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Repression of nicotinic acetylcholine receptor expression by antisense RNAs and an oligonucleotide

(*Xenopus* oocytes/*Torpedo*/hybrid-arrested translation/membrane currents/chloride channels)

K. Sumikawa and R. Miledi

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA 92717

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ABSTRACT **Four antisense RNAs, synthesized from cDNA clones coding for the four subunits of the acetylcholine receptor of *Torpedo* electroplaques, were used to study their effect on the expression of functional *Torpedo* acetylcholine receptors in *Xenopus* oocytes. All antisense RNAs inhibited the appearance of functional receptors in the oocyte's surface membrane for at least 1 week. This inhibition was specific because the antisense RNAs did not block the expression of the Cl^- channels, also encoded by *Torpedo* electroplaque mRNA. Experiments with incomplete antisense RNAs and a synthetic oligonucleotide indicate that covering the ribosome binding site or the initiation codon in the mRNA is not a necessary requirement for efficient blocking. Thus, the use of antisense RNAs combined with the *Xenopus* oocyte system provides a novel approach to screen cDNA libraries for the genes coding for multisubunit neurotransmitter receptors.**

It was recently discovered that gene expression in prokaryotes and in various cells, including *Xenopus* oocytes and mammalian cells, can be selectively inhibited by antisense RNA, that is, RNA that is complementary to a target RNA (see ref. 1 for a review). This inhibition sometimes involves a hybridization between an antisense RNA and its counterpart mRNA, which results in an inhibition of mRNA translation. Thus, antisense RNAs can be used for identifying a gene product of interest and studying its function as well as its role in early development.

To examine the applicability of antisense RNAs to the study of neurotransmitter receptors, which are key molecules in synaptic communication and also may play an important role in the formation of synaptic connections (2, 3), we have examined the effect of antisense RNAs on the functional expression of the multisubunit nicotinic acetylcholine (AcCho) receptor (AcChoR) of the electric organ of *Torpedo* in *Xenopus* oocytes.

MATERIALS AND METHODS

Plasmids. Full-length *Torpedo* AcChoR cDNA clones (4, 5) were provided by T. Claudio (Yale University; α, β, γ, and δ subunits) and S. Heinemann (Salk Institute; γ subunit). The cDNA inserts were excised from vectors and inserted into plasmids pSP64 (γ-subunit cDNA) or pGEM4 (α-, β-, and δ-subunit cDNAs). For *in vitro* transcription the resulting plasmids were linearized by digestion with *Hin*dIII (α), *Xba* I (γ and δ), or *Nae* I (β) and were used as templates.

mRNA Preparation. Total RNAs were extracted either from *Torpedo* electric organ or cat denervated muscles, and poly(A)$^+$ mRNAs were obtained by oligo(dT)-cellulose chromatography as described (6).

***In Vitro* Transcription.** The bacteriophage SP6 or T7 RNA polymerases were used to synthesize antisense RNAs in the presence of the cap analog GpppG by using 10 μg of linearized DNA as template as described (7–9). After synthesis, RNase-free DNase was added to a concentration of 1 unit per μg of DNA. Following phenol/chloroform extraction, the RNA was recovered by precipitation with ethanol and finally was dissolved in distilled water for injection into the oocytes.

Translation in *Xenopus* Oocytes. *Xenopus* oocytes were injected (*ca.* 50 nl) with *Torpedo* mRNA ($\approx$50 ng) alone or together with antisense RNA ($\approx$10 ng) and cultured at 16°C in modified Barth's medium containing gentamicin (0.1 mg/ml) and nystatin (50 units per ml) as in ref. 6. In some experiments, the oocytes were incubated in the presence of [^{35}S]methionine (1 mCi/ml; 1 Ci = 37 GBq).

Immunoprecipitation. For identification of the AcChoR subunits, ^{35}S-labeled oocyte translation products were heated to 100°C in 1% $NaDodSO_4$/5 mM EDTA for 3 min and were diluted with 4 volumes of buffer (60 mM Tris chloride, pH 7.6/6 mM EDTA/190 mM NaCl/1.25% Triton X-100). After addition of rat antisera raised against $NaDodSO_4$-denatured AcChoR and incubation for at least 12 hr at 4°C, immunocomplexes were adsorbed to protein A-Sepharose gel. The gels were then processed as described (10) and analyzed by $NaDodSO_4$ gel electrophoresis (11).

Electrophysiology. This was carried out as described (6, 12) with the oocyte membrane potential clamped at -60 mV. Atropine ($0.5-1.0$ μM) was used to block any possible muscarinic responses to AcCho (12).

Other. Restriction enzyme digestions were carried out as instructed by the supplier. The oligonucleotide was synthesized by using an automatic DNA synthesizer (Applied Biosystems, Foster City, CA).

RESULTS

Effect of Antisense RNAs on the Expression of *Torpedo* AcCho Receptors. It is well known that the AcChoR of *Torpedo* is a heteropolymer composed of five subunits of four different types α_2, β, γ, and δ (for reviews, see refs. 13–16). To obtain sufficient quantities of the four subunit-specific antisense RNAs, the cDNAs of the AcChoR subunits were inserted into plasmid vectors containing a phage SP6 promoter or both SP6 and T7 promoters. *In vitro* transcription of linearized plasmids by either SP6 or T7 polymerases (see *Materials and Methods*) generated pure preparations of the subunit-specific antisense RNAs (Fig. 1*A*). Typically, we obtained 1–2 μg of capped antisense RNA per μg of DNA.

We have shown (11, 17, 18) that injection of *Torpedo* electroplaque mRNA into *Xenopus* oocytes leads to the synthesis of the receptor subunits and to the incorporation of

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor.

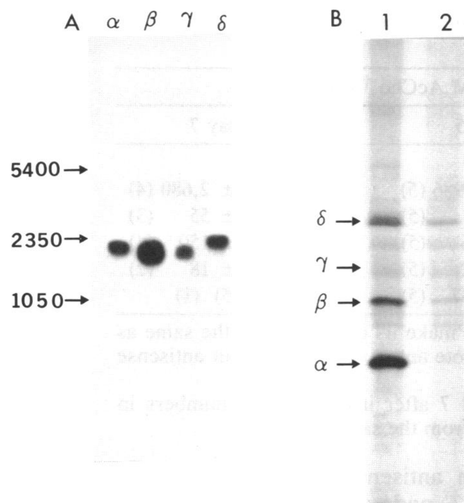

FIG. 1. (*A*) Agarose gel electrophoresis analysis of AcChoR subunit-specific antisense RNAs synthesized *in vitro*. The length standard was *Hin*dIII-digested phage PM2. (*B*) $NaDodSO_4$ gel electrophoretic analysis of the AcChoR subunits synthesized in oocytes and immunoprecipitated with antibodies raised against $NaDodSO_4$-denatured AcChoR. The immunoprecipitates were obtained from 10 oocytes injected with *Torpedo* mRNA alone (lane 1) and *Torpedo* mRNA with antisense α-subunit RNA (lane 2). The positions of the AcChoR subunits purified from *Torpedo* electric organ are marked by arrows.

functional *Torpedo* AcChoR in the oocyte's surface membrane. Therefore, we used *Xenopus* oocytes to examine the effect of each subunit-specific antisense RNA on the translation of all four subunit mRNAs and on the expression of functional AcChoRs.

To study the effect of antisense RNA on specific mRNA translation, oocytes were injected with whole *Torpedo* mRNA plus α-subunit antisense RNA and incubated in the presence of [^{35}S]methionine. The translation products were immunoprecipitated by using polyclonal antibodies against *Torpedo* AcChoR and then were separated by $NaDodSO_4$ gel electrophoresis. As a control, the *Torpedo* mRNA was translated in oocytes in the absence of antisense RNAs and processed as the test sample. The β and δ subunits were present in both samples, while the α subunit was seen only in the control sample (Fig. 1*B*). These results suggest that the α-subunit antisense RNA blocks specifically the translation of the α-subunit mRNA. In both samples, the presence of the γ subunit was not very obvious probably because of proteolysis during sample preparations, since the γ subunit is susceptible to proteolitic degradation (19). The amount of β and δ subunits in the test oocytes appeared to be less than those in the control oocytes, suggesting that in oocytes the unassembled subunits are degraded more rapidly than assembled ones, as happens in tissue-cultured muscle cells (20). However, the possibility that the α-subunit antisense RNA somehow inhibits the translation of the β- and δ-subunit mRNAs is not excluded.

To study the expression of functional AcChoRs, we measured the response to AcCho in oocytes injected with *Torpedo* mRNA alone or with one of the subunit antisense RNAs. Oocytes injected with *Torpedo* mRNA alone gave large smooth inward membrane currents in response to bath application of AcCho (Fig. 2), and the mean amplitude of the currents increased from 3,950 to 10,530 nA with longer times after mRNA injection (Table 1). In contrast, the amplitude of the currents elicited by AcCho applied to the oocytes injected with any of the antisense RNAs was greatly reduced (Fig. 2). Mean values of AcCho-activated currents at 3, 5, and 7 days after injection are shown in Table 1. At all the times examined, the strongest inhibition was consistently obtained with the α-subunit antisense RNA. In the experiment shown in Table 1, the mean currents obtained from oocytes injected with both whole *Torpedo* mRNA and the α-subunit antisense RNA were only 0.3–0.7% of that from oocytes injected with *Torpedo* mRNA alone; in other experiments, an even larger inhibition was observed. A smaller, but still large, inhibition was exerted by the β-, γ-, or δ-subunit antisense RNAs. For example, the mean amplitude of the AcCho-activated current at the various times after injection with β-subunit antisense RNA was reduced to 2.9–4.3%; with the γ- and δ-subunit antisense RNAs, it was decreased to 0.9–3.9% and 4.3–9.8%, respectively. Interestingly, with increasing time after injection, the AcCho-activated currents appeared to escape partly from the inhibition caused by the β- and δ-subunit antisense RNAs. This point will be reported in more detail at a later date.

Effect of Antisense RNA on the Expression of *Torpedo* Electroplaque Cl^- Channels. We have shown (21) that injection of *Torpedo* electric organ mRNA induces the appearance of at least two types of membrane channels in *Xenopus* oocytes: an AcCho-activated channel and a voltage-activated Cl^- channel. The Cl^- channel is activated at

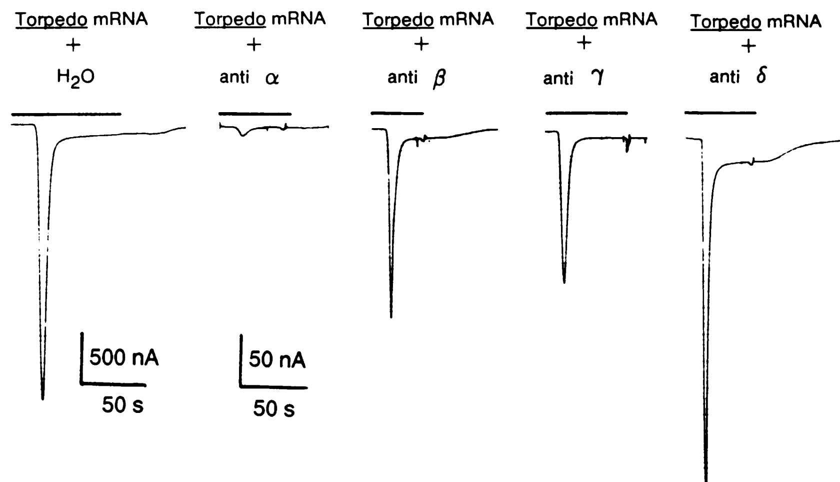

FIG. 2. AcCho-activated currents recorded in *Xenopus* oocytes injected with *Torpedo* mRNA alone or together with antisense RNAs. AcCho (100 μM) was applied by bath perfusion for the durations indicated by the bars. Atropine (0.5 μM) was used to block possible native muscarinic responses to AcCho.

Table 1. Membrane currents elicited by AcCho in *Xenopus* oocytes injected with *Torpedo* mRNA alone or together with synthetic antisense RNA

Injection*	Current elicited by 100 μM AcCho,† nA ± SEM		
	Day 3	Day 5	Day 7
Torpedo mRNA			
+ H_2O	3,950 ± 2,263 (5)	7,340 ± 1,986 (5)	10,530 ± 2,680 (4)
+ anti-α RNA	10 ± 6 (4)	20 ± 9 (5)	77 ± 55 (3)
+ anti-β RNA	115 ± 33 (4)	271 ± 56 (5)	456 ± 150 (4)
+ anti-γ RNA	152 ± 98 (4)	63 ± 22 (5)	147 ± 18 (2)
+ anti-δ RNA	170 ± 63 (4)	722 ± 237 (5)	(405) (1)

*Water was added when *Torpedo* mRNA was injected alone to make its concentration the same as when injected with antisense RNA. Anti-α (β, γ, δ) RNAs denote anti-α (β, γ, δ)-subunit antisense RNAs.

†Mean peak amplitudes of current elicited on days 3, 5, and 7 after injection. The numbers in parentheses refer to the number of oocytes. All oocytes were from the same donor.

potentials more positive than about −50 mV and is inactivated progressively at more negative potentials.

To test further whether the expression of functional AcChoRs was inhibited in a specific manner by the antisense RNAs, we also tested the oocytes for the expression of the Cl^- channels. Merely by inserting a microelectrode into the oocytes, it became evident that the antisense RNAs did not block the expression of the Cl^- channels because the resting potential was low and close to the chloride equilibrium potential, as is the case with oocytes injected with *Torpedo* electroplaque mRNA alone (21). Furthermore, the membrane conductance, measured from the current required to double the membrane potential from −20 to −40 mV, also showed that the Cl^- channel was well expressed. For example, in one experiment the membrane conductance was 18.5 ± 3.9 μS (mean ± SEM) in oocytes injected with *Torpedo* mRNA alone and 17.8 ± 3.3 μS when they were coinjected with Torpedo mRNA and α-subunit antisense RNA. Moreover, in both cases the current–voltage relation had the nonlinear behavior previously described (21). In contrast, the membrane conductance of control oocytes injected with combined synthetic α-, β-, γ-, and δ-subunit sense mRNAs was 3.1 ± 0.5 μS, and the current–voltage relation was fairly linear as in noninjected oocytes.

Length of Antisense RNAs and Potency of Repression. To examine the length of antisense RNA required for repressing the expression of functional AcChoRs, α-subunit antisense RNAs of different lengths were synthesized by using linearized DNAs at different restriction sites as illustrated in Fig. 3*A*. Each antisense RNA, having the same 5′ end but different 3′ ends, was injected into oocytes together with whole *Torpedo* electroplaque mRNA, and the oocytes were tested electrophysiologically. The antisense transcripts from the DNA linearized with *Hin*dIII or *Pvu* II abolished almost completely the expression of functional AcChoRs, and this inhibition appeared to be stable for more than 1 week (Fig. 3*B*). The *Hin*dIII antisense RNA (about 1810 bases) would cover all of the protein coding sequence and some of the 5′ and 3′ untranslated portions of the α-subunit mRNA, whereas the antisense RNA from the *Pvu* II-digested DNA template (about 1500 bases) would leave the 5′ untranslated sequence and 60 codons of protein coding sequence of the α-subunit mRNA exposed. In contrast, the antisense RNA truncated at the *Pst* I site (about 420 bases), which would cover only the 3′ untranslated region and about 1/11th of the protein coding sequence of the α-subunit mRNA, was less potent in inhibiting the expression of AcChoRs, and the inhibition was significantly reversed 9 days after injection. Thus, it is not necessary to cover the 5′ untranslated region and/or the sequence around the initiation codon to block the translation of the α-subunit mRNA; but hybridization to the 3′ untranslated sequence and/or the small portion of 3′ coding sequence is not sufficient for potent repression.

Antisense RNA Concentration and Repression Potency. To determine the amount of antisense RNA required to block the expression of functional AcChoRs, different amounts of the complete α-subunit antisense RNA were injected into the oocytes. About 10 ng of the antisense RNA per oocyte was

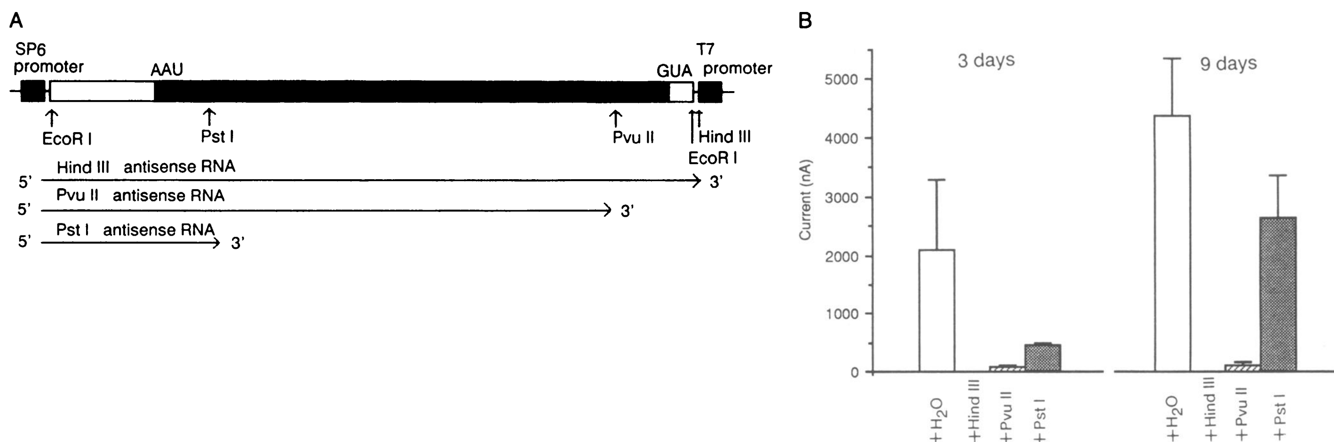

FIG. 3. (*A*) Schematic representation of linearized plasmid used as a template for synthesis of partial-length antisense RNAs. (*B*) Effect of different-length antisense α-subunit RNAs on the mean sizes of AcCho-activated membrane currents in *Xenopus* oocytes. The oocytes were injected with *Torpedo* mRNA alone or with antisense RNAs and were examined 3 or 9 days after injection. Responses were measured from records similar to those in Fig. 2. In each frame, columns (left to right) give measurements from oocytes injected with *Torpedo* mRNA alone or *Torpedo* mRNA with *Hin*dIII antisense RNA (no current), *Pvu* II antisense RNA, or *Pst* I antisense RNA. Each column represents the mean ± SEM of three to six determinations.

sufficient to block the expression of AcChoR by 99.2% in the oocytes that had been coinjected with about 50 ng of whole *Torpedo* mRNA (Fig. 4). Assuming that about 2.4% of the total mRNA in *Torpedo* electric organ is AcChoR mRNA (22), 50 ng of total mRNA would contain about 0.48 ng of the α-subunit mRNA and 0.24 ng of each of the other subunit mRNAs.

Nevertheless, 1 ng of antisense RNA was still very potent in blocking the appearance of functional AcChoRs (by >95%); even with 0.1 ng of antisense RNA, the expression was reduced to 22.1% of the control value. It should be noted that, in the last instance, the concentration of antisense RNA would be much lower than that of the target-sense mRNA, whereas repression in other systems frequently requires the antisense RNA to be in great excess. The unexpectedly large inhibition with 1 ng (and particularly with 0.1 ng) of antisense RNAs may be accounted for, at least partly, if in the oocyte an excess of the α subunit is required for the efficient assembly of functional AcChoRs, as is the case in muscle AcChoR (23).

Repression by a Synthetic Oligonucleotide. To assess further the regions of the α-subunit mRNA that can be covered for efficient inhibition of the expression of functional AcChoR, we synthesized an oligonucleotide (3′ CTTTGTGCAAACCAACGAT 5′) that is complementary to the coding sequence for amino acid residues 4–10 of the α subunit. Injection of the oligonucleotide together with whole *Torpedo* mRNA into the oocytes almost completely abolished the appearance of functional AcChoRs (Fig. 5). The mean AcCho-activated current was only 27 nA, as compared to about 2000 nA in control oocytes injected with *Torpedo* mRNA alone. Since it has been shown that injection of a large amount of DNA into *Xenopus* fertilized eggs is toxic (24, 25), it could be thought that the inhibition we observed was due to unspecific toxic effects. However, this oligonucleotide was much less effective in blocking the functional expression of cat muscle AcChoRs (about 47% inhibition) in oocytes injected with denervated cat muscle mRNA (see ref. 6). Furthermore, oocytes injected with *Torpedo* mRNA and the oligonucleotide were still able to express the Cl^- channel as efficiently as oocytes injected with *Torpedo* mRNA alone. Thus, it is very likely that the oligonucleotide inhibited the expression of the *Torpedo* AcChoR in a specific manner.

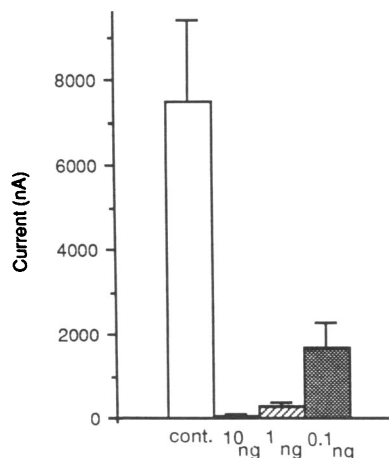

FIG. 4. Relation between the amount of anti-α-subunit RNA injected and the mean size of the AcCho-activated current in oocytes 4 days after injection. Bars (left to right) give measurements from the oocytes injected with *Torpedo* mRNA alone or *Torpedo* mRNA mixed with 10 ng, 1 ng, or 0.1 ng of anti-α-subunit RNA. Each column represents the mean ± SEM of five to seven measurements.

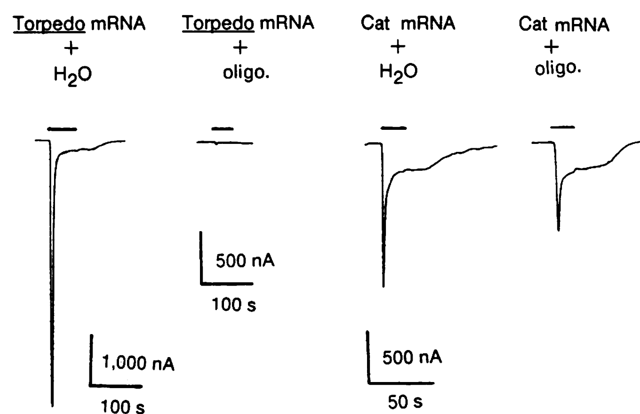

FIG. 5. Repression of AcChoR synthesis by an oligonucleotide. AcCho-activated currents in oocytes injected with *Torpedo* mRNA alone (≈50 ng), with *Torpedo* mRNA and oligonucleotide (≈150 ng), with cat mRNA (≈50 ng) alone, and with cat mRNA and oligonucleotide (≈150 ng). AcCho (100 μM) was applied by bath perfusion during the time indicated by bars. The oocytes were examined 3 days after injection.

DISCUSSION

We have shown previously that *Xenopus* oocytes injected with mRNA derived from *Torpedo* electric organ acquire functional AcChoRs and voltage-activated Cl^- channels on their surface membrane and that this depends on the translation of two different mRNAs (21). We now find that AcChoR subunit antisense RNAs block the expression of functional AcChoRs (Table 1) but not that of the Cl^- channels. This result alone indicates that the antisense RNAs specifically inhibit the expression of the AcChoR, a conclusion that is strengthened by the observation that receptors and channels expressed by other mRNAs were not inhibited by the *Torpedo* AcChoR subunit antisense RNAs. For example, when mRNA extracted from denervated cat muscle is injected into oocytes, it induces the appearance of muscle AcChoRs and voltage-activated Na^+ channels in the surface membrane (6, 26). This induction was not greatly affected by the injection of any AcChoR subunit antisense RNAs (unpublished results). Incidentally, this result suggests that the mRNAs coding for the *Torpedo* electric organ and cat muscle AcChoRs do not have sufficient homology to enable them to form very stable hybrid molecules between the *Torpedo* AcChoR antisense RNA and the cat muscle AcChoR mRNA.

It is known that an antisense RNA injected into *Xenopus* oocytes forms a hybrid molecule with the corresponding mRNA and prevents its translation (27, 28). Furthermore, it has been shown that the entire antisense RNA is not required to inhibit the translation of the mRNA, although it is most effective (see ref. 1). In the case of globin, a 45-base antisense RNA covering only the 5′ untranslated region of the mRNA and an antisense RNA that exposes only the 5′ untranslated region were as effective as the entire mRNA in blocking translation (27). In contrast, antisense RNAs that were complementary to the 3′ half of the protein coding sequence and/or 3′ untranslated sequence were unable to prevent translation. Therefore, it was suggested that the 5′ region of the mRNA must be covered by the antisense RNA to prevent translation effectively (27). A similar conclusion was reached with the thymidine kinase and chloramphenicol antisense RNAs (7). Our results (Fig. 3*B*) again suggest that an important antisense region for repressing the translation of the AcChoR's α-subunit mRNA is the 5′ region of the mRNA, but we show further that it is not necessary to cover the ribosome binding site or the initiation codon. For instance, the antisense RNA from the *Pvu* II-digested α-subunit cDNA, which does not cover the ribosome binding

site nor the AUG codon, was still quite effective in inhibiting the expression of functional AcChoRs. Interestingly the oligonucleotide (19-mer) designed to hybridize to the coding sequence for amino acid residues 4–10 of the α subunit was practically as effective as the entire antisense RNA.

In contrast to the observations mentioned above, a 270-base antisense RNA complementary to only the 3′ end of the mRNA coding for ribosomal protein L1 repressed translation as effectively as the entire (1300 bases) antisense RNA (28), whereas a 140-base antisense RNA that covered the 5′ untranslated region and the initiation codon was much less effective. These results appear to indicate that the accessibility of the target mRNA to the antisense RNA and its ability to form a stable hybrid molecule are critical factors for repressing translation, regardless of the region of complementarity.

Although all of the four subunit antisense RNAs that we used were effective in repressing translation, there was always some residual AcChoR activity, even when a large excess of antisense RNA was injected. Furthermore, the responses to AcCho appeared to increase with longer incubation times. This was most obvious in the case of the oocytes injected with the δ-subunit antisense RNA. At present we are unable to explain the basis for these observations. However, some residual activity might result if AcChoR molecules consisting of only three subunits are able to form functional receptors by themselves or by replacing the missing subunit with another subunit. In this context it should be noted that oocytes injected with combinations of three subunit-specific mRNAs that included the α-subunit mRNA have been shown to respond to AcCho, although the currents elicited were much smaller than those obtained with the complete AcChoR molecule (29). The largest response (about 10% of that of the complete AcChoR) was obtained from oocytes injected with the combination of α-, β-, and γ-subunit antisense mRNAs. This is consistent with our findings that the δ-subunit antisense RNA was the least effective in preventing the appearance of the functional AcChoR in the oocyte's surface membrane.

One of our objectives was to see how general is the inhibition of mRNA translation with antisense RNAs and to see if this could be applied to the study of oligomeric neurotransmitter receptors. We have demonstrated that the functional expression of the multisubunit AcChoR can be inhibited with any one of the subunit-specific antisense RNAs and that this inhibition was fairly stable for at least 1 week. Furthermore, we have shown that it is not necessary to use a full-length antisense RNA for efficient inhibition and that even a small synthetic oligonucleotide is effective in preventing translation.

Another important objective was to test the possibility of using antisense RNA for screening a cDNA library. A most efficient way of screening is to use hybridization probes such as oligonucleotides and antibodies. However, this requires the prior purification of the desired protein, which in the case of some neurotransmitter receptors is very difficult. Since *Xenopus* oocytes are very sensitive detectors of specific mRNAs coding for neurotransmitter receptors and voltage-operated channels (6, 17, 26, 30, 31), they can be used to screen a cDNA library for the genes encoding their structure. For that purpose, a library can be constructed by using a vector containing the SP6 or T7 promoter, or both, to allow the synthesis of sense or antisense RNA by *in vitro* transcription. The sense RNAs can be directly injected (cf. refs. 9 and 29) into the oocytes to test their ability to express the desired receptor, or the antisense RNAs can be coinjected with the whole mRNA, containing the desired receptor mRNA, into the oocytes to examine their ability to inhibit the expression of the receptor. When the expression of a functional receptor requires the synthesis of more than one type of protein subunit, an approach with antisense RNA may be of advantage, since we have shown that any one of the subunit antisense RNAs will inhibit the expression of functional receptors. Furthermore, as we have shown, full-length cDNA clones are not a necessary requirement for inhibition by antisense RNA. In contrast, when using sense RNA, full-length cDNA clones must be obtained, and all subunit mRNAs may need to be injected into the oocyte for expression of functional activity. All this might hinder the use of sense mRNA screening in the cloning of some heterooligomeric receptors or membrane ionic channels.

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