Cloning the Acetylcholine Receptor Genes

The cloned genes give a first look at the full amino acid sequences of the proteins that form the receptor for a neurotransmitter

Within the past few years, neurobiologists have begun adding the techniques of molecular biology to their tools for exploring the nervous system. A recent case in point is the cloning of the genes coding for all four of the proteins that make up the acetylcholine receptor in the electric organ of the *Torpedo* fish.

This receptor is perhaps the best understood of the neurotransmitter receptors. These structures, which are located on the membranes of neuronal target cells, specifically recognize and bind the chemicals released by firing neurons, thus converting a chemical signal to a response in the target. For example, binding of acetylcholine to its receptors on *Torpedo* electric organ cells causes them to fire. The receptors are also located on muscle cells, which contract in response to the neurotransmitter, and on brain cells. Despite their central role in the transmission of nerve impulses, much is still unknown about the synthesis, assembly, and operation of receptors, even the one for acetylcholine.

Having the cloned receptor genes in hand should open up a number of lines of investigation that would otherwise be difficult, if not impossible. For example, before the gene cloning, no complete amino acid sequence was available for any of the acetylcholine receptor proteins, primarily because it was too difficult to get enough of the scarce material to analyze. But it is relatively easy to determine the nucleotide sequences of genes, from which the amino acid sequences of the protein products can be deduced. This has now been done for the cloned genes, giving a first look at the complete structures of the four proteins.

Partial sequences of the *Torpedo* receptor proteins were available, however, and were instrumental in the success of the cloning work. The *Torpedo* receptor has a molecular weight of about 250,000. The four protein subunits are designated α, β, γ, and δ. There are two α subunits and one each of the other three in the receptor. A few years ago, Michael Raftery, Michael Hunkapiller, Catherine Strader, and Leroy Hood of the California Institute of Technology determined the sequences of the first 54 to 56 amino acids (counting from the amino terminals) of each of the four subunits.

Eric Barnard of Imperial College, London, and Katumi Sumikawa and his colleagues at the G. D. Searle Research Laboratories in High Wycombe, England, have now cloned the gene coding for the α subunit from *Torpedo marmorata*. Shosaku Numa of Kyoto University and his colleagues have cloned the genes for the α, β, and δ subunits from *T. californica*. And James Patrick, Stephen Heinemann, and their colleagues at the Salk Institute have cloned the gene for the γ subunit of *T. californica*.

In cloning, investigators make a library of cDNA's (complementary DNA's), copied by the enzyme reverse transcriptase from the messenger RNA's (mRNA's) of the appropriate cells. Picking out the cDNA coding for a specific scarce protein from the many hundreds in the library is a major problem. It was here that knowledge of the partial sequences proved helpful.

The sequences of the possible mRNA's that direct the synthesis of the protein segments can be predicted and the corresponding DNA segments are synthesized to be used as probes to fish out the desired clone from the cDNA library. (Because the genetic code is degenerate, there will be several possible mRNA and DNA sequences.)

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Both the British and Japanese groups used the knowledge of the partial amino acid sequences of the receptor subunits to produce short synthetic DNA fragments that helped them to identify the cDNA clones corresponding to the proteins. They then determined the nucleotide sequences of these clones.

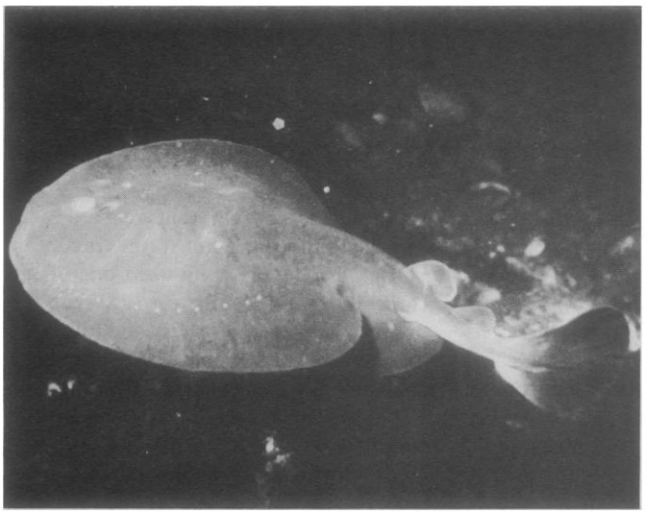
The Salk group used a different method to pick out the right cDNA's for sequence determination. In their initial screen of the library, which contained 960 clones, they identified 31 clones as receptor gene candidates by looking for clone sequences that were more abundant in electric organ cDNA than in cDNA from *Torpedo* brain. They then examined 14 of these to see which hybridized with mRNA that directed the synthesis of protein that reacted with specific antibody against the receptor. Patrick and Heinemann used one of the clones that passed this test to screen the original library, ultimately identifying a total of six clones that appeared to be copies of the same messenger.

All the investigators confirmed that they had in fact identified clones of genes coding for receptor proteins by comparing the amino acid sequences specified by the DNA's with those determined by the Caltech workers.

There was excellent agreement between the complete sequences determined for the two α subunits. "The sequences determined in Japan and in Britain agree with a few minor differences due to the species difference," Barnard says. The sequences of the four subunits of the *T. californica* receptor differed from one another, but there were regions of homology, confirming what was already suspected from the partial sequences. On average, about 35 to 40 percent of the amino acids of the proteins were homologous, and where substitutions did occur one amino acid was often replaced by another that was chemically similar. The homologies are distributed throughout the protein molecules but are most pronounced in the beginning two-thirds, according to the Numa group. These findings suggest that the genes may have originated by the duplication of a common ancestor gene.

In contrast to the similarities in the amino acid sequences, there was no detectable hybridization between the DNA's coding for the α and γ subunits, Heinemann says. This does not mean that the two genes are not derived from a common ancestor, however. "Because the code is degenerate, the DNA sequence can change during evolution without changing the amino acid sequence," Heinemann explains.

Somewhat surprisingly, the α subunit clone sequenced by the Japanese workers turned out to code for a protein containing 437 amino acids, not including the signal sequence which is 24 amino acids in length. (Signal sequences, which are attached to the beginning of proteins, help to direct the newly synthesized molecules to their final destinations in or out of the cell and are then clipped off.) Both the British group and the Salk workers, who have recently



John McCosker, Steinhart Aquarium

Torpedo californica

This ugly beast is a favorite source of acetylcholine receptors and now of genes.

sequenced an α subunit clone, obtained the same result. A protein with that many amino acids would have a molecular weight slightly in excess of 50,000, whereas the published values are around 40,000 for the intact protein, including the carbohydrate residues. All the receptor subunits carry carbohydrates.

Either the published values are incorrect which is possible because the methods for determining protein molecular weights sometimes give inaccurate results, especially for glycoproteins, or the mRNA for the α subunit or the protein itself may undergo processing. The Numa group notes two locations, one at amino acids 313 and 314 and the other at amino acids 330 and 331, that would be especially susceptible to protein-hydrolyzing enzymes. If the molecule were clipped there, the size of the protein would be consistent with a final molecular weight of 40,000. The β subunit, with 469 amino acids and a calculated molecular weight of almost 54,000 is also somewhat larger than expected; its molecular weight is usually given as 50,000 for the glycoprotein.

The gene for the γ subunit, according to Patrick and Heinemann, codes for a protein of 489 amino acids with a molecular weight of 57,000. The Japanese workers find that the δ subunit consists of 501 amino acids; its molecular weight is about 57,500. These figures are consistent with the previously determined values of 60,000 and 65,000 for the mature γ and δ proteins with their carbohydrate residues.

The ultimate goal is to use the structural information now being acquired to achieve a better understanding of how neurotransmitter binding to a receptor signals the cell to make a response. Researchers have already found in the case of the acetylcholine receptor, that binding opens an ion channel that permits movement of small ions through the membrane, thus depolarizing the cell. There is no good picture of how this happens, however.

Knowledge of how the receptor proteins fold themselves during insertion into the membrane and combine with one another to form a complete receptor should help in this regard. As Patrick points out, "If you know the amino acid sequence of a protein, you can deduce something about how it folds up." For example, he and Heinemann have identified in the α subunit protein four segments that are very hydrophobic and likely to be imbedded in the membrane and also segments that are more hydrophilic and thus likely to be on the inner or outer membrane surfaces.

Additional features of interest include the amino acids to which the sugar residues are attached and the binding site for acetylcholine itself. The Numa and Barnard groups have located the same asparagine residue as a possible site of carbohydrate attachment in the α subunit, and the Salk workers have identified three asparagine residues that are likely sites of attachment in the γ subunit.

Acetylcholine binds to a region of the subunit that must be located on the outer cell surface. The Numa group has made the suggestion, based on the new structural data plus earlier evidence, that a sequence of 25 amino acids that centers around residue 135 of the α subunit may be the site of acetylcholine binding. They find this sequence to be very highly conserved in the α, β, and δ subunits.

It should be possible to pinpoint the amino acids participating in acetylcholine binding, opening of the ion channel, and other functions of the receptor by producing specific alterations in the genes and determining how this affects the workings of the receptors. "It is the most unequivocal way of determining which parts are needed for which function," Barnard says of this idea. The roles of the individual subunits can be assessed, now that all four genes are in hand, by introducing the genes singly or in various combinations into cells, such as *Xenopus* eggs, that do not normally make receptors.

According to Barnard and Sumikawa, when receptor protein mRNA is injected into *Xenopus* eggs, the messenger is translated. The resulting receptor proteins insert themselves into the egg cell membrane where they assemble to form a functional receptor, which both recognizes acetylcholine and opens an ion channel in response to it.

Because of their relative scarcity, comparatively little is known about the acetylcholine receptors of mammals. The *Torpedo* gene clones may help remedy this situation by serving as probes to identify and clone the mammalian genes, Heinemann points out. "We already know that the *Torpedo* DNA clones hybridize with mammalian DNA," he says, although the Salk workers do not yet know whether the hybridizing mammalian DNA codes for acetylcholine receptor proteins. Barnard and Sumikawa have found that their *Torpedo* clones do not hybridize with mRNA for acetylcholine receptor proteins that was prepared from cat muscle. Even if the *Torpedo* clones cannot be used as probes for the mammalian genes, investigators can apply similar cloning approaches to the mammalian genes.

At present, no one knows how many genes there are for acetylcholine receptor proteins or how their expression is controlled during development. For example, there are indications that the receptors of immature muscle, which are distributed more or less uniformly over the cell surface, differ from those of mature tissue, which are clustered under the nerve endings. The cloning work should help to clarify the molecular nature of these differences. In addition, comparison of the sequences of receptor proteins from various species and tissues should shed light on the evolutionary history of the proteins.—**JEAN L. MARX**

Additional Readings

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