DEPARTMENT OF THE ARMY
HEADQUARTERS, US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000

REPLY TO
ATTENTION OF

OCT 3 0 2013

Freedom of Information/
Privacy Act Office

Mr. John Greenewald
[redacted]

Dear Mr. Greenewald,

This letter is in response to your Freedom of Information Act (FOIA) requests dated September 24, 2013 and letter of referral from Defense Technical Information Center dated October 23, 2013. In this response, DTIC released one document to the US Army Medical Research and Materiel Command (USAMRMC) for review and release.

Release of document ADP008883 entitled "Cloning of the Human Brain (TTX-Sensitive) and the Human Cardiac (TTX-Insensitive) Na + Channel cDNAs" is attached.

Your request was processed in accordance with the Freedom of Information Act (FOIA), 5 United States Code (U.S.C.) § 552.

Sincerely,

Sandra J. Rogers

Sandra J. Rogers
Freedom of Information/Privacy Act Officer
U.S. Army Medical Research and
Materiel Command

DEFENSE TECHNICAL INFORMATION CENTER
8725 JOHN J. KINGMAN RD, STE 0944
FT. BELVOIR, VA 22060-6218

IN REPLY
REFER TO: DTIC-R (FOIA 2013-170)

OCT 23 2013

MEMORANDUM FOR CDR, USAMRMC

SUBJECT: Freedom of Information Act (FOIA) Request

Reference: Freedom of Information Act (FOIA) request from Mr. John Greenewald, (attachment 1)

Release of document ADP008883, entitled, "*Cloning of the Human Brain (TTX-Sensitive) and the Human Cardiac (TTX-Insensitive) Na+ Channel cDNAs*", can only be released by the appropriate controlling activity. The controlling activity currently identified on the document is U.S. Army Medical Research and Development Command. Therefore, we are forwarding this request to you for processing and direct response back to Mr. Greenewald. We have notified him of this action (attachment 2). A copy of document ADP008883 is provided at attachment 3.

Should your review of the above document result in a determination to delimit it (make available to the public) or a determination that the distribution statement should be changed, please advise this office in writing so we may mark our records accordingly.

The category of request was "other." To date, Mr. Greenewald has incurred no assessable fees for services from DTIC. Please call me at (703) 767-9204 if you have any questions.

FOR THE ADMINISTRATOR:

MICHAEL A. HAMILTON
FOIA Program Manager

Attachments:
As stated

Hamilton, Mike CIV DTIC R

From: John Greenewald, Jr. <john@greenewald.com>
Sent: Tuesday, September 24, 2013 12:49 PM
To: FOIA
Subject: FOIA REQUEST

To whom it may concern,

This is a non-commercial request made under the provisions of the Freedom of Information Act 5 U.S.C. S 552. My FOIA requester status as a "representative of the news media." I am a freelance television producer often working on documentaries related to my FOIA requests, my work is commonly featured throughout major news organizations, and I freelance writer for news sites as well. Examples can be given, if needed.

I hope a full fee waiver for this request can be considered, because all documents received will be available FREE OF CHARGE in their entirety on http://www.theblackvault.com. This site, run entirely by me, has been around for 17 years, and literally served more than 8,000 people a day on average. I have a unique way of disseminating this information, and can offer additional details, if needed.

I prefer electronic delivery of the requested material either via email to john@greenewald.com or via CD-ROM via postal mail. Please contact me should this FOIA request should incur a charge.

I respectfully request a copy of the following document:

Title: (U) Cloning of the Human Brain (TTX-Sensitive) and the Human Cardiac (TTX- Insensitive) Na+ Channel cDNAs

View Full Text: (pdf) - 554 KB -

https://www.dtic.mil/DOAC/document?document=ADP008883&collection=ac-tr&contentType=PDF&citationFormat=1f

File:/UL/p008883.pdf

Accession Number: ADP008883

Personal Author(s): Hartmann, Hali A ; Helm, Amelia T ; Brown, Arthur M

Corporate Author: BAYLOR COLL OF MEDICINE HOUSTON TX

Report Date: 13 May 1993

Att 1

Pages:11 Page(s)

Report Number: XA - USAMRDC (XA)

Monitor Series: USAMRDC

Contract/Grant/Transfer Number: DAMD17-91-C-1093 (DAMD1791C1093)

Thank you so much for your time, and I am very much looking forward to your response.

Sincerely,

John Greenewald, Jr.

[redacted]

[redacted]

Sincerely,

John Greenewald, Jr.

The Black Vault

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DEFENSE TECHNICAL INFORMATION CENTER
8725 JOHN J. KINGMAN RD, STE 0944
FT. BELVOIR, VA 22060-6218

IN REPLY REFER TO: DTIC-R (FOIA 2013-170)

OCT 23 2013

Mr. John Greenewald
[redacted]

Dear Mr. Greenewald:

This is in response to your email dated September 24, 2013, requesting information under the Freedom of Information Act (FOIA) (enclosure 1). Under Department of Defense rules implementing the FOIA, published at 32 CFR 286, your request was categorized as "other."

The document that you have requested, ADP008883, entitled, "*Cloning of the Human Brain (TTX-Sensitive) and the Human Cardiac (TTX-Insensitive) Na+ Channel cDNAs",* is limited to U.S. Government agencies and their contractors only; therefore, your request has been forwarded to the organization below for processing and direct response back to you. Please direct all future correspondence related to document ADP008883 to.

CDR, USAMRMC
ATTN: MCMR-SG
504 Scott Street
Fort Detrick, MD 21702-5012

To date, there are no assessable fees for services from DTIC. Please understand that other members of the public may submit a FOIA request for copies of FOIA requests received by this office or the names of those who have submitted requests. Should such occur, your name and, if asked for, a copy of your request will be released; however, your home address and home telephone number will not be released. Other private citizens who have obtained your name by using such a request may contact you. However, correspondence from the DoD about your request will be on official letterhead. If you have any questions, please call me at (703) 767-9204. Thank you for your interest in obtaining information from DTIC.

Sincerely,

MICHAEL HAMILTON
FOIA Program Manager

Enclosure

Atc 2

Cloning of the Human Brain (TTX-Sensitive) and the Human Cardiac (TTX-Insensitive) Na^+ Channel cDNAs

Hali A. Hartmann, Amelia T. Helm, and Arthur M. Brown

Department of Molecular Physiology and Biophysics
Baylor College of Medicine, Houston, TX 77030

Abstract

Tetrodotoxin and saxitoxin, two of the most potent toxins known to man, could potentially be used as biological warfare agents. Lethality could result from respiratory and cardiovascular failure due to the blockage of ion conduction through sodium channels. Presently, there are no antidotes to protect U.S. soldiers after exposure. Brain and skeletal (innervated) muscle have a high affinity for these toxins; while cardiac tissue sodium channels are relatively insensitive. In addition, kinetic difference also exist between these two classes of sodium channels. The structural basis for these differences can elucidated mainly through the combined disciplines of molecular biology and electrophysiology. The full-length cDNAs for these two proteins have been cloned and are in preparation for expression in xenopus oocytes and a mammalian cell expression system. The toxin phenotype will then be elucidated by the electrophysiological analysis of mutant cDNAs prepared by site-directed mutagenesis. From this data base, structures of possible antidotes may be determined, analogous compounds identified, and their effects tested by electrophysiological methods.

This work was supported by the U.S. Army Medical Research and Development Command under Contract DAMD17-91-C-1093.

COMPONENT PART NOTICE

THIS PAPER IS A COMPONENT PART OF THE FOLLOWING COMPILATION REPORT:

TITLE: Proceedings of the Medical Defense Bioscience Review (1993) Held in Baltimore, Maryland on 10-13 May 1993. Volume 3.

TO ORDER THE COMPLETE COMPILATION REPORT, USE AD-A275 669.

THE COMPONENT PART IS PROVIDED HERE TO ALLOW USERS ACCESS TO INDIVIDUALLY AUTHORED SECTIONS OF PROCEEDING, ANNALS, SYMPOSIA, ETC. HOWEVER, THE COMPONENT SHOULD BE CONSIDERED WITHIN THE CONTEXT OF THE OVERALL COMPILATION REPORT AND NOT AS A STAND-ALONE TECHNICAL REPORT.

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INTRODUCTION

Tetrodotoxin and saxitoxin are among the most potent toxins known to man, as nanomolar quantities in the nervous system block conduction in sodium channels and can cause death from resultant respiratory and cardiovascular failure. These toxins could be used as biological warfare agents. Presently, there are no known pharmacological antidotes which would protect soldiers after exposure. Mechanical life-support equipment to support the respiratory and cardiovascular systems could be employed in the event of toxin poisoning; however, such measures would not typically be available in a field situation.

Through the combined disciplines of molecular biology and electrophysiology, it may be possible to elucidate exact location and structure of the receptor sites for tetrodotoxin and saxitoxin. The first phase of this research project is to obtain the cDNAs for both human brain and cardiac sodium channels.

Brain and peripheral nerve sodium channels are tetrodotoxin sensitive (TTX-S) and have a high affinity for the toxins. Conversely, cardiac sodium channels have low affinity for the toxins or are insensitive (TTX-I). The wildtype cDNAs will be expressed in xenopus oocytes and in a mammalian expression system for electrophysiological studies. In the second phase, the structural basis for these differences in toxin phenotype and other structural properties will be elucidated through site-directed mutagenesis experiments on residues in and/or near the ion conducting pathway. Finally, from this data base, possible antidotes could be designed. For example, analagous compounds may be identified or site-specific antibodies could be made which serve as vehicles for antagonists.

We report here the cloning of the cDNAs for the full-length human brain and cardiac sodium channels. The experimental design for site-directed mutagenesis and the expression in the mammalian cell line, HEK-293 (human embryonic kidney) is discussed.

MATERIALS AND METHODS

Human Heart Sodium Channel. A human fetal heart library from Clonetech (oligo(dT)-primed) has been screened with probes designed de novo which were homologous to the S5-S6 linker domain I and the III-IV linker of rat heart sodium channel cDNA (2 x 10^9 cpm/µg DNA). Standard molecular biology protocols were followed according to Maniatis et al., (1990), Clonetech (1992), and Amersham (1992). The probes were hybridized to the filter lifts at 64 C O/N in 1x SSPE (0.15M NaCl, 0.01M $NaH_2PO_4H_2O$, 1mM EDTA). The filters were washed under low stringency conditions with 2x SSC for 1 hr at 33C (0.3M NaCl, 30mM Na_3 Citrate, pH 7.0) followed by a higher stringency wash in the 2x SSC at 53 c for 5 min. From these 0.5 x 10^6 pfu screened, five positive clones were isolated. The lambda DNA was isolated from the phage according to the Sephaglas PhagePrep kit (Pharmacia) and purified by Magic Lambda Prep (Promega). The clones were ligated into the pBluescript SK+ phagemid at the EcorI sites after initial sequence data from the PCR products ligated into the TA vector (Invitrogen). Two of the clones had high homology to the S5-S6 linker region of domain I and the III-IV linker segment of the rat heart cDNA. Random-labeled probes were made de novo from these two clones and 1

million pfu of the fetal heart library were screened. Hybridization and wash conditions were similar except temperatures were adjusted for the new Tm's of the probes. Eight positive sodium channel clones were found; these clones were mainly cDNAs of the same segments found from the first screen. The total cDNA isolated after the second screening corresponds to nucleotide 646-1910 and nucleotide 3780-6070. The initiation codon is at position 151 and the termination codon at position 6201.

A third library screening of 2.4 million pfu was done with four 49mer (base pair) probes synthesized de novo from oligonucleotides homologous to the following adult cardiac sodium channel nucleotides (Gellens et al., 1992): probe #1 - pos.317 to 365 (5'end), probe 2 - pos.2487 to 2535, probe 3 - pos.3294 to 3332, and probe 4 - pos.6412 to 6190. From this screening, at least 18 positive sodium channel cDNAs were isolated. Partial clones were isolated; two clones aligned with residues from 1900 to 3780 and one clone aligned with residue 5478 to 7576. The remaining positive clones were short fragments representing the 3'end half of the cDNA. The total fetal heart sodium channel cDNA isolated thus far corresponded to the adult heart sodium channel cDNA nucleotide sequence 320-7526. Therefore, approximately 170 bases were needed at the extreme 5'end (inclusive of the initiation codon) for a complete open-reading cDNA.

The library had been screened three times for a total of approximately 4×10^6 pfu without successfully cloning the 5'end of the clone. To expedite the completion of a full-length cDNA of the human heart sodium channel, we used the polymerase chain reaction (PCR) to clone the 5'end. From frozen biopsy tissue of diseased adult ventricular myocardium (Baylor College of Medicine, Department of Cardiology), myocytes were isolated by enzymatic digestion (collagenase) and total RNA was extracted by a modification of the guanidinium method of the RNA Stat kit (Tel-Test "B", Inc). First-strand cDNA was made following the protocol of the GeneAmp kit (Perkin Elmer). Primers were designed from oligonucleotides to match positions 123 to 151 and 2535 through 2507 of the published adult cardiac sodium channel sequence. Standard PCR techniques were used for these PCR reactions (Ehrlich).

Figure one shows the nine clones we have used to construct the full-length cDNA. The front half of the clone was constructed as follows: PCR #1 was ligated to clone #28 at a BsabI site at position 2261, clone #28 was ligated to clone #32 at the EcorI site at 2382, clone #32 joined clone #42 at the Eco0109I site at 3059, and clone #62 joined #42 at the BspEI site at residue 3420. The back half of the clone was ligated together at the following residues: the KpnI site at 4375 joined clones 8 and 11, and the BsaaI site at 6000 in clones 11 and 25. The front and back halves were joined at the NdeI site at 3890 in clones 8 and 62.

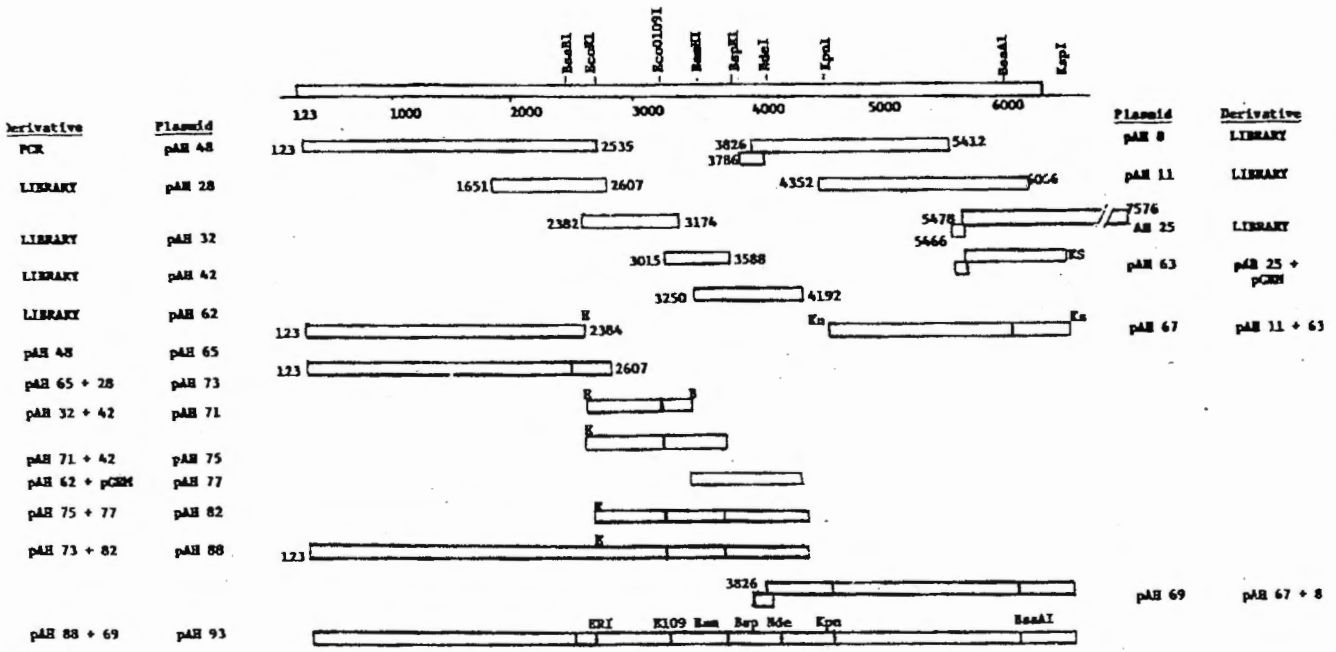

FIGURE 1

Assembly of Human Cardiac Sodium Channel cDNA. Nine clones from the fetal heart library and the PCR product from adult ventricular myocardium were used to assemble the full-length clone. The restriction sites at the top of the chart were used to assemble the partial clones.

Shown in Figure two is the alignment of our full-length human cardiac sodium channel with that published by Gellen et al.,(1992). The PCR product has four amino acid changes from the published clone. Valine at amino acid residue 120 is now an isoleucine and is in the intracellular segment prior to the S1 in domain 1, alanine at residue 180 is a glycine in our PCR product and is located in the intracellular loop between S2 and S3 of domain 1. Two residues in the large loop between domain I and II have been changed: the arginine at residue 552 has been changed to glycine and the threonine at residue 559 has been changed to an alanine. In clone 42 there are five amino acid differences: the histidine at residue 987 is now a glutamine, the glutamine at 1027 is now an arginine, the tryptophan at residue 1085 is an glycine, arginine at residue 1087 is an glutamine, and the glycine at residue is an alanine. All of these residues are located in the intracellular loop between domain II and III. Our clone does not have the second glutamine at residue 1077. There appears to be a sequence error at this residue in the Gellen clone as alignment of that sequence with all known sodium channel cDNAs to date necessitates a gap at this residue for alignment (personal observation).

Human Brain Sodium Channel. Standard molecular biological techniques were used to prepare (from frozen brain tissue) the randomly-primed human brain library in lambda gt10 phage (Maniatis et al., 1990). The cDNA clones were flanked with EcorI linkers and ligated into the pBluescript SK(-) phagemid (Stratagene). The library was screened with P^{32} ATP end-labeled probes made from restriction fragments of the rat brain type III sodium channel (see above for procedures). Approximately 50 positive clones were isolated, from which partial sequence data showed homologies with the three rat brain cDNAs. These ranged in size from 0.5kb to 4.0kb. From double-stranded sequencing of these positive clones by the dideoxy chain-termination technique (United States Biochemical), five clones were chosen which had the greatest alignment with rat brain type II sodium channel (87% homology) and most of these clones overlapped with each other to a substantial degree. 97.2% of the coding region of the human brain sodium channel type II was represented by these five clones. Two of the clones were most likely digested into two clones by EcoRI during the creation of the library. The last 150 bases were never screened successfully from this library; there was also an EcorI site located here as well as in the rat brain type II clone.

We were able to complete the full-length human brain type II sodium channel by PCR, since the primary sequence of a human brain type II sodium channel had recently been published by Ahmed (et al., 1992). The amino acid sequence of the DNA segments needed to complete our full-length clone were identical with our rat brain type II clone, with the exception of one amino acid in the last 50 amino acids before the stop codon. This difference was corrected to that of the published human sequence in the design of one of the forward PCR primers. Forward and reverse primers were designed which included the nearest unique restriction site where the PCR fragments would join with the respective clones. A few extra bases were then included for stable attachment of the enzyme during digestion prior to ligation to the clone (Hartmann et al, 1992). The PCR fragments were filled in with T4 DNA polymerase to ensure blunt-ends prior to enzyme digestion.

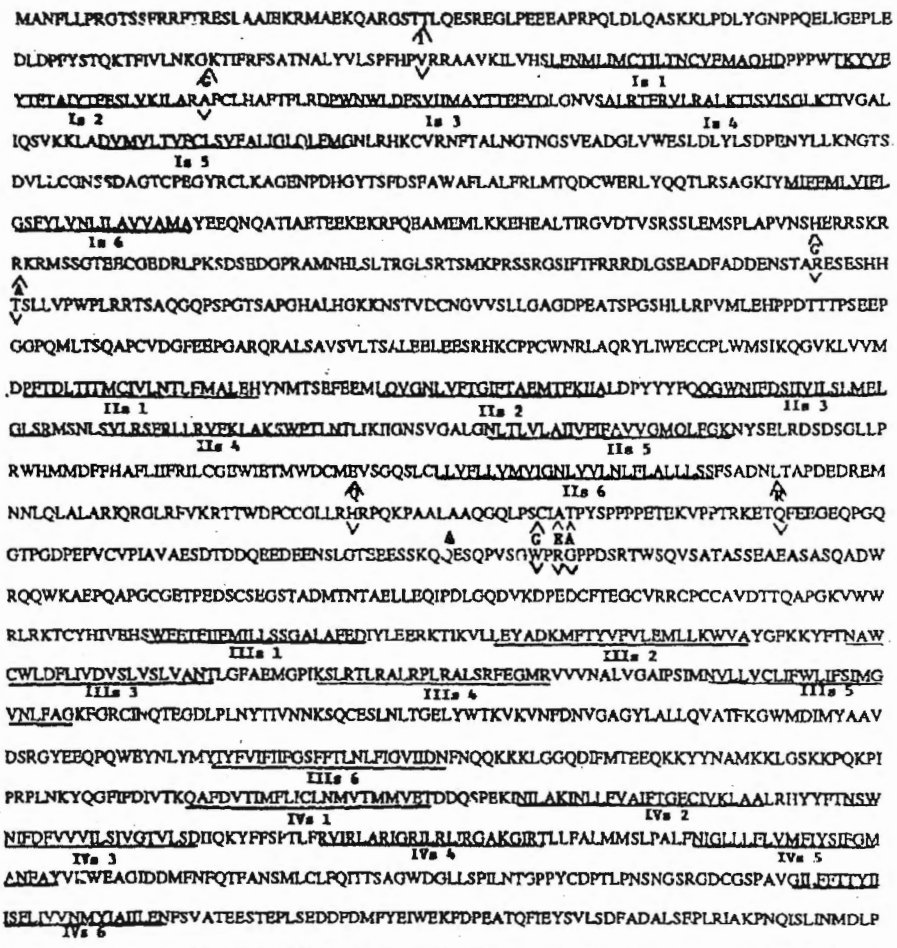

FIGURE 2

Amino acid sequence of human cardiac sodium channel cDNA. The amino acid sequence of our full-length clone is aligned with that published by Gellen et al. The nine amino acid differences found in our clone are shown above (◇). The (▲) symbol denotes the glutamine residue absent in our clone and probably results from a sequence error in the published clone. The open-reading frame consists of 2016 residues.

To date, over half of the full-length human brain sodium channel has been assembled before the final linkage of the front half of the clone with the back half (Fig. 3). Clone 58 is joined to clone 17 through a BglII site at position 3353; clone 17 will be ligated to clone 14b through two different SpeI sites, 3436 and 3625, by way of two PCR fragments (#1 and #2); clone 14b will ligate to clone 1a by the BpmI site at 4345; and clone 1a will be linked to clone 47 by the BstxI site at 4859; and the last 50 amino acids to the stop codon and 400 bases of the 3'untranslated region are linked to clone 47 by the EcorI site at 6077 through PCR fragment #3. The 5'untranslated region (156 bases) has been deleted, as there are several initiation codons before the codon surrounded by the eucaryotic initiation sequence (Kozac sequence; Kozac, 1981). These methionines will produce short and nonsense peptides when translated if they are retained in the cDNA; therefore, the 5'untranslated region will include only the Kozac sequence. This step was also completed by PCR (PCR #4).

Our full-length brain sodium channel cDNA is 99.8% homologous to the published human brain type II cDNA. There are only three amino acid differences: L524R, A1325V, and L1768V (Fig. 4). Residue 524 is in the intracellular loop between domain I and II, residue 1325 is at the C-terminus end of transmembrane segment IIIS4, and residue 1768 is located in transmembrane segment IVS6.

The assembly of the full-length clones have been done in the pGEM-3 plasmid vector (Promega). The pBluescript phagemid vector has been problematic in recombination and deletions of DNA in other cloning efforts by others in our department. We have used a new strain of bacteria created by Stratagene, the SURE strain, whose genotype is highly recombination-deficient and thus decreases the frequency of these events.

DISCUSSION

The efforts of the first phase of this research project have mainly been expended on the preparation of the full-length human cardiac and brain sodium channel cDNAs. Cloning lengthy cDNAs from cDNA libraries can be a laborious effort if the library is not of high quality and contains only short cDNAs. This situation is usually caused by a low-quality reverse transcriptase. Unfortunately, we encountered this problem in the cloning of the cardiac cDNA; i.e., a library with short cDNAs and over-representation at the 3'end of the sodium channel cDNA, while the 5'end was underrepresented. However, we were fortunate to have access to human cardiac biopsy tissue, and were thus able to produce high quality total RNA to synthesize first strand cDNA and then PCR the missing 5'end of the cDNA.

During the preparation and assembly of the two full-length human clones, we did preliminary experiments on the rat brain type IIa sodium channel cDNA. Other laboratories have encountered substantial rearrangement and deletion problems with this clone during subcloning procedures, e.g., mutagenesis, low yields, and deletion problems during growth of the plasmid in liquid culture (personal communication). We now have established protocols for mutagenesis in these lengthy cDNAs and have successfully completed several mutations. The Amersham system, developed from the method of Ekstein, et al. (1985), is a reliable procedure. We are presently working on mutagenesis experiments by PCR, which, if successful, will be a more efficient procedure. The presence of infrequent unique

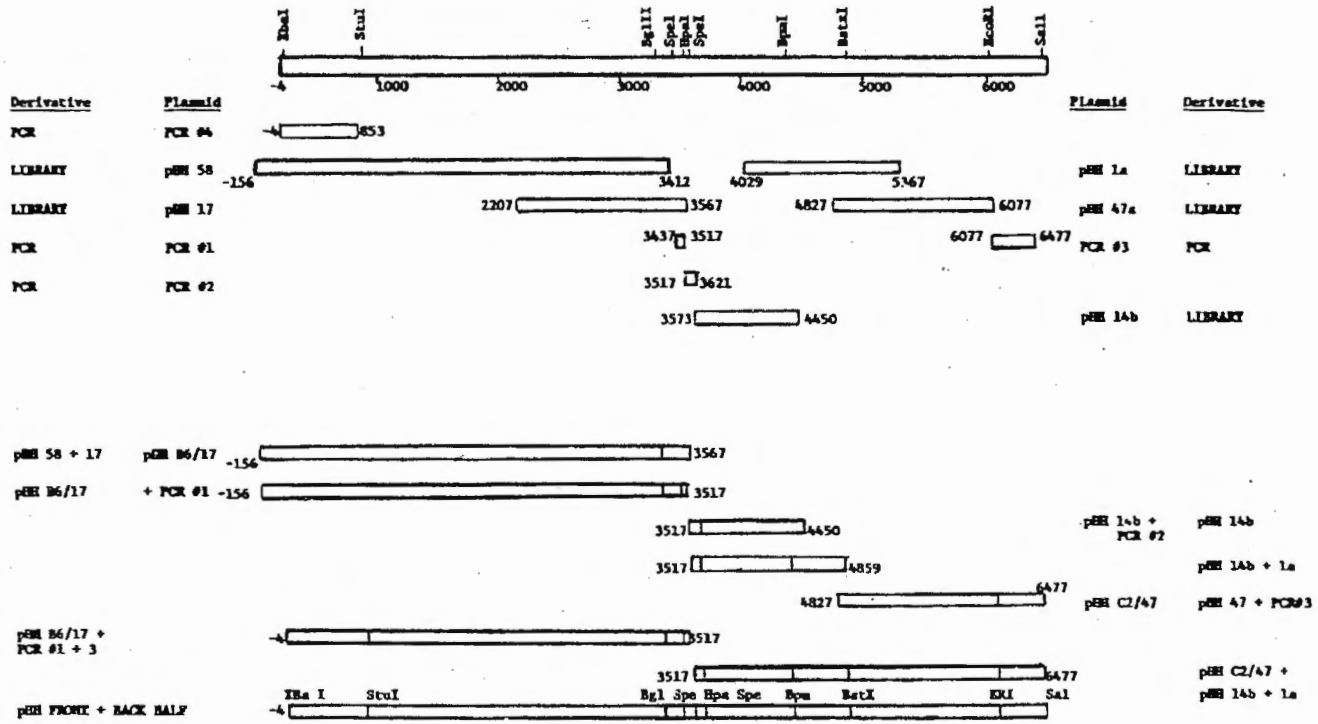

FIGURE 3

Assembly of the Human Brain Sodium channel cDNA. Five clones from the human brain library and four PCR products of rat type IIa brain cDNA were used to assemble the full-length clone. Restriction sites at the top of the chart joined the clones.

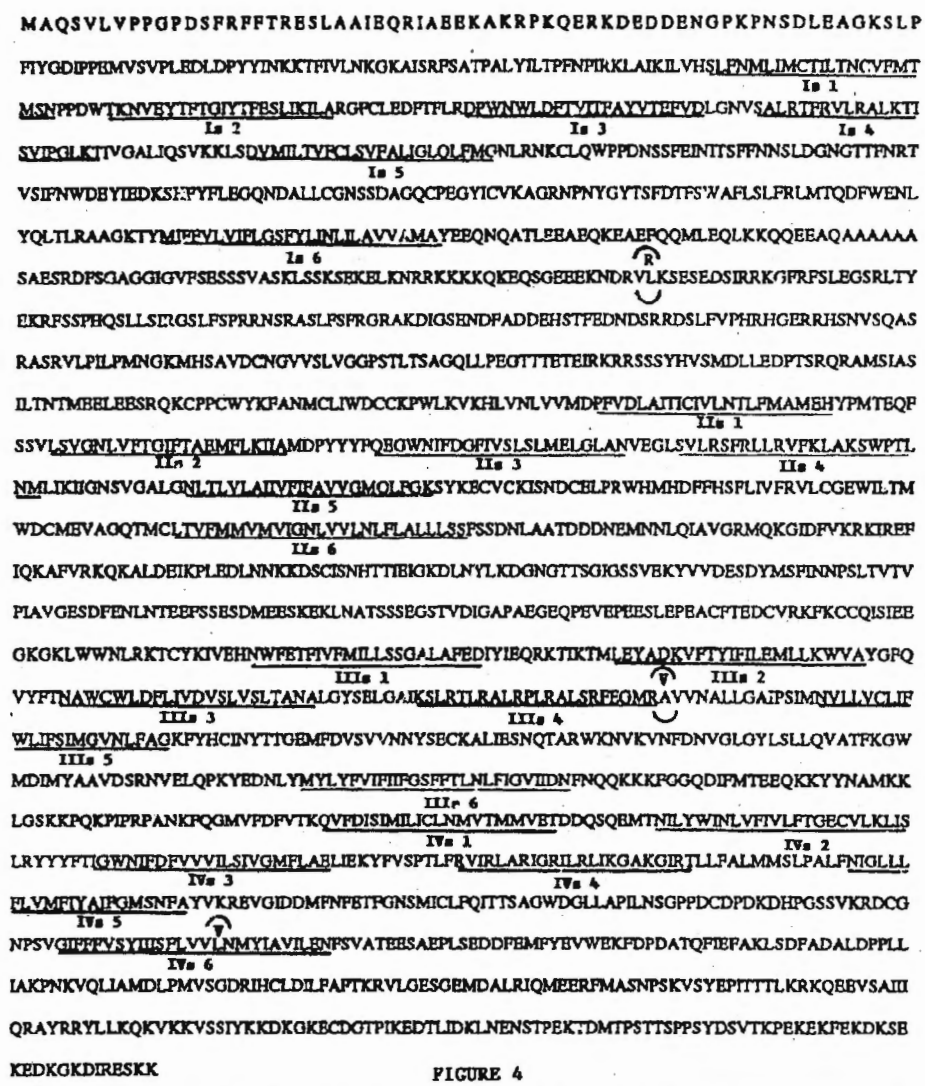

FIGURE 4

Amino acid sequence of human brain sodium channel cDNA. The amino sequence of our clone is aligned with that published by Ahmed et al. Three amino acid differences found in our clone are shown above (◌). The open-reading frame consists of 2005 residues.

restriction sites in a long cDNA necessitates long PCR products (or long inserts to subclone in the Amersham procedure). The tendency of Taq polymerase to incorporate mismatched bases increases with PCR products over 1kb (Erlich, 1986). We are currently experimenting with Vent polymerase (New England Biolabs), which is a high-fidelity polymerase that should reduce incorporation of mismatched bases. If this procedure is successful, mutagenesis experiments on the sodium channel cDNAs can proceed rapidly.

Glycosylation, and perhaps other post-translational events, are well-documented to occur in the sodium channel in vivo. Other subunits of the sodium channel protein are recently documented to interact with the alpha subunit and have effects on kinetics (Kyle et al., 1993; Chanine et al., 1993). In addition to performing electrophysiological studies on sodium currents from our clone in oocytes, we are presently cloning our full-length clones into the pRC/CMV mammalian expression vector for transfection into the mammalian HEK cell line (human embryonic kidney cells) for stable expression. The cells are transfected with lipofectin, a cationic liposome, and resistant clones are selected with G418, genectin. After two to three weeks, the resistant clones become visible and pure clones are selected for electrophysiological studies.

REFERENCES

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Gellens, M.e., George, A.L.Jr., Chen, L., Chanine,M., Horn, R., Barchi, R.L., and Kallen, R.G. (1992) Primary structure and functional expression of the human cardiac tetrodotoxin-insensitive voltage-dependent sodium channel. Proc. Natl. Acad. Sci. USA 89:5564-5582.

Erlich, H.A. (1989) PCR Technology: Principles and applications for dna amplification, Stockton Press, New York, New York.

Ahmed, C.M.I., Ware,D.H., Lee, S.C., Patten, C.D., Ferrer-Montiel, A.V., Schinder, A.F., McPherson, J.D., Wagnew-McPherson, C.B., Wasmuth, J.J., Evans, G.A., and Montall, M. (1992) Primary structure, chromosomal localization, and functional expression of a voltage-gated sodium channel from human brain. Proc. Natl. Acad. Sci. USA. 89:8220-8224.

Hartmann, H.A., Kirsch, G.E., Drewe, J.A., Taglialatela, M., Joho, R. and Brown, A.M. (1991) Exchange of ion conducting pathways between two related K+ channels. Science 251:942-944.

Nakamaye, K. and Eckstein, F. (1986) Nucl. Acids Res. 14:9679-9688.

Kozac, M. (1981) Possible role of flanking nucleotides in recognition of the AUG initiation codon by eukaryotic ribosomes. Nucl. Acids Res. 9:5233-5252.

Kyle,J.W., Chang,S.Y., Satin,J., Fang, J.M., Fozzard, H.A., and Rogart, R.B. (1993) Rat brain β1 Na channel subunit interacts with rat brain IIa, skeletal muscle (μ1), and rat heart (RH1) α-subunits. Biophys. J. 64:376a.

Chanine, M., Bennett, P.B., Horn, R. and George, A.L. (1993) Functional expression of the human skeletal muscle sodium channel. Biophys. J. 64:B5a.