Transport proteins	
Na,K-ATPase	[77, 164, 165, 196, 229]
Na^+-glucose cotransport	[81, 90, 242]
Glucose carrier	[20, 103, 181]
Anion-exchanger (band 3)	[18, 27]
Receptors	
Substance K receptor	[139]
Substance P receptor	[258]
Adrenergic receptors ($\alpha 2$, $\beta 1$, $\beta 2$)	[54, 55, 109, 110]
Muscarinic receptors (I,II,III,IV)	[3, 28, 56, 114, 115]
Thyroid-stimulating hormone	[179]
Serotonin ($5HT_{1c}$)	[97]
Serotonin ($5HT_2$)	[183]
Mas-oncogene (angiotensin)	[95]
NGF receptors	[197]
Low density lipoprotein receptor	[180]
Rhodopsin	[105]
Atrial natriuretic peptide clearance receptor	[57]

membrane and to allow sealing between glass pipette and membrane.

Expert advice on the practical aspects of the oocyte expression system is available, including handling of the frogs, isolation of follicles, removal of follicle cells, maintenance of follicles and oocytes in culture, and microinjection of cDNA into the nucleus or mRNA into the cytoplasm [35, 73, 75, 112]. Information on the fate of injected mRNA in the oocyte [120, 209] and on the electrophysiological properties of the native follicles and oocytes [40] have been expertly reviewed. The latter review also contains a valuable discussion of the use of the oocyte as an expression system for ion channels.

The experimental procedures involved in the functional expression of membrane proteins in the *Xenopus* oocyte are illustrated in Fig. 1. Total RNA is extracted from cell cultures or from whole tissues. In many cases crude total RNA is sufficient for obtaining a functional signal from the expressed proteins. However, the amount of mRNA thereby entering the cell will be very small. This is due to the

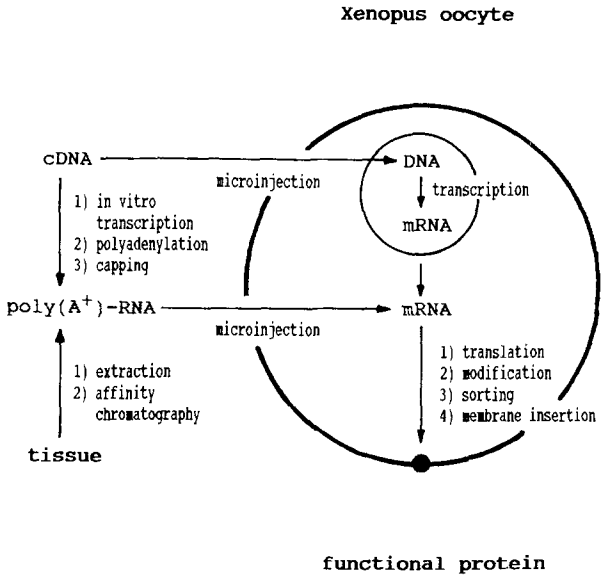

Fig. 1. Scheme illustrating functional plasma membrane protein expression in the *Xenopus* oocyte

small percentage of mRNA in total RNA and due to the limited concentration of RNA that can be handled with a microinjection pipette. At concentrations exceeding 2 mg/ml, the viscosity of the solution makes precise delivery into the oocytes impossible. Better expression is usually possible by using purified poly(A^+)-RNA, obtained from total RNA by affinity chromatography (e.g. 12). If cDNA coding for a protein is available, it is transcribed in vitro, to obtain the corresponding RNA. Capping and polyadenylation may contribute to the stability of the message in the oocyte [45]. Approximately 50 nl of a RNA solution is microinjected into the cytoplasm at the vegetal pole of either intact follicles of naked oocytes. Alternatively, cDNA (10 nl) is microinjected directly into the nucleus [34, 112].

While injection of cDNA or of pure mRNA derived from cDNA will often result in detectable activity of the expressed protein within hours, mixtures of mRNA from crude sources may require culturing of the injected oocytes for several days, depending on the sensitivity of the assay (*see below*).

It may be added here that many laboratories have experienced problems in securing a steady supply of morphologically intact, healthy oocytes, characterized by a dark brown to black pigmented, animal hemisphere and a yellowish, vegetal hemisphere. In fact, upon first examination, many female frogs have either few mature oocytes or cells displaying "bold" patches on the animal hemisphere. Morphologically altered cells are usually not stable in culture and cannot be used in expression experiments. Often the same donor animal, two weeks after the first surgery, delivers morphologically intact oocytes (E. Sigel, *unpublished observations*). Removal of part of the ovary appears to stimulate maturation of new oocytes. Reusing the same animals for up to 15 oocyte donations, therefore, not only helps to keep the number of experimental animals low, but also secures a supply of good quality oocytes.

Notes of Caution

If a new membrane protein is to be expressed, the oocyte should be checked for the presence of any endogenous protein, which could mask the newly expressed function. For example, microinjection of cDNA derived pure mRNAs coding for the two subunits of the Na-K-ATPase from *Torpedo californica* resulted in a functional signal only two- to sevenfold higher than in noninjected oocytes, due to the high endogenous Na-K-ATPase activity [164, 196].

Control experiments carried out in the presence of inhibitors of transcription, usually actinomycin D, may help to exclude the possibility of activation of an endogenous *Xenopus* gene by the introduction of mRNA into the oocyte. It should be noted here that the oocyte also contains a large endogenous pool of nontranslated mRNA [4], and no control experiments are available to exclude, *a priori,* activation of such an endogenous species of mRNA, or the contribution by the oocyte of one or several protein subunits to form a functional multisubunit protein. While the latter possibility is difficult to exclude, circumstantial evidence is available from a large number of experiments to render the former possibility unlikely:

—Membrane proteins expressed from crude mRNA(s) are always present in the tissue of origin of the mRNA(s). Expression is usually only observed if the tissue of origin is a rich source for the particular mRNA(s).

—The properties of the newly expressed proteins are similar to those found in the tissue of origin; e.g. oocytes injected with cDNA derived mRNAs coding for *Torpedo* electroplax and bovine skeletal muscle acetylcholine receptors, express ion channels whose single channel kinetic properties are reminiscent of those in the native tissues ([191], further discussed below).

—In many cases where cDNA coding for functional proteins was altered by using in vitro mutagenesis, the consequent functional alterations were always in line with faithful expression.

In spite of all the circumstantial evidence available, any result obtained with the oocyte system should be critically evaluated for the possibility of the activation of endogenous mRNA, or for the contribution by the oocyte itself of a subunit to an expressed functional protein.

Post-translational processing and proper positioning of the membrane proteins are often faithfully carried out by the oocyte. Thus, secretory proteins are usually secreted, and other proteins are targeted to the correct intracellular location [209]. However, it should be noted that in some cases proteins have been shown to differ in the extent of glycosylation [209]. In an interesting study, glycosylation of the α-subunit of the *Electrophorus* electrocyte sodium channel in the native tissue and in the mRNA-injected oocyte were compared [233]. In this case, the α-subunit was only partially glycosylated in the oocyte. The functional implications of this observation are not clear.

While the molecular properties of membrane proteins are often reproduced precisely after expression in the oocyte, the expressed protein may interact with protein partners different from those *in situ*. This is illustrated by the finding that the liver vasopressin receptor interacts with pertussis-toxin sensi-

tive G-proteins *in situ*, but with pertussis-toxin insensitive G-proteins after expression in the oocyte [159]. Alternatively, an important interaction partner may lack in the oocyte, for example, a G-protein for a receptor.

The host environment may also have a specific influence on the newly expressed protein, e.g., resulting from the phospholipid composition of the plasma membrane or from the activity of regulatory enzymes, that may both differ between oocyte and tissue of origin. These parameters may also differ between different batches of oocytes. Thus, the steady-state activity of protein kinase C in different batches has been found to be variable [202]. It is also known (for review *see* Dascal [40]), that certain endogenous receptors are only present in oocytes of some individual donor animals, and, in addition, underlie seasonal variation.

Newly Expressed Proteins Can Be Detected Structurally or Functionally

A factor that facilitates detection of newly expressed membrane proteins is the relatively low abundance in the oocyte plasma membrane of channels, transport systems and receptors. Follicles show physiological responses to many agents, e.g. adrenaline, serotonin, dopamine, purines, muscarinic agonists (for review *see* [40]), and peptide hormones such as corticotropin-releasing factor, arginine-vasopressin and cholecystokinine [160] but, upon removal of the follicular cell layers, most of these responses are lost. The denuded oocyte only responds to cholecystokinine [160], while the other receptors seem to reside on the follicular cells, which are electrically coupled to the oocyte. The plasma membrane has also a Na-K-ATPase [119], sodium-dependent amino acid carriers [98], sodium-dependent and sodium-independent glucose carriers [249], a glucose uptake system, which can be activated by insulin-like growth factor I [96], a small endogenous calcium conductance [43], a voltage- and calcium-dependent chloride conductance [15, 149], and other voltage-dependent conductances, displaying rather unusual activation properties (reviewed in [40]). A further ion channel is evident from single channel recordings: stretch-activated channels similar to the ones described in cultured chick skeletal muscle cells [65]. This nonselective cation channel in the oocyte shows some spontaneous activity and is further activated by applying negative pressure to the patch [145]. Overlap of the spontaneous activity of this channel with the activity of newly expressed channels causes some problems in single channel recordings, and care should be taken not to confuse channel opening of stretch-activated channels with newly expressed ion channels.

The amount of newly expressed membrane protein after injection with crude mRNA varies, depending on the expressed protein, and is also related to the abundance of the specific mRNA in the tissue of origin and to the degree of integrity of the mRNA preserved during the isolation procedure. The relative expression of different membrane proteins has also been reported to depend on the handling of the isolated mRNA. Repetitive freezing and thawing, e.g. of total chick forebrain mRNA dissolved in a buffered solution, resulted in a gradual loss of sodium channel expression, while the expression of the GABA receptor channel remained unaffected by this treatment [198].

In the absence of an endogenous oocyte protein with the same function expression of a surrogate protein often exceeds the limit of detection by a large factor. Surprisingly, pure mRNA(s) frequently do not result in much higher functional signals than those obtained with qualitatively optimal preparations of crude total tissue mRNA, which may contain thousands of different mRNA species. The reason for this is not clear. Even expression of some cloned species of cDNA, may only result in very small signals. For example, of the three different sodium channel α-subunits that have been cloned from rat brain, only type II [11, 163] and type III [225] are efficiently expressed in the oocyte and result in substantial ion currents, whereas less than 1% of that current was observed after expression of the type I sodium channel [163]. In this case additional subunits may be needed for efficient expression.

Copy numbers of newly expressed proteins exceeding 10^{10} in a single oocyte have recently been reported for the case of the cloned intestinal sodium-glucose cotransporter [242]. But, usually, not more than 5×10^8 membrane protein molecules are expressed, after injection with pure nucleic acids. If injected with crude mRNA, a maximum of 10^7 copies per cell is usually not exceeded. Hence, these numbers restrict structural and functional detection.

Structural confirmation of expression of new membrane proteins has usually relied on identification of biosynthetically labeled proteins, either by immunoprecipitation [e.g., 35] or after affinity purification [e.g., 216]. IgE-receptors have been visualized by binding of IgE-coated, fluorescent microspheres [127]. Evidence for the presence of newly expressed proteins has also been obtained by measuring specific binding of a ligand of high specific radioactivity (e.g., 216).

Functional detection of newly expressed receptors coupled to the production of second messengers has the advantage in that the signal is amplified, but

relies on the presence in the oocyte, either by co-expression or by endogenous availability, of the signalling chain distal to the receptor. Thus receptors expressed from cDNA coding for human adrenergic $\beta 1$ and $\beta 2$ receptors interacting with endogenous G_s-proteins have been functionally detected by measuring agonist-induced adenylate cyclase activation [54, 109]. The oocyte has proven particularly suitable for the detection of newly expressed receptors that activate the IP_3/calcium second messenger pathway, making use of an amplified reporting system, endogenous to the oocyte. All these newly expressed receptors (Table 1) elicit upon activation, after a lag time of several seconds, an oscillatory current response, similar to that of the endogenous muscarinic acetylcholine receptor (reviewed in detail in [40]). Comparable responses are obtained after microinjection of calcium [41, 150] or IP_3 [168] into the oocyte. The responses are inhibited by previous injection of EGTA into the cells [41, 173]. Thus, all available evidence is in line with a signalling chain: receptor—G-protein—phospholipase C—IP_3—calcium-release from endogenous compartments. The released calcium activates endogenous chloride currents [15, 150], which can be monitored electrophysiologically. Changes in cytosolic calcium concentrations have also been measured as fura-2 signals [228], as light emission from aequorin-injected cells [192], or as increased radioactive calcium efflux [254]. Ligand-induced IP_3 production has been measured directly from small pools of oocytes [142, 167]. All functions in the signalling chain distal to the receptor also occur endogenously in the oocyte, as activation of receptors, expressed after injection with a cDNA-derived, pure mRNA coding for the substance K receptor [139], or the serotonin 1c receptor [97], is sufficient to generate ligand-induced chloride currents. Use of this reporting system has led to the discovery of a new brain receptor acting via the IP_3/calcium pathway. Expression of whole rat brain mRNA has shown that quisqualate cannot only open a subtype of glutamate receptor channels, but also activates a receptor interacting with the above signalling chain [173, 214, 215].

Newly expressed ion channel currents can be recorded using the conventional whole cell voltage-clamp technique or at the single channel level by the patch-clamp technique [145]. The first method is particularly sensitive. It may be estimated that about 5,000 expressed ion channels are sufficient to create a significant current signal, assuming a single channel current amplitude of 1 pA, and an open probability close to 1.0. In contrast, single channel recording requires a much higher level of expression. This is due to the necessity of finding single channel molecules, using a pipette with a tip opening area of about 4 μm^2 on an oocyte surface measuring about 2×10^7 μm^2 [145]. Therefore, assuming random distribution in the surface membrane, patch clamping may be applied conveniently only to oocytes expressing at least 10^6 channels. Alternatively, ion fluxes through channels have been measured using flame emission spectroscopy [6], or by radioactive tracer flux [33].

Radioactive tracer flux studies have been undertaken in order to probe newly expressed membrane transport proteins (*see* Table 1). The presence of electrogenic carriers in the surface membrane of the oocyte has been confirmed by electrophysical techniques, either measuring substrate-induced ion currents [242] or membrane potential changes [8].

Applications of the Expression System

Expression of Crude, Tissue-Derived mRNA

Crude mRNA-induced expression has opened a valuable approach to the study of the types of questions reviewed in this section. In addition, many studies on the biogenesis, role of post-translational modifications, targeting and regulation of membrane proteins (reviewed in later sections) have been performed using crude mRNA expressed in a simple, foreign environment, where many experimental factors can be better controlled than *in situ*.

Functional Expression Yields Information on Size and Abundance of the Coding mRNA

If a functional protein can be detected after injection of crude, total mRNA isolated from a tissue, the mRNA may then be fractionated using sucrose density gradient centrifugation or agarose gel electrophoresis, to obtain mRNA of defined size [e.g., 80, 220, 221]. Size-selected subfractions represent the starting point for expression cloning (*see below*). It should be borne in mind that this holds true only for single subunit proteins or proteins whose subunits are coded for by mRNAs of very similar size. In the worst case, a size fractionation of mRNA could result in the complete loss of expression of the function, if a small molecular weight mRNA is essential to complement a large component mRNA during functional expression. Consequently, after separation steps of low resolution, as sucrose density gradient centrifugation, a peak broadening for multi-subunit proteins is observed, compared to single subunit proteins.

Size fractionation cannot only help to establish the molecular size of the mRNA coding for a defined

functional protein, but has also been used to obtain evidence for the existence of multiple isoform mRNAs coding for such a function. Thus, expression of crude, size fractionated mRNA from neonatal and adult rat spinal cord, and cerebral cortex, indicated existence of two different forms of the glycine receptor, that differ in their functional properties [1]. While the adult cerebral cortex form was found to be predominantly coded by 18S mRNA, a very high molecular weight fraction was found to code for most of the adult spinal cord receptor [1]. Similar observations were made for the rat brain A-type potassium channel [188]. Information on the role of the small molecular weight component(s) known to be tightly associated with the large α-subunit of the voltage-dependent sodium channel [31] has been obtained from fractionation experiments. Sumikawa et al. [22] found that large molecular weight mRNA was sufficient to drive synthesis of voltage-dependent sodium channels. However, size fractionation of the mRNA of rat and rabbit brain and subsequent injection of a high molecular weight fraction into oocytes resulted in the expression of a sodium current with threefold slower inactivation than that observed after injection with total mRNA [111]. This fast inactivation could be restored by co-injection of a small molecular weight fraction mRNA [111]. Similar findings were made when the cloned rat brain α-subunit was supplemented with crude small molecular weight mRNA [11]. These observations are further discussed in a later section.

The size of the functional signal obtained by mRNA-induced expression is dependent on the amount of specific mRNA injected. This has been used to quantify relative amounts of mRNA in different tissues coding for this function. Thus, information on the ontogeny in rat brain [30] and on the distribution in mouse brain [9] of the ligand-gated GABA and glycine channels has been obtained. Changes in mRNA levels coding for the nicotinic acetylcholine receptor and for the sodium channel have been found after denervation of cat skeletal muscle [176]. Quantitation of mRNA also helped to elucidate the mechanism of homologous desensitization of the receptor for thyrotropin-releasing hormone [171]. Exposure of GH3 cells to the agonist led to a rapid decrease in mRNA coding for the receptor, as shown by quantitation of expression in oocytes after isolation of mRNA from untreated and hormone-treated cells [171]. Similarly, it has been shown that the mRNA coding for rat myometrium potassium channel is induced by estrogen treatment and regulated throughout the estrous cycle and during pregnancy [25]. The use of functional expression for the quantitation of a given mRNA species may be useful in cases where the cDNA is not yet available. But this approach suffers from some shortcomings: isolation of mRNA, even from aliquots of the same tissue, may be variable in quality, protein isoforms cannot be differentiated, and the cellular origin of the mRNA is not known. At least for those membrane proteins that have been cloned, *in situ* hybridization and, to some extent, Northern blot analysis promise much higher specificity and resolution for the quantitation and localization of specific mRNA.

Table 3. Plasma membrane proteins cloned with the help of the functional assay in the *Xenopus* oocyte (functional expression cloning)

Membrane protein	References
Na^+-glucose cotransporter	[81]
Serotonin receptor ($5HT_{1c}$)	[97]
Substance K receptor	[139]
Substance P receptor	[258]
K^+ channel (kidney)	[230]
K^+ channel (brain delayed rectifier)	[53]
Kainate receptor channel	[87]

Functional Expression Cloning Using a Direct Approach

Many membrane proteins, especially receptors and ion channels in the central nervous system, only represent a tiny fraction of the total membrane protein. This makes isolation by biochemical methods and, therefore, a conventional cloning approach difficult. The latter depends on the availability of primary protein sequence information or of highly specific antibodies. Cloning with the help of a functional assay in the *Xenopus* oocyte was pioneered by Noma et al. [166]. Supernatant of crude mRNA-injected oocytes was used for the detection in a bioassay of the activity of newly synthesized and secreted IgG1 induction factor. This cloning approach is now called functional expression cloning and has been rapidly applied to the cloning of several plasma membrane proteins (Table 3). The strategy of functional expression cloning is illustrated in Fig. 2. Functional expression obtained with total mRNA from a given source confirms the presence of mRNA coding for the protein in question. mRNA can then be fractionated according to size, the active fraction identified, and, after reverse transcription, used for the generation of a cDNA library. This library is then transcribed into polyadenylated mRNA, capped and expressed in the oocyte. Subsequent appearance of

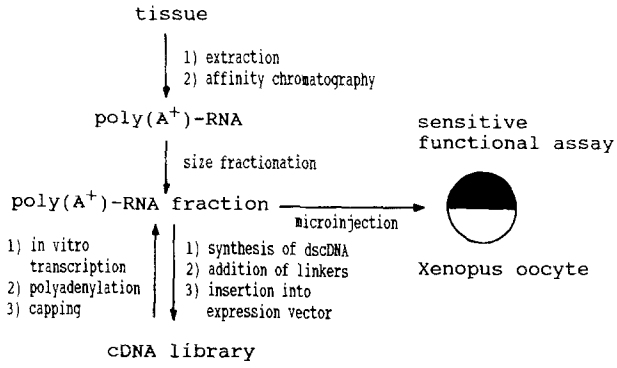

Fig. 2. Scheme illustrating functional expression cloning. For further explanation *see* text. Once the cDNA library results in a positive response in the functional assay, the clone is identified by progressive subdivision of the library. *dscDNA:* double stranded cDNA

the function in the oocyte confirms the presence of a full length clone in the library. The clone coding for the function may then be identified by progressive subdivision of the library.

Several technical features have been exploited in order to enhance the probability of functional detection of a signal from cDNA library derived mRNA. For the expression cloning of the sodium-glucose transporter the total tissue mRNA was size fractionated using a preparative electrophoresis technique [80], resulting in a considerably enriched fraction of mRNA, which was used as starting material for the generation of the library [81]. Unidirectional cloning was used in conjunction with sensitive, direct electrophysiological detection in the case of the rat brain delayed rectifier potassium channel [53], and the kainate receptor channel [87]. Several groups [97, 133, 139, 258] made use of an amplified signal in the form of an ionic current that is provided by the oocyte for functional detection of receptors linked to IP_3 production (*see above*).

Direct functional expression cloning has the advantage over classical strategies in that the danger of sequencing "false positive" clones, due to cross-reactivity of antibodies or nucleotide probes, can be avoided. Furthermore, full-length clones will directly be obtained. However, it should be pointed out that functional expression cloning, using the direct, straightforward approach, is restricted to the cloning of single subunit proteins. As discussed above, multisubunit proteins may not be expressed in a functional form, after fractionation of the nucleic acids coding for the different subunits. Unless single subunits of a multisubunit protein produce an assayable function, more complex strategies have to be used for functional expression cloning of these proteins.

Functional Expression Cloning Using an Indirect Approach

The indirect approach to functional expression cloning makes use of antisense nucleotide inhibition of expression (*see below*). This strategy has first been chosen by Lübbert et al. [133] in an attempt to clone the $5HT_{1c}$ receptor. Size-selected mouse brain mRNA, capable of directing synthesis of this receptor in the oocyte, was used to generate an antisense cDNA library by directional cloning. Single-strand antisense cDNA of portions of the library were then hybridized with the starting mRNA and separated on a sucrose density gradient, and the unhybridized mRNA was screened for a depletion in the ability to direct expression of the receptor in the oocyte, indicating the presence of antisense cDNA in the corresponding portion of the library. It was also shown that the mRNA isolated from the positive hybrid portion was able to direct functional expression of the receptor. The drawback of this indirect approach is illustrated by the fact that these attempts resulted in the isolation of a partial length clone only [133], whereas the direct positive approach to the same receptor led to the isolation of a full-length clone coding for this single subunit protein [97]. However, for multi-subunit proteins and for single subunit proteins coded for by mRNA longer than about 6 kB, for which generation of a full length cDNA library is difficult, this may be the strategy of choice.

Functional expression cloning has provided an unexpected approach that holds a lot of promise, but only the future will tell how generally this approach can be applied to the cloning of those proteins that at present cannot be cloned using conventional techniques.

Oocyte Expression Can Be Inhibited by Antisense Nucleotides

Translation of mRNA in *Xenopus* oocytes has been shown to be inhibited by complementary, antisense RNA [79, 143] or DNA [102]. Prehybridization of crude mRNA with antisense RNA or cDNA against part of the coding mRNA, prior to injection into the oocyte is sufficient to inhibit expression. This has been documented by many groups. For instance, it has been shown that an oligonucleotide complementary to the coding sequence for amino acid residues

4–10 of the α-subunit of the nicotinic acetylcholine receptor channel is sufficient to suppress expression of this multi-subunit protein complex [217]. Antisense RNA directed against either of the four different subunits inhibited functional expression [217]. Antisense oligodeoxynucleotides of 60 to 80 bases length induced arrest of translation of sodium channels [132]. Similar experiments have been used to show the close relationship of the dihydropyridine receptor cloned from rat skeletal muscle, which cannot be functionally expressed in the oocyte [231], with a dihydropyridine-sensitive heart calcium channel. It has been demonstrated that mRNA-induced expression of rabbit and rat heart calcium channels, but not dihydropyridine-insensitive calcium channels, may be suppressed by complementary oligonucleotide probes to cDNA sequences coding for the rat skeletal muscle dihydropyridine receptor [131]. Antisense oligodeoxynucleotides directed against the mRNA coding for the cloned strychnine binding subunit of the glycine receptor [194] inhibit expression of glycine receptor channels by adult but not by neonatal spinal cord mRNA [2], confirming earlier observations of a heterogeneity of glycine receptor channels [1].

Limitations of the Approach

In addition to the problems inherent to oocyte expression experiments, expression of total mRNA isolated from a tissue of choice has additional limitations:

—A membrane protein of interest may not be expressed in functionally detectable amounts, perhaps due to low abundance of the specific mRNA(s) in the source tissue. Often the relative extent of expression of two proteins in the native tissue is comparable, but only functional expression of one of them may be achieved. The reason for this is far from clear. Stability of the mRNA during isolation, storage and after injection into the oocyte cytoplasm, and translational efficiency may differ for the respective mRNAs. Some polypeptides may be improperly modified, folded or assembled, and therefore not reach the plasma membrane.

—When functional expression is obtained, it is often not clear what the cellular origin of the protein is, because every tissue is made up of a number of different cell types.

—Several newly expressed proteins may interact in a way that is not typical for the native tissue, because the proteins may originate from different cells or are localized differently in the same cell type.

Some of these shortcomings can be circumvented when pure mRNA coding for a defined protein is available.

Expression of cDNA or cDNA Derived mRNA

Identity of cDNA Clones Can Be Confirmed by Expressing the Encoded Function

Conventional cloning using antibodies or partial primary sequence information of the protein to be cloned is subject to many pitfalls that result in "wrong clones." Confirmation of the identity of the coded protein, if possible with a functional assay, is therefore a primary goal after establishing a cDNA sequence. The *Xenopus* oocyte has served as a test tube for a large number of cloned plasma membrane proteins (*see* Table 2). In most cases the cDNA was transcribed in vitro, the RNA capped, poly(A^+)-tailed and microinjected into the cytoplasm of the oocyte. Ballivet et al. [14] used instead direct nuclear injection of cDNAs coding for the chick neuronal acetylcholine receptor subunits. cDNAs were linked to a heat shock promotor and the oocytes exposed to high temperature after nuclear injection, in order to initiate transcription. This procedure circumvents in vitro preparation of RNA. Ymer et al. [257] used cytomegalovirus promoter, thereby omitting the need to expose the oocytes to elevated temperature.

The minimal structure needed to carry out a partial function can only be investigated in functional expression experiments. This is illustrated here for the voltage-dependent sodium channel for which biochemical work has demonstrated tight interaction of the large molecular weight rat brain α-subunit with one or two smaller subunits [31] although the functional role of these additional subunits remains unclear. Functional expression of the cloned α-subunit of type II rat brain sodium channel, and subsequent functional characterization [163, 210, 211] indicates that biosynthesis and targeting to the surface membrane are successfully carried out by the oocyte. Furthermore, membrane potential-dependent gating of the induced channel differs only slightly in the steady-state inactivation from previous measurements on rat peripheral nerve and rat twitch muscle [211]. The conclusion that expression of the α-subunit is sufficient to form a voltage-activated channel was also reached by hybridization selection of specific mRNA from crude rat brain mRNA, using a partial length cDNA clone selected by immunoscreening from a rat brain cDNA library [63]. However, time-dependent inactivation of the current expressed from the cDNA coding for a rat brain type IIa α-subunit, which differs from the type

II α-subunit in only six amino acid positions, is threefold slower than observed after expression of total rat brain mRNA [11]. Normal inactivation behavior could be restored by co-injection of crude, small molecular weight mRNA from rat brain [11]. This confirms observations discussed in a previous section, obtained with mRNA fractionation experiments. However, it is not clear from both of these experiments, whether the small molecular weight fraction contributes really a protein subunit essential for the functional expression of the native kinetic properties of the channel, or whether it codes for a factor necessary for the proper biosynthesis of the sodium channel. In the case of the rabbit heart dihydropyridine-sensitive calcium channel there is also evidence that the α1-subunit alone is sufficient for the expression of dihydropyridine-sensitive barium currents through calcium channels in the oocyte [148]. Biochemical evidence had previously indicated the presence of an additional large and several small subunits [31]. Co-expression of the heart α1-subunit with the α2-subunit cloned from rabbit skeletal muscle led to a threefold enhancement of the ion current detectable in the oocyte surface membrane [148]; however, the mechanism of this stimulation of expression is not clear. The oocytes also display a small endogenous current component [43], and it is difficult to exclude the possibility that endogenous peptides associate with the newly expressed large subunits. It is hoped that in the future, after cloning of the DNA coding for the small subunits of the sodium and calcium channels and co-expression with the large ones, the role of the small subunits will be clarified.

Expression experiments of the cloned cDNAs coding for the bovine skeletal muscle nicotinic acetylcholine receptor have led to the clarification of a long-standing problem in developmental biology. It has been known for some time that embryonic muscle contains a type of nicotinic channel that differs in single channel kinetics and amplitude from the adult form. After cloning of different isoforms of the subunits coding for this channel, expression of different combinations, namely of $\alpha\beta\gamma\delta$ and $\alpha\beta\delta\varepsilon$, resulted in the functional expression of channels with single channel properties reminiscent of adult and embryonic forms of the channel [155]. *In situ* hybridization confirmed that the switch from the embryonic (extrajunctional) to adult (junctional) form is most likely due to a switch from the γ-subunit to the ε-subunit. Thus, change in a single subunit of a pentameric channel composed of four different subunits is sufficient to create an altered function.

It should be borne in mind that functional expression does not necessarily give information on the subunit composition of a functional membrane protein *in situ*. This is illustrated here for the case of the cloned $GABA_A$ receptor, of which many subunit isoforms have been cloned so far. However, it is not known what subunit isoforms must be combined in order to obtain a functional channel exhibiting properties as observed in cultured neurones and faithfully expressed in the *Xenopus* oocytes after injection with crude brain mRNA (*see* Table 1). Single cloned subunit isoforms express very little [21, 203] current, with the exception of the rat β1-subunit [203]. The latter results in the formation of an anion-selective conductance, which is blocked by picrotoxin, a well-known GABA channel blocker. However, this channel is not gated by GABA, but opens spontaneously in its absence [203]. Formation of such an abnormal, nongated channel is suppressed by co-expression of an α-subunit isoform. Combinations of α- together with β-isoforms express large GABA-gated chloride currents [125, 126, 128, 195], but the Hill coefficient for GABA gating indicates, that only one, as compared to normally two, GABA molecules are needed to open the channel [125, 126]. Furthermore, in these cloned and expressed GABA channels the barbiturate pentobarbital is able to elicit current amplitudes similar to those of GABA alone [137; E. Sigel, P. Malherbe, and H. Möhler, *in preparation*). This is in contrast to the channel properties obtained from crude brain mRNA, where only a small pentobarbital current is observed [175]. Combined expression in 293-cells of the human α1-, β1- with a γ2-subunit restores the effects of allosteric modulatory drugs that act at the benzodiazepine binding site [184], which cannot be observed in currents expressed with combinations of α- and β-subunits. In the case of the cloned rat subunits expressed in oocytes, co-expression of the γ2-subunit only partially restores the benzodiazepine effects [138] and for a full recovery replacement of β1- by the β2-subunit is essential (E.S., P.M., H.M., *in preparation*). Furthermore, no α-subunit is needed for a full benzodiazepine responsiveness (E.S., P.M., H.M., *in preparation*). However, the above α-β-γ-subunit combinations also result in channels that show a Hill coefficient of one for the GABA gating. In summary, expression experiments need to be combined with additional methodological approaches, to get information on the true *in situ* composition of the various isoforms of the GABA receptor channel. Nevertheless, expression of subunit isoform combinations help to establish minimal structural requirements for a given functional property.

It is often possible in the oocyte expression system to study functions that are much more complex than those encoded by the introduced, cDNA-derived genetic information. The newly expressed pro-

tein may interact in a meaningful way with components endogenous to the oocyte. The case of receptors activating the IP_3 cascade has been discussed in detail in earlier sections. Functional coupling of newly expressed adrenergic receptors to adenylate cyclase endogenous to the oocyte has also been shown [109]. Interestingly, the expressed human β2-adrenergic receptor retains agonist-induced desensitization [109]. Newly expressed receptors may not only interact with oocyte G-proteins, but also with the machinery controlling receptor internalization. For example, when cloned human LDL-receptor is expressed in oocytes it is rapidly internalized following exposure to the ligand [180].

Structure-Function Relationships Can Be Studied by Expression of Species Hybrids

The most simple form of genetic manipulation that does not require cDNA alteration is the expression of species hybrid forms of multi-subunit proteins. This approach has been used to show that the δ-subunit from mouse BC3H-1 cell line could replace the δ-subunit, but not any of the other subunits of the nicotinic acetylcholine receptor from *Torpedo californica* electroplax [253]. Later all 16 possible species hybrids have been expressed and investigated for efficiency of expression and voltage dependence [140, 260]. This approach is particularly attractive when two species express multi-subunit proteins that differ in their functional properties. In this case it is possible to identify the subunit that confers this difference to the protein complex. Thus, *Torpedo* and bovine acetylcholine receptors display different single channel kinetics, the opening times being short in the former and long in the latter. Expression of different hybrid forms of this four subunit protein resulted in the assembly of functional ion channels and showed that the bovine δ-subunit is sufficient to confer the slow kinetics to the *Torpedo* receptor channel [191]. The cat receptor shows slower desensitization of the current response to acetylcholine than the *Torpedo* receptor [219]. Co-expression of crude cat muscle mRNA with pure cDNA derived mRNA coding for individual subunits of the *Torpedo* receptor identified the γ-subunit as sufficient to confer rapid desensitization to the cat receptor [219].

Structure-Function Relationships Can Be Studied Using in Vitro Mutagenesis

The use of *in vitro* mutagenesis in combination with functional expression has contributed to the understanding of the functional architecture of membrane proteins. Hybrid formation between different domains of homologous proteins (chimeric polypeptides) permits the identification of important functional features within a single polypeptide. Single point mutations of interesting residues lead to the understanding of protein function at an even higher resolution. Studies of altered protein structures are evidently limited to the observation of those mutant proteins whose biosynthesis and surface membrane insertion remains unaffected.

Species hybrid experiments with the multisubunit protein acetylcholine receptor were carried out with the aim to identify the subunit that confers the smaller channel conductance (as observed for the bovine channel in an extracellular medium having a low divalent cation concentration) to the *Torpedo* channel that displays a larger conductance under these conditions [92]. This work led to the conclusion that the bovine δ-subunit is sufficient to produce the smaller conductance [92]. In an attempt to further pinpoint the responsible structure, the experiments were then extended to the study of chimeric δ-subunits. This led to the finding that a short amino acid sequence, including the putative transmembrane segment M2 and short adjacent portions, of the bovine δ-subunit are sufficient to produce the smaller channel conductance [92]. Single amino acid substitution of negatively charged residues and glutamine residues in the vicinity of transmembrane segment M2 on all subunits forming the *Torpedo* receptor have led to the identification of three putative rings of negative charges that determine the single channel conductance [91]. Independent evidence, supporting a functional allocation of the M2 portion as a part of the ion channel pore, has been obtained [122]. Replacement of serine residues within the M2 region by alanine on the α-, or δ-subunits led to a decrease in the single channel outward current amplitude and to a decrease in the residence time of an open channel blocker [122]. Deletions and single point mutations of the cDNA coding for the α-subunit have been investigated in order to determine effects on the expression of the mutant receptors in the surface membrane, by measuring bungarotoxin binding, and effects on functional properties by electrophysiological techniques [156]. Five putative transmembrane sequences have been replaced individually by a transmembrane sequence from vesicular stomatitis virus glycoprotein, showing that only the transmembrane sequence M4 may be replaced without impairing functional expression [236]. Replacing cysteins 416 and 420 of the γ-subunit by phenylalanines resulted in little alteration of channel expression and channel activity, arguing against the presence of the amphipathic helix in a tightly paced transmembrane domain [182].

Very elegant work on the voltage-dependent so-

dium channel contributed to our knowledge on the voltage-dependent gating of ion channels. Single amino acid substitutions and subsequent functional characterizations of the mutants led to the identification of positive charges in the putative transmembrane segment S4, which are involved in channel activation and of a putative cytoplasmic portion of the protein which is important for channel inactivation [210].

In the *Drosophila* shaker mutant A-type potassium channel, replacement of glutamate 422 by lysine lead to a decrease in the affinity of the potassium channel for the channel outer-mouth ligand, charybdotoxin [136]. Careful interpretation of these findings helps to assign the topology of the channel protein in the membrane.

The Na,K-ATPase of rat kidney, which displays low affinity for cardiac glycosides, may be converted to a high affinity form, upon replacing the first two transmembrane segments of the α-subunit with the ones of the *Torpedo* ATPase, which in the intact form displays high binding activity for the same drugs [165].

Chimeric proteins made of human adrenergic β1- and β2-receptors, that are both known to couple to the same effector systems *in situ,* have been engineered and functionally expressed [55]. In these chimeric proteins, the nature of the binding site was established with binding studies using subtype-specific antagonist displacement of an iodinated ligand. This approach led to the identification of the transmembrane helices responsible for subtype-specificity [55]. The effect of truncations and insertions of the human β2-adrenergic receptor on extent of expression, ligand binding properties, ligand-induced activation of adenylate cyclase endogenous to the *Xenopus* oocyte, and homologous desensitization have also been described [109].

Type I and type II muscarinic acetylcholine receptors, which correspond to the pharmacologically defined M1 and M2 receptors, couple *in situ* to phosphoinositide formation and adenylate cyclase inhibition, respectively [56, 115]. Characterization of type I/type II hybrids led to the identification of a putative cytoplasmic region, between the proposed transmembrane sequences V and VI, involved in the selective coupling to the respective effector systems [114].

The rate of internalization of the human LDL-receptor expressed in the oocyte is greatly reduced after replacing tyrosine-807 by a cysteine, a mutation known to slow down incorporation of the receptor into coated pits in mammalian cells [180]. Thus, signals governing receptor-mediated endocytosis in the oocyte are similar.

This discussion is neither complete, nor is it thought to imply that the *Xenopus* oocyte is the only expression system, in which such structure function studies can be performed. Many similar experiments have been carried out using transfected cells. Alternative techniques have also helped to identify functionally important structural features and some of the above mutagenesis studies only helped to confirm previously reached conclusions. An approach using in vitro mutagenesis has clearly a great potential for contributing to our understanding of the functional architecture of membrane proteins. However, experience made with bacterial transport proteins, e.g. the light-driven proton pump, bacteriorhodopsin [104], makes clear that the use of a whole range of complementary experimental approaches is important. As long as there are no standard procedures available for the crystallization of membrane proteins, such combined strategies will have to be used to approach a clearer understanding of the molecular mechanisms involved in membrane protein function.

Biosynthesis, Functional Properties and Modulation of Membrane Proteins Can Be Studied in a Foreign Environment

Translation, Assembly, Modification and Sorting

The oocyte expression system has been used to study all aspects of the biosynthesis of membrane proteins. Insertion and folding in the endoplasmic reticulum of single polypeptide chains has been investigated, using engineered hybrids to probe the effects of topogenic sequences [187]. Injection into the oocyte of a cDNA derived RNA, coding for the β-subunit of the Na,K-ATPase from *Xenopus* kidney A6 cells, has been shown to result in the enhanced conversion of the α-subunit of the oocyte Na,K-ATPase from a trypsin-sensitive form to trypsin-insensitive form, resulting in an enhanced expression of the protein in the plasma membrane [58]. The effect of variations in the stoichiometry of the mRNAs coding for individual subunits of the *Torpedo* nicotinic acetylcholine receptor on functional channel formation in the surface membrane led to the conclusion that the relative abundance of the α-transcript may control the rate of receptor assembly [29]. Assembly of the multi-subunit nicotinic acetylcholine receptor and pathway to the plasma membrane have been probed by immunoprecipitation of biosynthetically labeled subunits of the *Torpedo* receptor from different subcellular fractions [218]. While assembly of incomplete receptors was not greatly affected by the lack of one of the four subunits, the integration into the surface membrane was strongly reduced. Inhibition of N-glycosylation by tunicamycin had the same effect [218]. The impor-

tance of glycosylation for assembly was also tested in another study. The single site for potential N-glycosylation, asparagine 141, in the cloned α-subunit of the *Torpedo* receptor was replaced by aspartic acid [156]. This change resulted in an apparent inability to assemble with the three co-expressed polypeptides that usually form a functional channel and suppressed incorporation of the receptor into the surface membrane, as measured by bungarotoxin binding and a functional assay [156]. The role of glycosylation for functional expression in the surface membrane has also been tested for various other proteins. Inhibition of core glycosylation using tunicamycin strongly inhibited the appearance of functional sodium channels [198, 223] and GABA channels in the surface membrane, while little effect on the appearance of calcium and potassium channels and kainate channels was observed [223]. Functional expression of the cloned *T. californica* Na,K-ATPase was also not affected by tunicamycin [229]. Appearance in the surface membrane of the cloned human LDL-receptor remained unaffected by deletion of the region that normally undergoes O-linked glycosylation [180]. Preventing glycosylation has different effects on functional expression in the oocyte, depending on the individual protein.

Newly expressed membrane proteins are inserted into the oocyte plasma membrane quantitatively in the correct orientation. This was shown by external application to, and micro-injection of GABA and glycine into, brain mRNA-injected oocytes. While the neurotransmitters failed to open channels from the inside, large currents were observed upon application to the outside [174].

The oocyte is a highly asymmetric cell. Therefore, it is not surprising that an early report mentioned an apparently asymmetric localization of newly expressed cat nicotinic acetylcholine receptor channels. Pressure application of agonist elicited larger currents at the vegetal pole than at the animal pole [153]. Furthermore, it has been claimed that the muscarinic acetylcholine and thyrotropin-releasing hormone receptors, that are linked to the production of the second messenger IP_3, are expressed at higher density on the animal pole than at the vegetal pole [169]. These experiments were carried out using pressure application of agonist onto the surface of the oocyte. The latter results should, however, be interpreted with caution. The receptor in these cases is linked through a series of intermediate events to a reporting system (*see above*). Nonrandom distribution of any of the components involved in signal transmission, distal to the receptor, would conceivably result in a locally different response upon application of agonist. It has indeed been shown that injection of IP_3 at the animal pole elicits much larger currents than at the vegetal pole [19, 135], whereas conflicting reports have been published on an asymmetry of the response to injection of calcium [135, 150]. Dreyfus et al. [44] studied the distribution of a membrane-associated butyrylcholinesterase after injection with cDNA-derived mRNA. Sectioning and labeling, using immunofluorescent techniques, indicated that the protein was expressed at about a twofold higher density at the animal pole than at the vegetal pole. Co-injection of total mRNA from brain or skeletal muscle, but not liver, promoted formation of enzyme aggregates [44]. In this case it is not known whether the enzyme is formed as an integral membrane protein or whether it is anchored in the membrane via a glycolipid. In recent experiments, we used localized superfusion of the oocyte with either agonists, to detect the distribution of several ligand-gated ion channels, or channel blockers, to detect voltage-gated ion channels. During the initial phase of mRNA-induced expression, channels were found to be almost exclusively inserted into the animal hemisphere, while at later stages of expression the relative density of expression was found to be threefold higher in the animal pole area than in the vegetal pole area (A.B. Peter, H. Reuter and E. Sigel, *in preparation*). Using drugs that interfere with the function of microtubules (cholchicin) or actin filaments (cytochalasin D), we have found that these cytoskeletal structures are essential for a nonrandom delivery of the ion channels to the surface membrane of the oocytes. Neither of the two anti-cytoskeletal drugs strongly interfered with the amount of active channels expressed, but led to random insertion into the surface membrane (E. Sigel, V. Niggli and A.B. Peter, *in preparation*).

Functional Properties

Determination of some functional properties of membrane proteins *in situ* may be difficult, and more accessible to experimentation, after "transplantation" into the oocyte. This is exemplified in the investigation of the voltage dependence of ion carrier proteins. In the oocyte, the membrane potential can easily be set at a defined level using the two-electrode voltage-clamp technique, and carrier mediated fluxes have been measured simultaneously in single oocytes [119]. Thus, voltage dependence of the *T. californica* electroplax Na-K-ATPase [196] and of the electroneutral transport mediated by the anion exchange protein (band III) from mouse erythrocytes [64] have been determined. Voltage dependence of electrogenic carrier-mediated current has been directly determined in the case of the intestinal sodium-glucose cotransporter [242].

Modulation by Drugs

The study of drug actions on membrane proteins is sometimes difficult *in situ*. It can be difficult to control membrane potential and agonist and drug concentrations precisely and at the same time. This is especially the case of the study of neuronal membrane proteins. For example, tests to check the action of well-characterized inhibitors to confirm expression of a defined protein is a matter of routine. But beyond these simple tests, oocyte expression has been used to quantitatively describe drug actions to a detail difficult to achieve *in situ* or in relevant model systems, such as cell cultures. Thus, the action of the barbiturate pentobarbital [175], the channel blocker *t*-butylbicyclophosphorothionate [243], the anthelminthic avermectin B_{1a} [200], the tremorigen aflatrem (255), and allosteric, modulatory compounds, that act at the benzodiazepine binding site [201], on the GABA-gated chloride current have been described in detail. As far as data on the *in situ* action of these drugs are available, the oocyte faithfully reproduces these properties. A detailed study at the single channel level, of the action of the alkaloid veratridine on the voltage-dependent sodium channel expressed in the oocyte [199], led to similar conclusions as did later work performed in cell culture [17]. The action of diphenylhydantoin on the human fetal brain sodium channel has also been investigated [238]. The action of dihydropyridines and ω-conotoxin have been used for the characterization of newly expressed calcium channels from rat heart [43], rat myometrium [52], and *Torpedo* electric lobe [240], respectively. Calcium channels expressed from rat brain respond to neither of the two inhibitors [121]. Interestingly, pentobarbital has also been found to inhibit expressed human brain calcium channels [72]. Moreover, extensive pharmacological characterization has been carried out with newly expressed glutamate channels, with the aim to differentiate and characterize NMDA (N-methyl-D-apartate), quisqualate and kainate-activated channels (*see* Table 1) and the quisqualate receptor acting via IP_3 [214, 215]. The $5HT_{1c}$ receptor has been identified as a putative site of action of the antidepressant drug imipramine [237]. Forskolin, often used as a specific activator of the adenylate cyclase, has been shown to alter the gating of the *Torpedo* nicotinic actylcholine receptor by a direct mechanism [252].

The characterization of human membrane proteins with respect to details of drug action and modulation by post-translational modifications is of high medical interest. Human fetal mRNA has been shown to result in the expression of functional ligand- and voltage-gated ion channels [69]. Besides the study by Tomaselli et al. [238], who investigated the action of the drug diphenylhydantoin on human sodium channels, and Gundersen et al. [72], who have described pentobarbital action on brain calcium channels, little use has so far been made of this potential, except for those human proteins that have already been cloned.

In summary, the *Xenopus* oocyte system is often useful to determine drug action and has a potential as a screening system for new drugs. Functional characterization of structurally defined entities also leads to a new classification of receptor and channel subtypes at a much higher level than with conventional techniques. Agonists, antagonists, modulators and identity of the second messenger produced may be studied unperturbed by the presence of other protein isoforms.

Modulation by Second Messengers

Protein kinase C activators inhibit the signalling chain leading to IP_3 release in rat brain mRNA-injected oocytes [93, 101, 157]. Phorbol ester stereospecifically [93, 157] inhibits the chloride current signal elicited by acetylcholine (muscarinic response), serotonin and quisqualate. While currents induced by IP_3 injection into the cytoplasm were stimulated by the same treatment, the current responses elicited by calcium or the G-protein activator GTP-γS, were unaffected [101]. These findings suggest a site of action of protein kinase C, either proximal to or at the G-protein. Injection of cAMP and cGMP led also to inhibition of the chloride current responses [93].

Differential regulation of calcium currents, expressed from mRNA obtained from different tissues, has been observed. While the slowly inactivating channel expressed after injection with rat heart mRNA is stimulated by activators of protein kinase A [43], the channel expressed from brain mRNA responds to activation of protein kinase C [121, 202]. cAMP injection has also been found to enhance a sustained, ω-conotoxin sensitive current component expressed from rat brain mRNA [100].

Cross-talk between several newly expressed chick neuronal membrane proteins has been demonstrated [202]. Activation of the mRNA-induced quisqualate receptor has been shown to stimulate the activity of the barium current through calcium channels and to inhibit sodium currents and the GABA-gated chloride current, all co-expressed in the same oocyte. Experiments with activators of protein kinase C, diacylglycerol and phorbol esters indicate that cross-talk occurs via activation of protein kinase C [202]. Support for a modulation of sodium currents was provided by injection into the oocyte of nonhy-

drolyzible analogs of GTP, known to activate G-proteins. In such experiments the sodium current expressed from rat brain was similarly inhibited [33]. These observations made in the foreign expression system provided the first functional evidence for the modulation of sodium channels and GABA-gated channels by G-protein-mediated signalling pathways.

Inter- and Intraspecies Comparison in a Standard Environment

Comparison of the properties of membrane proteins fulfilling the same functions in the same species in different tissues, or at different times of ontogeny in the same tissue, and comparison between the same tissues in different species, is generally difficult. This is due to the different requirements of different cell types for maintaining their functions. Also, the membrane composition and the regulatory state of the cells to compare may be very different. The oocyte expression system enables comparison of membrane proteins under standardized conditions, inserted into a membrane of identical composition, in cells with the same regulatory state. Due to the possible variability of oocytes obtained from different donor animals (*see above*), such comparisons should, however, be carried out directly on the same batch of oocytes.

Species differences, known from measurements in the native tissues, were evident after expression of cloned bovine and *Torpedo* nicotinic acetylcholine receptors [191]. As mentioned above, these channels differ in their kinetic behavior and conductance at the single channel level. In addition, embryonic (extrajunctional) and adult (junctional) forms of the nicotinic acetylcholine receptor have been known to differ in their single channel properties, and, as detailed above, this is evident after expression of the corresponding subunit combinations [155].

Such a comparison can also be carried out by researchers cut off from the supply of cloned material, or when the protein in question has not been cloned yet, by studying mRNA-induced proteins. For example, two types of glycine current, expressed mainly in rat spinal cord and rat cerebral cortex, respectively, were found to differ in their desensitization properties and sensitivity to glycine [1]. mRNA derived from innervated and denervated cat skeletal muscle induced sodium channels displaying differential sensitivity to the channel blocker tetrodotoxin [176]. Chick muscle mRNA-induced sodium channels have been compared with those induced by chick brain mRNA [198]. Both preparations led to expression of channels with short open times, as recorded by the patch-clamp technique at the single channel level. In addition, oocytes injected with muscle mRNA had a second type of sodium channel characterized by longer open times [198]. The measurements do not necessarily suggest the presence of two different sodium channels in chick skeletal muscle, since regulation of the muscle sodium channel could be different from the brain type, and the fast and slow form could be due to a different regulatory state.

Concluding Remarks

The oocyte of *Xenopus laevis* is the best studied system for the characaterization of all stages of embryogenesis. In recent years the oocyte has taken over a new role as a test tube for the study of the biogenesis, functional architecture and modulation of plasma membrane proteins. Only with the wide use of the oocyte has it been appreciated to what extent the signals governing these processes are conserved throughout evolution. The possibility to perform biochemical experiments on a single or only a few cells is attractive. In a wide number of different approaches the *Xenopus* oocyte will remain a standard tool when functional expression of integral membrane proteins is required.

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Note Added in Proof

Recently, an mRNA coding for an α-subunit of *Xenopus* skeletal muscle nicotinic acetylcholine receptor (AchR) channel has been shown to be expressed in *Xenopus* oocytes [Hartmann, D.S., Claudio, T. 1990. *Nature (London)* **343**:372–375]. Suppression of either transcription of this endogenous mRNA, using actinomycin D, or translation, using antisense RNA to α-subunit of *Xenopus* AchR was shown to suppress ion channel formation induced by foreign mRNAs coding for the *Torpedo* AchR βγδ-subunit combination [Buller, A.L., White, M.M. 1990. *Mol. Pharmacol.* **37**:432–428]. Thus, results obtained in subunit deletion studies [116, 253], carried out to establish the minimal number of *Torpedo* subunits needed to form a functional AchR require reinterpretation.