

Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes

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A complementary DNA encoding a voltage-gated chloride channel from *Torpedo marmorata* electric organ was cloned by expressing hybrid-depleted messenger RNA in *Xenopus* oocytes. The predicted protein has a sequence of 805 amino acids containing several putative membrane-spanning domains. Expression of the protein in *Xenopus* oocytes shows that it is sufficient for channel function.

CHLORIDE channels are present in the plasma membranes of most cells and participate in various cellular functions, such as regulation of cell volume¹, transepithelial transport^{2,3} and stabilization of membrane potential in muscle⁴. There is a large variety of different Cl⁻ channels^{2,5}, and their importance is underscored by diseases such as cystic fibrosis, where mutations in the cystic fibrosis gene product⁶ block the cyclic AMP activation of epithelial Cl⁻ channels (for reviews, see refs 5, 7). Further, in certain forms of myotonia a decrease in plasma membrane Cl⁻ conductance leads to an increase in excitability⁸.

Identification and purification of Cl⁻ channels has been hampered by the absence of high-affinity ligands, with the exception of those binding to postsynaptic glycine receptors and GABA receptors. These ligand-gated Cl⁻ channels, which have been cloned^{9,10}, probably belong to a different class of channels. Recently, putative Cl⁻-channel proteins from kidney and trachea have been partially purified¹¹, but it is unclear which of the main proteins (of relative molecular mass 97K, 64K, 40K and 27K) represents a Cl⁻ channel. Monoclonal antibodies raised against *Necturus* gallbladder cells have been reported to inhibit Cl⁻ conductance¹². One of these antibodies reacts against proteins of *M_r* 219K and 69K, which have been suggested to be components of a Cl⁻ channel.

We used the electric ray *Torpedo* as a model system to isolate a Cl⁻ channel. The electric organ of *Torpedo californica* is an abundant source of a voltage-gated Cl⁻ channel (refs 13, 14; for review see ref. 15). This channel is thought to ensure the high conductance of the non-innervated membrane of the electrocyte necessary for efficient current generation caused by Na⁺ influx through the acetylcholine receptor at the innervated membrane. Previous attempts¹⁶ (including our own¹⁷) to identify the channel protein by using chemicals that block the *Torpedo* channel¹³ and presumably bind covalently to it, have failed because of the low binding specificity of the inhibitors used. In the absence of biochemical data on the protein(s) constituting this channel, we decided to rely only on function by using an expression cloning approach. By contrast to other Cl⁻ channels¹⁸, the channel from *Torpedo* electric organ can be expressed easily in *Xenopus* oocytes^{17,19}. We have now cloned the complete cDNA encoding this channel and show that a single protein subunit of calculated *M_r* ~ 89K, which contains several putative membrane-spanning domains, is sufficient for functional expression in *Xenopus* oocytes.

Size of active mRNA

A Cl⁻ channel from *T. californica* electric organ has been

expressed in *Xenopus* oocytes^{17,19}. We confirmed that a similar Cl⁻ conductance is expressed using RNA from *T. marmorata*, a closely related species. Consistent with the presumed abundance of the channel, small amounts (~5 ng) of total, non-poly(A)⁺ selected RNA were sufficient to cause a fivefold increase in oocyte plasma membrane conductance 2 days after injection. As described for *T. californica*¹⁹, the newly expressed channel is not calcium-dependent, unlike the endogenous *Xenopus* oocyte Cl⁻ channel(s)^{20,21}.

We determined the size of the mRNA(s) responsible for channel expression by size-fractionation using denaturing gel electrophoresis. A single mRNA size class of 9–10 kilobases (kb) is sufficient for functional expression in oocytes (Fig. 1). Mixing RNA from that fraction with others of lower sizes does not cause a further increase of membrane conductance. The difference in size to that published previously (~6 kb)¹⁹ for *T. californica* may result from species differences or from the use of non-denaturing sucrose gradients¹⁹.

Complementary DNA cloning

Our results are compatible with a single protein subunit being sufficient for channel function. A 9-kb mRNA may (but need not) encode a very large protein. In that case, a direct expression cloning approach using RNA transcribed *in vitro* from cDNA clones^{22,23} is difficult because of technical limitations in obtaining complete cDNA. We therefore chose a hybrid-depletion approach²⁴ in which cDNA size is not critical and which can also succeed if the channel is a complex of several essential subunits.

A tightly size-selected, functionally active mRNA fraction (Fig. 1) was used to construct a cDNA library in a plasmid vector. Single-stranded DNA derived²⁵ from small groups of clones was used to deplete the corresponding mRNAs from total *Torpedo* electric organ RNA which was then injected into *Xenopus* oocytes. A group of clones containing the desired cDNA should then cause attenuation or disappearance of Cl⁻-channel expression. The current flowing through nicotinic acetylcholine receptors, which are efficiently co-expressed by electric organ RNA¹⁹, was used as an internal control (Fig. 2b–e). After screening about 3,000 clones, we identified two groups of clones which reproducibly reduce Cl⁻-channel expression without interfering with acetylcholine receptor function (see Fig. 2c). Both groups contain 2–3 clones attenuating Cl⁻ currents, several of which have repetitive DNA sequences, recognize a broad smear on northern blots and probably exert nonspecific effects (by hybridizing to repetitive RNA sequences in untranslated regions of a large subset of the RNA population). Another clone (DC10) suppressing Cl⁻-channel expression (Fig. 2e), however, has a cDNA insert of 305 base pairs (bp) with a single open reading frame and no repetitive sequences. This insert hybridizes to a band of 9–10 kb in northern blots, which is most abundant in electric organ. We used this clone as a probe to isolate larger clones from a second cDNA library. From the number of clones detected, we estimate the corresponding mRNA to be abundant (≥0.1%), consistent with the electric organ being an abundant source of Cl⁻ channels. This probably implies that we have repeatedly overlooked correct clones during

the screening procedure. Indeed, the degree of functional depletion achieved with the initial clone is rather weak (Fig. 2e). Non-overlapping parts from clones identified by hybridization with clone DC10 were then used in additional hybrid-depletion assays and hybrid-arrest experiments using anti-sense oligonucleotides²⁶. This always led to a specific inhibition (by 70 to 90%) of Cl⁻-channel expression, excluding non-specific hybridization.

Our hybrid depletion approach could result in the cloning of a protein other than the channel itself—an enzyme involved in some essential post-translational processing, for example. Thus, we had to show that the isolated clone indeed encodes the channel, or at least a subunit of a possible channel complex.

Channel expression

Three clones with cDNA inserts ranging from 2.7 to 3.2 kb were

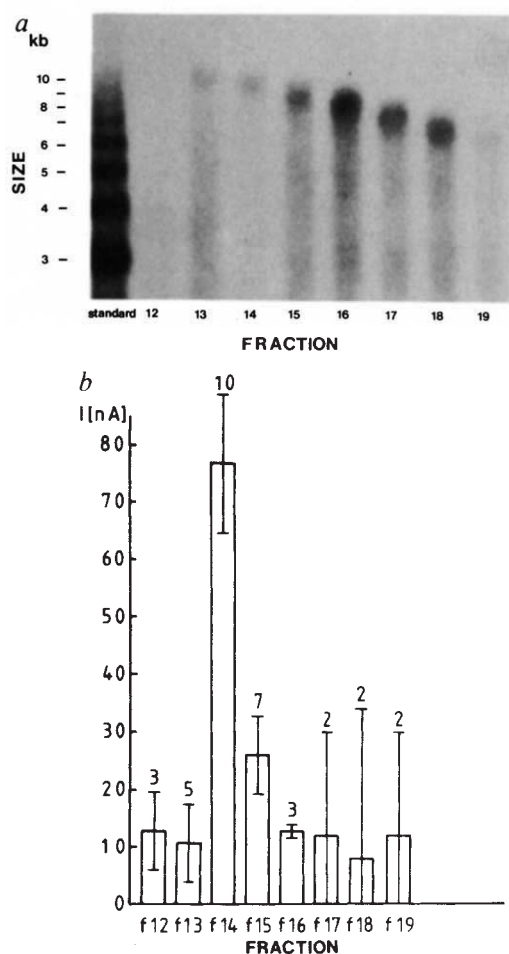


FIG. 1 Functional expression of size-fractionated mRNA. *a*, Northern blot analysis of size-fractionated RNA probed with labelled oligo(dT) to detect all mRNAs; *b*, Functional expression of the mRNA fractions shown in *a* in *Xenopus* oocytes. The mean differential current necessary to clamp the oocytes from -80 to -10 mV, from which the corresponding current of non-injected control oocytes has been subtracted, is plotted against the fraction number. Numbers above the columns, number of oocytes measured; bars, s.e.m.

METHODS. About 60 μ g of poly(A)⁺-selected RNA from *T. marmorata* electric organ was size-fractionated by denaturing electrophoresis on a 0.8% agarose gel as described²⁴. The size of the resulting fractions was analysed by northern blot analysis using ³²P-labelled oligo(dT) as a probe²⁴. The size standard is a denatured end-labelled 1-kb DNA ladder (BRL). About 1/600 of the RNA from single fractions was injected into *X. laevis* oocytes which were obtained and manipulated as described⁴². Current-voltage relationships of injected and control oocytes were obtained by two-electrode voltage clamp 2 days after injection. The command voltage was varied between -100 and 0 mV using the program shown in Fig. 2 (inset).

isolated from a random-primed cDNA library using the initial clones as probes. Partial sequence analysis (showing a candidate initiator ATG at the 5' end and stop codons in all reading frames at the 3' end) indicated that they contain the complete coding region, so we transcribed RNA *in vitro* from all three of them. Injection of any of these RNAs into oocytes causes the expression of a large Cl⁻ conductance (Fig. 2f) similar to that observed with injection of total *Torpedo* electric organ RNA (Fig. 2b), demonstrating that a single polypeptide encoded by this cDNA is sufficient for Cl⁻-channel activity.

Ion-substitution experiments show that the increase in conductance is carried by chloride ions (Fig. 2f). Current-voltage relationships were measured (using the command voltage program of Fig. 2, inset), and the hyperpolarization induced activation of the channel resulted in an outwardly rectifying current similar to the one elicited by total RNA (Fig. 3). The resting membrane potential ($I=0$) is shifted towards the Cl⁻-equilibrium potential of *Xenopus* oocytes (-20 to -30 mV)²⁰. When extracellular Cl⁻ concentration is decreased, the resting potential shifts further towards the new, more positive Cl⁻-equilibrium potential. Currents are reduced, especially at hyperpolarizing voltages, presumably because Cl⁻-influx is limited by reduced availability of chloride (half-saturation for the *T. californica* channel occurs at 75 mM Cl⁻)¹⁵. Further, 1 mM DIDS (4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid) and 1 mM diphenylamine-2-carboxylate²⁷ (DPC), known inhibitors of Cl⁻ channels, cause a large inhibition of this conductance and shift the resting potential back to values close to those of uninjected controls. This result was observed for channels expressed either from total RNA or from *in vitro* RNA. The effect of DPC was fully reversible after wash-out, but as described¹³ for the reconstituted *T. californica* channel, the inhibition by DIDS is irreversible, presumably because of covalent binding. In contrast to the reconstituted channel, however, low concentrations of DIDS ($\leq 10^{-4}$ M) are ineffective within a few minutes. The chloride conductance induced by *in vitro* RNA is not dependent on either extra- or intracellular Ca²⁺, as oocytes injected with EGTA and measured in Ca²⁺-free saline containing 1 mM EGTA also display the typical chloride conductance (our unpublished results). This virtually excludes the possibility that we have cloned an activator of the endogenous oocyte Ca²⁺-dependent Cl⁻ channel^{20,21}.

The time- and voltage-dependence of currents induced by cRNA was determined by stepping the voltage for 25 s to values between 0 and -100 mV from either a depolarizing holding potential (-10 mV; Fig. 3c), or from a hyperpolarizing potential (-100 mV; Fig. 3d). Starting from -10 mV, a voltage-induced activation of currents is observed for voltages more negative than about -40 mV (Fig. 3c). This activation is slow (of the order of several seconds), and the tail-currents indicate that subsequent inactivation after stepping back to -10 mV is even slower. By stepping from a holding potential of -100 mV to more positive voltages, we determined the time-dependent current-voltage relationship of channels activated by hyperpolarization (Fig. 3d). Depolarizing to -90 or -80 mV induces only small currents, which then increase with larger depolarizations. This leads to an overall outward rectification of currents after hyperpolarization-induced activation (Fig. 3a, b). These latter current-voltage relationships were obtained after passing several times through the staircase program shown in Fig. 2 (inset), which activates the channel by its hyperpolarizing steps.

Chloride conductance expressed from total electric organ RNA shows the same complex voltage-dependence (our unpublished results). The channel is slowly activated by hyperpolarization. Once activated, current flow increases with depolarization, leading to an apparent outward rectification. This compares well with the characteristics of the reconstituted *T. californica* channel (see ref. 15). This channel is activated slowly (within seconds) when the *cis* side of the bilayer is made negative. Once activated, however, the probability of the channel being open rapidly

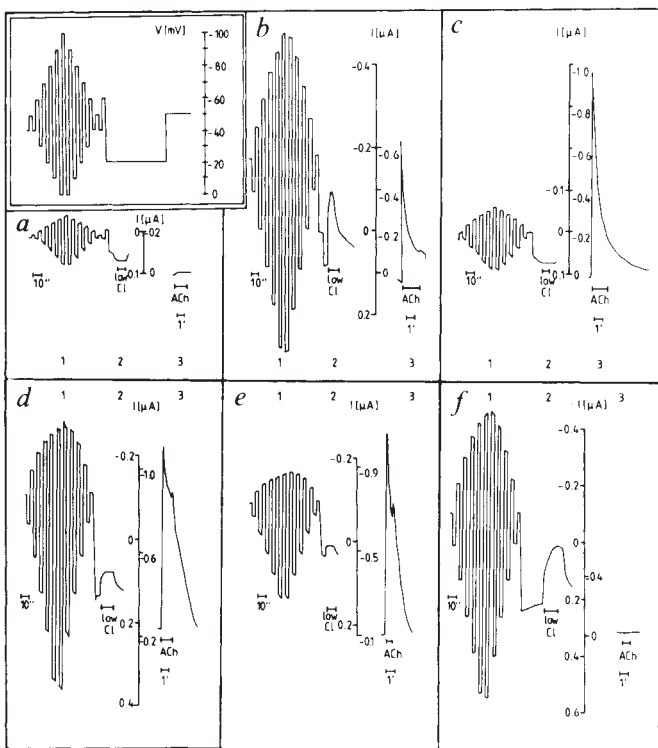


FIG. 2 Expression cloning assays. During the screening procedure, three tests were performed on each oocyte using two-electrode voltage-clamp technique: (1) determination of current-voltage curves by varying the command voltage in steps (4 s each) of 10 mV between -100 and 0 mV (inset, top left). (2) Direct determination of Cl^- current induced by changing bath $[\text{Cl}^-]$ from 104 to 7 mM (replaced by cyclamate; bars) at $V = -20$ mV (Cl^- current is larger at depolarized V). (3) Measurement of current elicited by 1 mM acetylcholine (ACh) (in the presence of 10^{-5} M atropine to block endogenous muscarinic receptors; bars) at $V = -50$ mV. Tests are indicated by numbers in all panels. Note different current and timescales. Inset, Command voltage program used during voltage clamp experiments, chosen to yield an easily recognizable 'fingerprint' of channel activity during the screening procedure. **a**, Uninjected control oocyte with current-voltage relationship typical for native oocytes: lowering $[\text{Cl}^-]$ causes no detectable Cl^- current (the small current is caused by liquid junction potentials⁴³); acetylcholine has no effect. **b**, Oocyte injected with ~ 10 ng total electric organ RNA. An outward-rectifying current typical for the *Torpedo* Cl^- -channel is observed: reducing bath $[\text{Cl}^-]$ leads to an inward current (underestimated owing to the presence of liquid junction potentials⁴³) application of acetylcholine leads to a large, inactivating inward current. **c**, Oocyte injected with electric organ RNA depleted with 60 μg single-stranded DNA (ssDNA) mixture from group DC (12 clones), the positive group finally identified; expression of Cl^- conductance is largely reduced (tests 1 and 2), while the acetylcholine-induced current is fully present. **d**, Oocyte injected with RNA depleted with ~ 5 μg single-stranded (ss) DNA derived from DC8, a single, negative clone from group DC: both Cl^- channels and acetylcholine receptors are fully expressed. **e**, Oocyte injected with RNA depleted with ~ 5 μg ssDNA derived from DC10, the final positive clone from group DC; reduction of Cl^- current and full response to acetylcholine. We typically used 5 μg ssDNA when screening clones in groups of 12; larger inhibition (80–90%) of Cl^- currents was obtained when hybrid-depleting with 50 μg ssDNA from clone DC10. **f**, Oocyte injected with cRNA from 7134, a full-length clone identified by hybridization with clone DC10. Large Cl^- currents are observed, and no response to acetylcholine. **METHODS**: Random primed cDNA⁴⁴ was prepared from the active mRNA fraction 14 of Fig. 1, ligated to adaptors compatible with the *Bst*XI overhang in CDM8 (ref. 45), size selected, and ligated into *Bst*XI-cut plasmid vector pBst. This vector was constructed by deleting the *Bst*XI site in pBluescriptKS(+) (Stratagene) and inserting the stuffer fragment from CDM8 (ref. 45) into the single *Xho*I site. After transformation of *Escherichia coli* XL1-blue (Stratagene) about 1.2×10^6 clones were obtained. A second library in pBst was made by ligating size-selected cDNA (obtained by random-primed synthesis with RNaseH-free Moloney murine leukaemia virus reverse transcriptase (BRL) from 1.0 μg non-fractionated poly(A)⁺ RNA) into pBst. Transformation of *E. coli* XL1-blue by electroporation gave about 6×10^6 primary transformants. The first library was screened by hybrid-depletion assays and the second one with radiolabelled cDNA fragments. For hybrid depletion, ssDNA was prepared²⁵ from groups of 12 clones (~ 60 μg total) and used with 20 μg total electric organ RNA as described²⁴. The single-stranded DNA was not linearized and no poly(dA) was included. The final depleted RNA was taken up in 8 μl H_2O , 50 nl of which were injected into each oocyte. Capped *in vitro* RNA was synthesized using T3 RNA polymerase from 7134 after linearizing with *Xba*I. About 0.5 ng RNA were injected per oocyte, which were measured after 1–2 days in ND96 saline (ref. 42) at room temperature.

increases with more positive voltage on the *cis* side. Assuming that the cloned and the reconstituted channels are identical, this suggests that the *cis* side of the bilayer corresponds to the cytoplasm. This could explain the lack of an extracellular effect of micromolar concentrations of DIDS in our system: the reconstituted channel is inhibited from the *cis* side¹³.

Protein structure

Figure 4 shows the 2,674-nucleotide sequence of the cDNA insert of clone 7134, one of the clones eliciting chloride conduct-

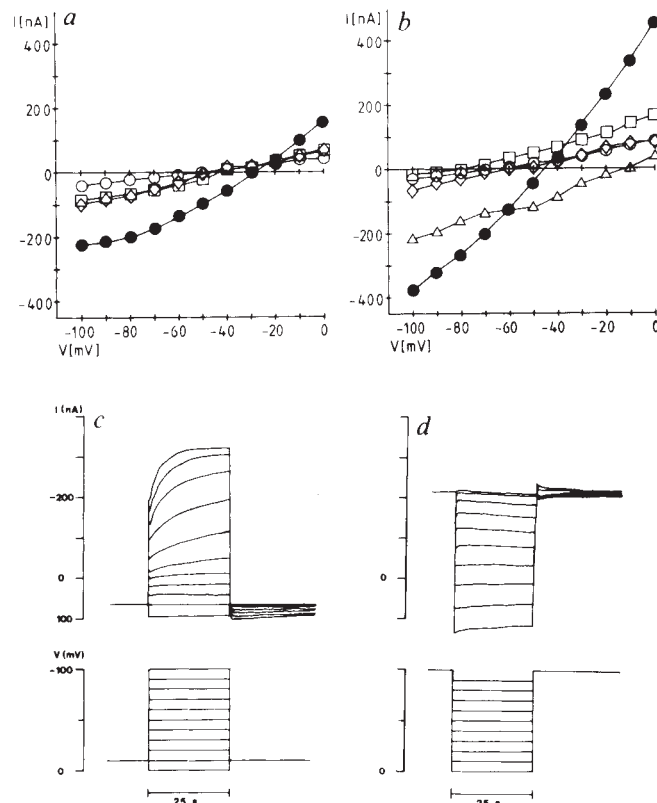


FIG. 3 Electrophysiological characterization of chloride conductance expressed from total RNA (a) or cRNA (b, c, d). **a, b**, Current-voltage relationships of chloride conductance expressed from total electric organ RNA (a) or cRNA derived from chloride channel clone (b). The current necessary to clamp the oocytes to the indicated voltages is shown for: (○) uninjected controls; (●) oocytes injected with total (a) or synthetic mRNA (b), measured in ND96 saline. (◇) RNA-injected oocytes, measured in ND96 containing 1 mM DIDS. (□) RNA-injected oocytes, measured in ND96 containing 1 mM diphenylamine carboxylate. (△) cRNA-injected oocyte, measured in low-chloride saline (96 mM NaCl replaced by sodium cyclamate in ND96). **c, d**, Time- and voltage-dependence of chloride currents expressed from cRNA. In **c**, oocyte potential was held depolarized at -10 mV for ≥ 4 min and then stepped for 25 s to the values indicated at the bottom. This led to a slow activation of currents for potentials more negative than -40 mV. In **d**, the conductance was kept activated by holding for ≥ 1 min at -100 mV, and the potential was then stepped for 25 s to more positive values. This demonstrates the apparent outward rectification of macroscopic currents of the channel activated by hyperpolarization.

METHODS. Current-voltage relationships (a, b) were determined using two-electrode voltage clamp two days after injecting ~ 10 ng total electric organ mRNA (a) or ~ 0.5 ng synthetic mRNA derived from clone 7134 (b). The command voltage was varied according to the program in Fig. 2. Results obtained from single, representative oocytes are shown. As DIDS acts irreversibly, it was applied last. The resting membrane potentials of the batch of oocytes used for b were more negative than those of a, explaining the shift of the curves towards more negative values of V . This may also explain why the reversal potential observed with cRNA injected oocytes (b) is more negative than the one in a, as in the presence of a larger K^+ conductance expressed in parallel with a large Cl^- conductance intracellular Cl^- concentration will equilibrate to yield a more negative equilibrium potential. When liquid junction potentials⁴³ are taken into account, the curve for low-chloride medium (△) will be shifted by ~ 10 mV towards more positive values. The fact that in b the resting potential in the presence of DPC is slightly hyperpolarized versus controls might be due to inhibition of unspecific cation channels by DPC (ref. 2). In c and d, conventional two-electrode voltage clamp was performed on oocytes (from a different batch) one day after injection of capped *in vitro* RNA (~ 0.4 ng) from clone 7134.

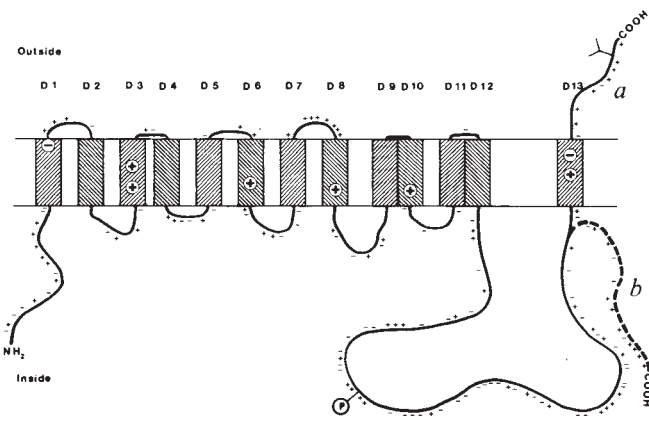


FIG. 6 Proposed membrane topology for the *Torpedo* chloride channel. For *a*, it is assumed that all hydrophobic domains indicated in Fig. 5 cross the plasma membrane, and that the N-terminal part of the protein is located intracellularly. The potential glycosylation site at amino-acid 801 could then be used (Y), as could be the phosphorylation site at 600 (P). *b*, domain D13, which (with D3 and D8) displays intermediate hydrophobicity, is assumed not to cross the plasma membrane, leading to a model with 12 membrane spans. Strongly charged amino acids (Arg, Lys, Glu, Asp) are indicated by their charges.

hydrophilic pore. One such alternative is shown in Fig. 6*b*, in which D13 is assumed not to cross the membrane. In this case, 12 membrane spans would be predicted, with both N and C termini on the cytoplasmic side.

Several putative membrane-spanning domains contain residues with charged side chains. Although D1 and D13 contain negative charges, positive charges (Arg or Lys) are present in domains D3, D6, D8, D10 and D13, and histidines are found in D4, D9, D11 and D12. This could provide a selectivity filter for chloride ions, possibly in conjunction with positive charges just next to putative membrane-spanning domains such as the highly positively charged region between D7 and D8.

Discussion

Expression from cRNA proves that a single polypeptide is sufficient for the function of the *Torpedo* chloride channel, while our hybrid-depletion and hybrid-arrest experiments suggest that we have cloned the major chloride conductive pathway from the electric organ which can be expressed in *Xenopus* oocytes. The protein has up to 13 putative membrane-spanning domains, which are considered to be a hallmark of ion channels and other transport proteins. Database searches show that it is a novel protein while matrix comparisons³² revealed no significant simi-

larity to glycine¹⁰ and GABA receptors⁹, which are ligand-gated Cl⁻ channels or band 3 (ref. 48), the electroneutral anion exchanger of erythrocytes.

Many cloned cation-selective channels probably belong to a superfamily of ion channels. Members of a large gene family of potassium channels originally identified³³⁻³⁵ as transcripts of the *Drosophila shaker* locus have six putative membrane-spanning domains, whereas the voltage-dependent Na-channel³⁶ and the dihydropyridine-sensitive Ca-channel³⁷ have four repeats of six such domains. The cGMP-gated cation channel³⁸ of the retinal rod photoreceptor may also belong to the same superfamily³⁹. The chloride channel described here does not fit easily into this group. Although omission of one of the more questionable membrane-spanning domains (such as D13; Fig. 6*b*) may give two times six membrane spans, there are no clear equivalents to the charged S4 domains (repeats of Arg-X-X, where X are mainly hydrophobic amino acids, and Arg may be replaced by Lys) present in this superfamily. This segment is thought to provide the voltage-sensor. In view of the presence of a similar segment in the cGMP-gated channel, which is only weakly voltage-sensitive, other interpretations are possible³⁹, and an entirely different voltage-sensitive potassium channel⁴⁰ has no such segment. Although the channel described here is voltage-dependent, only one stretch (positions 75-81) is at all similar to a S4 segment. This stretch, in being located after the first transmembrane segment, is atypical. Voltage-dependence could be caused by charges found in several putative transmembrane domains of the *Torpedo* channel.

We cannot state unequivocally whether the Cl⁻ channel we have cloned is the same as the one studied in the lipid-bilayer system¹³⁻¹⁵. The chloride selectivity of the present channel, its voltage-dependence (slow activation by hyperpolarization and, once activated, increase in conductivity with depolarization), together with the abundance of its mRNA, seem to argue for such an identity. The situation will be clarified by single-channel analysis of channels expressed from the present cDNA.

On the basis of biophysical data, the *Torpedo* chloride channel as observed in reconstitution was suggested¹⁴ to be a homodimer (named 'double-barreled channel'). This suggestion could be clarified by crosslinking studies. A similar 'double-barreled' Cl⁻ channel has recently been described⁴¹ in the mammalian kidney which displays nearly the same peculiar voltage-activation as that described here on the macroscopic level. These findings, together with the presence of a potential cAMP-dependent phosphorylation site in the Cl⁻ channel reported here, may increase our chances of isolating the epithelial, cAMP-regulated Cl⁻ channel involved in cystic fibrosis using our cDNA as a probe. □

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