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Messenger RNA coding for only the α subunit of the rat brain Na channel is sufficient for expression of functional channels in *Xenopus* oocytes

(hybrid selection/sucrose-gradient fractionation/voltage clamp)

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ABSTRACT **Several cDNA clones coding for the high molecular weight (α) subunit of the voltage-sensitive Na channel have been selected by immunoscreening a rat brain cDNA library constructed in the expression vector λgt11. As will be reported elsewhere, the amino acid sequence translated from the DNA sequence shows considerable homology to that reported for the *Electrophorus electricus* electroplax Na channel. Several of the cDNA inserts hybridized with a low-abundance 9-kilobase RNA species from rat brain, muscle, and heart. Sucrose-gradient fractionation of rat brain poly(A) RNA yielded a high molecular weight fraction containing this mRNA, which resulted in functional Na channels when injected into oocytes. This fraction contained undetectable amounts of low molecular weight RNA. The high molecular weight Na channel RNA was selected from rat brain poly(A) RNA by hybridization to a single-strand antisense cDNA clone. Translation of this RNA in *Xenopus* oocytes resulted in the appearance of tetrodotoxin-sensitive voltage-sensitive Na channels in the oocyte membrane. These results demonstrate that mRNA encoding the α subunit of the rat brain Na channel, in the absence of any β-subunit mRNA, is sufficient for translation to give functional channels in oocytes.**

The initial depolarization event in the propagation of an action potential in nerve and muscle is due to the opening of voltage-sensitive Na channels. This channel–protein complex has been purified from *Electrophorus electricus* electroplax membranes (1), rat muscle (2), chicken cardiac muscle (3), and rat brain (4). As isolated, the electroplax protein consists of a single large subunit of M_r 260,000 (5–7) encompassing 1820 amino acids (8). Similarly, the chicken heart protein comprises one subunit of M_r 230,000–270,000 (3). However, as isolated, the rat brain Na-channel complex contains one large subunit (α) with a M_r of ≈260,000 as determined by $NaDodSO_4$/PAGE, and two associated small subunits, β1 and β2, of M_r 36,000 and 33,000, respectively (9, 10). The rat skeletal muscle channel also contains both α and at least one β subunit (11, 12). In adult rat brain, the α and β2 subunits are covalently attached by disulfide bonds (9). Both the rat and eel protein complexes have been reconstituted into phospholipid vesicles, with restoration of toxin-induced activation of Na permeability (13–16).

The cDNA coding for the electroplax protein has been molecularly cloned and its sequence has been determined (8). Furthermore, while the present manuscript was in the proof stage, the cloning and sequencing of cDNAs coding for rat brain Na channels was reported (17). This latter study shows that there are at least two related α-subunit Na-channel mRNAs in rat brain, denoted I and II, and there is possibly a third.

The functional significance of the small subunits in rat brain is not known. Schmidt *et al.* (18) present data suggesting that disulfide linkage of the α and β2 subunits, insertion into the cell-surface membrane, and attainment of a functional conformation are closely related late events in the biogenesis of functional Na channels in rat brain. However, Sumikawa *et al.* (19) have shown that when rat brain poly(A) RNA is fractionated by velocity centrifugation on a nondenaturing sucrose gradient, a high molecular weight fraction by itself was sufficient to induce Na-channel function when injected into oocytes. A similar experiment, albeit at a lower resolution, was carried out by Hirono *et al.* (35) with the same result. These results suggest that the small subunits are not necessary for channel function in oocytes.

In this report, we have isolated cDNA clones encoding portions of the coding region of the large subunit of the Na channel. We have used these clones to analyze a sedimentation fractionation experiment similar to that of Sumikawa *et al.* (19). We found that only those fractions that by hybridization contained the high molecular weight α-specific mRNA [9 kilobases (kb)] were translated in the oocyte to give functional channels. Furthermore, the fractions containing the α-subunit mRNA had undetectable quantities of low molecular weight RNA, as determined by hybridization with an unrelated probe that detects 2.2- and 2.4-kb brain mRNAs. We have also used the cDNA clones to hybrid-select α-subunit-specific mRNA. This mRNA induced synthesis of voltage-sensitive tetrodotoxin-inhibited Na channels when injected into *Xenopus* oocytes. These results indicate that α-subunit mRNA, in the absence of any β-subunit mRNA, is translated in *Xenopus* oocytes to give functional Na channels.

EXPERIMENTAL PROCEDURES

Isolation of Rat Brain RNA and Na Channel Clones. RNA was isolated from the brains of 4- to 14-day-old rats by the lithium chloride/urea procedure (20) or by a modification of the procedure of Chirgwin *et al.* (21) using guanidine hydrochloride. The procedures for construction of cDNA libraries in the vector λgt11 (22, 23) for immunological screening with an antibody to the rat brain channel (24) and for characterization of the clones at the sequence level will be described elsewhere.

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Abbreviation: kb, kilobase(s).

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RNA Transfer. RNA was electrophoresed through a 1.1% agarose gel containing 2.2 M formaldehyde at 3.3 V/cm for 8 hr as described (25). After electrophoresis, the gel was soaked in water for 20 min, then transferred to nitrocellulose or Hybond N (Amersham) (26). Hybridization was carried out at 63°C–68°C as described (27).

Preparation of SP6 RNA Transcripts. Labeled hybridization probes were synthesized from 1 μg of linearized template DNA in 20 μl with 500 μM each ATP, GTP, and UTP, and 100 μCi of [^{32}P]CTP (400 Ci/mmol; 1 Ci = 37 GBq; Amersham) (27, 28).

Hybrid Selection. Nitrocellulose filters (Schleicher & Schuell; 9-mm diameter) were loaded with 40 μg of DNA as described (29). Filters were air-dried, washed three times with 6× SSC (1× SSC = 0.15 M NaCl/0.015 M Na citrate) and baked for 2 hr at 80°C under vacuum. To release any DNA that was not properly bound, filters were immersed in 0.3 ml of H_2O and placed in a boiling water bath for 1 min. Prehybridizations were performed overnight at 65°C in 1 M NaCl/0.1 M Pipes, pH 6.4/2 mM EDTA/yeast RNA (300 μg/ml; type III; Sigma). For hybridizations, 100 μl of the same buffer was used, but the yeast RNA was replaced with 30 μg of poly(A) RNA from the brains of 4- or 14-day-old rats. Hybridization time was 1 or 3 hr. Afterwards, RNA remaining in the hybridization solution was precipitated twice with ethanol. The filters were washed three times with 0.2× SSC/0.2% $NaDodSO_4$ at 65°C and three times with 10 mM Tris·HCl, pH 7.5/2 mM EDTA at room temperature. RNA bound on the filter was eluted by shaking the filters in 0.3 ml of H_2O for 1 min in a boiling water bath. Subsequently, filters were quick-frozen in an ethanol/dry ice bath, thawed on ice, and RNA in the water was precipitated twice with ethanol together with 5 μg of yeast RNA as carrier. The final pellets of the bound RNA and of the RNA precipitated from the hybridization solutions were each dissolved in 3 μl of H_2O, and 0.06 μl of each was injected into *Xenopus* oocytes.

RNA Fractionation by Sucrose Gradient Sedimentation. Poly(A) rat brain RNA (150 μg in 100 μl of H_2O) was heated to 65°C for 3 min and then layered on a 6–20% linear sucrose gradient containing 15 mM Pipes, pH 6.4/5 mm EDTA/0.25% sarcosyl. The gradient was centrifuged in an SW 27.1 rotor at 24,000 rpm at 4°C for 18 hr. Thirty-four 0.5-ml fractions were collected and the RNA was precipitated with sodium acetate and ethanol. The RNA from every three fractions was then pooled for all further experiments. RNA gel blots utilized 1/20th of each pool per lane (equivalent to 7.5 μg of unfractionated RNA) and oocyte microinjections utilized 1/1200th of each pool per oocyte (equivalent to 125 ng of unfractionated RNA).

RNA Injections into Oocytes and Electrophysiological Procedures. These procedures were carried out as described (28). After removing follicle cells by incubating the oocytes for 3 hr in Ca^{2+}-free OR-2 solution (30) containing collagenase (2 mg/ml) (Sigma; type IA), 60 nl of RNA solution was injected into the cytoplasm by using a device similar to that described by Contreras *et al.* (31). The oocytes were kept in ND 96 (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes NaOH, pH 7.5) supplemented with penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 0.5 mM theophylline at room temperature until testing (usually 40 hr).

A standard two-microelectrode voltage clamp (model 8500, Dagan Instruments, Minneapolis, MN), was used to test the oocytes in a recording chamber continuously perfused with ND 96. The electrodes were filled with 3 M potassium chloride and had resistances of 0.5–1 mΩ. Holding potential was −80 mV. Stimulation and analysis procedures have been described (32).

RESULTS

Isolation and Characterization of Na Channel cDNA Clones. A rat brain cDNA library in the expression vector λgt11 was screened with antibody against the purified rat brain Na channel by standard methods (23, 24). Additional clones were isolated by hybridization screening of cDNA libraries with the initial clones. The isolation and characterization at the sequence level of the cDNA clones will be described elsewhere.

Sequence analysis revealed the expected moderate degree of homology with the eel electroplax sequence (8), ranging up to 80% at the nucleotide level and 100% at the amino acid level. Comparison with the subsequently published sequences of the rat brain Na channels (17) revealed that all of the clones isolated by us correspond to the rat channel II of Noda *et al.* (17). However, the clones used in this paper would all hybridize strongly to both rat channels I and II at the stringencies used.

RNA Gel Blots. The mRNA coding for the rat brain Na channel was identified by RNA gel blot hybridization. Highly labeled single-strand RNA probes, ≈400 nucleotides long and corresponding to the antisense strand, were synthesized in the SP6 system. As shown in Fig. 1, a combination of two antisense probes complementary to coding sequences in the amino-terminal and carboxyl-terminal regions of the rat II α subunit hybridized to an RNA species of 9.0 kb. The intensity of hybridization indicates that this is an RNA of low abundance. There is a second fainter component of 8.0 kb. Still fainter bands of 10.5 and 11.5 kb can be seen with appropriate exposures. In support of the interpretation that these bands code for a voltage-sensitive Na channel, we observed that the same probes hybridized to bands of comparable length but of lower intensity for RNA from rat heart and of still lower intensity for skeletal muscle (Fig. 1), but that no signal was seen with RNA from rat liver, kidney, or spleen (data not shown).

Sedimentation Analysis Shows That Na Channel Activity Is Encoded in a High Molecular Weight mRNA Component. Rat brain poly(A) RNA was fractionated by sucrose-gradient velocity sedimentation, similar to the experiment described by Sumikawa *et al.* (19). Each fraction was characterized for its content of α-subunit mRNA by gel electrophoresis under denaturing conditions, followed by hybridization of the gel blots to a Na-channel probe. The molecular lengths of the poly(A) RNA in the several fractions as well as the amount of poly(A) RNA in each fraction were measured by hybridization of the blots with ^{32}P-labeled poly(dT). The degree to which the several high molecular weight fractions were contaminated with lower molecular weight RNA was deter-

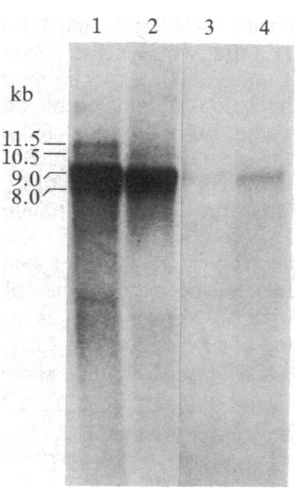

FIG. 1. RNA gel blots were hybridized to combined 5′- and 3′-specific Na-channel probes. Lane 1, 10 μg of rat brain poly(A) RNA isolated from 14-day-old rats. Lanes 2–4, 40 μg of total RNA isolated from 4-day-old rat tissue as follows: lane 2, brain; lane 3, skeletal muscle; lane 4, heart. Lanes 3 and 4 were autoradiographed for 4.5 times as long as lanes 1 and 2. This experiment was with a formaldehyde gel. Similar results have been obtained with glyoxal and CH_3HgOH gels. Length determinations are from RNA standards (Bethesda Research Laboratories).

mined by hybridization of the same blots with a cloned probe that was known to hybridize to two specific brain poly(A) RNA molecules of 2.2 and 2.4 kb.

The results are presented in Figs. 2 and 3. Fig. 2 *Left* shows that most of the discrete 9.0-kb α-subunit mRNA was in fraction D, which contained poly(A) RNA in the range of 7–12 kb (Fig. 2 *Right*) and 1.5% of the poly(A) RNA recovered from the gradient. This and other high molecular weight fractions contained no detectable amount of 2.2- to 2.4-kb poly(A) RNA (Fig. 2 *Center*). Many of the fractions showed hybridization to degraded Na-channel RNA of lower molecular weight. Comparison with the unfractionated RNA lane shows that some random degradation had occurred during sedimentation. We estimate by densitometric scans that ≈50% of the Na-channel hybridization is retained in the discrete 9.0- and 8.0-kb bands.

A quantity of 1/1200th of each fraction (see *Experimental Procedures*) was injected into each of several oocytes and the peak Na-channel current was measured. The data in Fig. 3 show that all of the Na-channel activity as assayed by oocyte injection resided in fractions C and D. As can be seen (Figs. 2 and 3), these two fractions contained full-length Na-channel mRNA. The specific activity in fraction D had been enriched at least 6-fold compared to unfractionated RNA, based on the size of the peak current and the amount of RNA in that fraction. No Na-channel activity was detectable in any of the lower molecular weight fractions E–K. No lower molecular weight RNA was detectable in fractions C and D (Figs. 2 and 3), indicating that the Na channels synthesized from mRNA in these fractions did not contain any subunits coded for by lower molecular weight RNAs.

Injection of Hybrid-Selected α-Subunit RNA Produces Functional Na Channels. Messenger RNA specific for the α subunit of the Na channel was isolated by hybrid selection with a single-strand antisense M13 DNA clone containing an 830-nucleotide insert from the 3′ half of the coding region. RNA that hybridized to the probe (bound) and RNA that did not hybridize to the probe (unbound) were injected into *Xenopus* oocytes and the oocytes were studied with a voltage-clamp apparatus 2 days afterwards. Fig. 4 presents averaged traces of voltage-clamp current showing that electrically excitable Na channels are induced in oocytes by hybrid-selected RNA. In this case, when the oocyte was depolarized from a holding potential of −80 mV to a test potential of −20 mV, the inward current increased (within the rise time of the apparatus, 2 msec) to a peak value of 120 nA and decayed over the next few milliseconds. The peak Na currents were associated with depolarizations to −10 or −20 mV, just as observed with nonselected RNA. The inward current was completely inhibited by 1 μM tetrodotoxin (Fig. 4). After washing out the tetrodotoxin, the Na current reappeared (data not shown). The effects of voltage, time, and tetrodotoxin on the response are all as observed by others (19, 33, 34) and provide definitive evidence for expression of functional Na channels by hybrid-selected RNA.

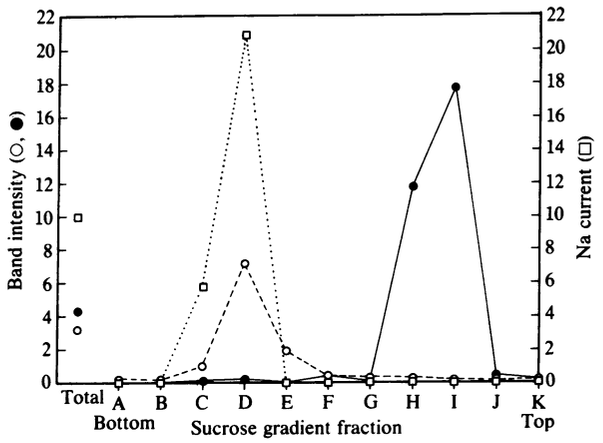

FIG. 3. Analysis of Na-channel content of sucrose-gradient RNA fractions. The autoradiographs in Fig. 2 were measured by densitometric scanning and integration with an LKB UltroScan XL Densitometer. (○) Area under the Na-channel bands (8 and 9 kb) in each fraction of the sucrose gradient in Fig. 2 *Left*. (●) Area under the low molecular weight bands (2.2 and 2.4 kb) in each fraction of the sucrose gradient in Fig. 2 *Center*. Band intensity is expressed as absorbance units × mm. (□) Average peak Na current expressed in *Xenopus* oocytes after injection of 1/1200th of each pooled fraction. The current is expressed as nA per ng of injected RNA, and each value represents the average result from three oocytes. The quantity of RNA injected was determined from the weight percentage of RNA in each sucrose-gradient fraction. This was calculated from the molar percentage and average size of each fraction, values obtained from densitometric scans of the autoradiograph in Fig. 2 (*Right*).

The results of these experiments are summarized in Tables 1 and 2. In the experiment detailed in Table 1, the Na channel electrophysiological response to depolarization was measured and compared to the current due to the outward flow of chloride ions in response to serotonin (10 μM), which was measured as a control. It should be noted that our later studies have shown that serotonin currents >1200–1500 nA are usually limited by saturation, (H.L., unpublished observations) so that large serotonin currents are actually underestimates of the amount of serotonin receptor encoding RNA. The vector control in Table 1 shows that under the conditions of hybridization used, Na-channel RNA was quite unstable, with only about 10–20% of the original activity remaining after incubation. Of the remaining activity, however, ≈37% was contained in mRNA bound by the filter containing the α-subunit insert. On the other hand, only ≈1% of the

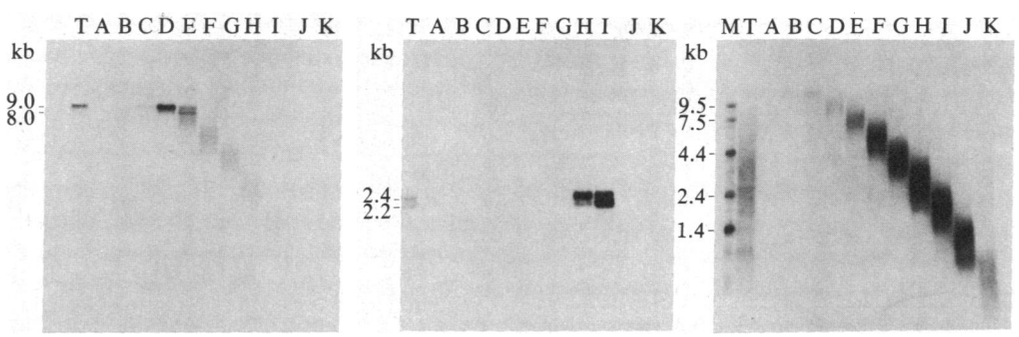

FIG. 2. Hybridization of sucrose-gradient fractionated RNA. Rat brain poly(A) RNA was fractionated, electrophoresed, and transferred to Hybond N as described in *Experimental Procedures*. Fractions are A–K and T (1 μg of unfractionated RNA). The filter was hybridized as follows: (*Left*) Na-channel α-subunit probe; (*Center*) brain low molecular weight probe; (*Right*) ^{32}P-labeled poly(dT) to detect mRNA. Exposure times were 2 hr (*Left* and *Center*) or 20 min (*Right*).

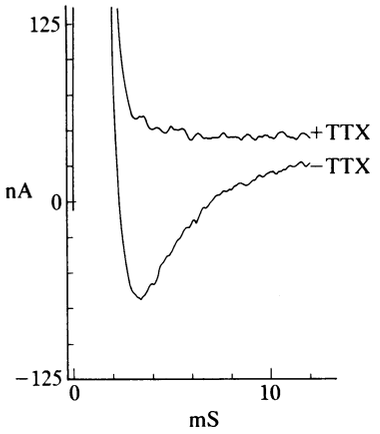

FIG. 4. Na-channel current induced by hybrid-selected RNA. Poly(A) RNA from the brains of 4-day-old rats was hybrid-selected with an antisense cDNA clone for the Na channel and injected into *Xenopus* oocytes. The oocytes were tested 40 hr later for voltage-sensitive Na currents. These tracings represent voltage-clamp currents associated with a jump from −80 to −20 mV. The transient upward deflection is the current required to charge the membrane capacitance upon depolarization. This is followed by the downward deflection, which is due to the inward flow of Na ions. The Na current was completely abolished in the presence of 1 μM tetrodotoxin (TTX).

serotonin activity was contained in the mRNA bound to the Na-channel probe.

In the experiments summarized in Table 2, the average Na-channel signal from mRNA bound to the hybridization filter was divided by the average signal from mRNA that did not bind to the filter to determine a bound-to-unbound ratio. As can be seen, this ratio of bound to unbound Na-channel mRNA activity was 3.1 for hybrid selection with a Na channel α probe, whereas the ratio was only 0.16 for hybrid selection with a vector control. These experiments further demonstrate that RNA selected by hybridization to a Na channel α-subunit insert is sufficient for expression of functional Na channels when injected into *Xenopus* oocytes.

DISCUSSION

We have isolated clones encoding the high molecular weight (α) subunit of the rat brain voltage-sensitive Na channel by immunological screening of cDNA libraries. The structural characterization of these clones will be reported independently.

Table 1. Na-channel and serotonin receptor expression by hybrid-selected RNA

RNA	Na-channel peak current,* nA	Serotonin peak current,* nA	Na/serotonin,† normalized ratio
Brain A+	664 ± 155(7)	1125 ± 377(4)	1.00(±0.40)
Bound (α insert)	37 ± 5(11)	12 ± 5(6)	5.20(±2.00)
Unbound (α insert)	64 ± 9(9)	1000 ± 184(8)	0.11(±0.03)
Unbound (vector)	107 ± 23(3)	1920 ± 650(3)	0.09(±0.04)

*Average peak values ± SEM for the number of oocytes surviving (in parentheses). Holding potential for the serotonin-induced current was −60 mV.

†Ratios were obtained by dividing Na peak current by serotonin peak current, and then normalizing so that the value for rat brain poly(A) (Brain A+) was equal to 1.0.

Table 2. Na-channel expression by hybrid-selected RNA

RNA	Na-channel peak current, nA* Exp. 1	Exp. 2	Bound/unbound†
Brain A+‡	313 ± 82(7)	288 ± 74(6)	
Bound (α insert)	17 ± 10(24)	63 ± 29(3)	3.10
Unbound (α insert)	0 (10)	38 ± 8(3)	
Bound (vector)	0 (10)	22 ± 8(6)	0.16
Unbound (vector)	31 ± 14(9)	113 ± 20(3)	

*Average peak values ± SEM for the number of oocytes surviving (in parentheses).

†Ratios were obtained by averaging all values for experiments 1 and 2 and then dividing the peak Na current for bound RNA by that for unbound RNA.

‡Rat brain poly(A) RNA.

Using the Na-channel clones, we have analyzed the fractionation of Na-channel mRNA by sucrose-gradient sedimentation. Sumikawa *et al.* (19) and Hirono *et al.* (35) had previously shown that after nondenaturing sucrose-gradient centrifugation of rat brain poly(A) RNA, Na-channel activity (as assayed by the oocyte injection assay) resided in a high molecular weight fraction. In a similar experiment in which we also tested the effectiveness of the size fractionation by hybridization with Na channel and other probes, we also found that injection of a high molecular weight fraction gave functional channels. We further demonstrated by RNA gel blots that this fraction was enriched in undegraded 9-kb Na-channel mRNA, that it contained only RNA in the range of 7–12 kb in detectable amounts, and, specifically, that RNA of the length that might be expected for a subunit of M_r 33,000–36,000 was not present in the fraction (see below).

To confirm that α-subunit RNA alone is sufficient to encode functional Na channels, we performed positive hybrid-selection experiments with an α-subunit antisense cDNA clone affixed to a membrane filter. These experiments demonstrated that α-subunit RNA selected by hybridization and injected into *Xenopus* oocytes did result in Na channels that showed the expected time response and voltage-sensitive characteristics and were inhibited by tetrodotoxin. However, rather small Na-channel activities were recovered by hybrid selection because of the instability of Na-channel RNA. Messenger RNA encoding the serotonin receptor was quite stable under the same hybridization conditions, and <1% of serotonin mRNA activity was recovered in the hybrid-selected fraction (Table 1). In addition, the ratio of hybridized to unhybridized mRNA encoding Na-channel activity was ≈20-fold greater when an α-subunit DNA probe was used compared to the vector DNA alone (Table 2). Therefore, it is quite unlikely that any other mRNA, for example Na-channel β-subunit mRNA, was present in the hybrid-selected material. Thus, α-subunit mRNA by itself was sufficient for the synthesis of functional Na channels in the oocyte.

The β subunits of the rat brain Na channel have M_r values of 36,000 and 33,000 when glycosylated and 23,000 and 21,000 when deglycosylated (10). Thus, the required coding length is <0.7 kb of RNA. Based on other precedents, it is improbable (but not impossible) that β-subunit mRNA could lie in the size range of 7–12 kb included in the active fraction. It is more likely that RNA encoding a protein of this size will be in the 1- to 3-kb size range. No RNA of this size could be detected in the sucrose-gradient fractions, which contained the RNA active for expression of functional channels in the oocyte

(Fig. 3). Therefore, the sedimentation experiment supports the hybridization-selection experiment in showing that α-subunit RNA by itself is sufficient for functional expression in oocytes.

What then is the role of the β1 and β2 subunits in the rat brain channel? The β1 subunit is covalently labeled by photoreactive derivatives of α- and β-subunit scorpion toxins, suggesting that it is located at or near the receptor sites at which these toxins modify Na-channel gating (36, 37). Selective removal of the β1 subunit from purified brain Na channels in detergent solution is accompanied by loss of saxitoxin binding activity (38) and of the ability to reconstitute neurotoxin-activated $^{22}Na^+$ influx on incorporation into lipid vesicles (D.J. Messner and W.A.C., unpublished data), suggesting that a complex of α and β1 subunit is required to maintain a functional state of the detergent-solubilized and purified Na channel. In contrast, selective removal of the β2 subunit has no apparent effect on the functional properties of purified Na channels. Schmidt *et al.* (18) offer evidence that disulfide-bond linkage of the β2 subunit with the α subunit occurs before the incorporation of intracellular α chains into the cell-surface membrane. This linkage is evidently unnecessary for insertion into the oocyte membrane. It is conceivable that the oocyte has endogenous polypeptides that play the role of the β subunits, but there is no evidence for this hypothesis. Further analysis of the physiological and pharmacological properties of Na channels produced by α-subunit mRNA in oocytes may reveal differences that result from the absence of β1 and β2 subunits.

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