Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 335–339, January 1991
Medical Sciences

Direct amplification of a single dissected chromosomal segment by polymerase chain reaction: A human brain sodium channel gene is on chromosome 2q22–q23

(chromosome microdissection/gene mapping)

JIAN HAN*, CHIUNG-MEI LU†‡, GEORGE B. BROWN†‡, AND THOMAS A. RADO§¶

*Laboratory of Medical Genetics, and Departments of †Psychiatry and Behavioral Neurobiology, ‡Biochemistry, and §Medicine, University of Alabama at Birmingham, Birmingham, AL 35294

Communicated by Ernest Beutler, September 24, 1990

ABSTRACT **We have devised a general strategy for gene mapping based upon the direct amplification of a target sequence within a single microdissected Giemsa-banded chromosomal segment using the polymerase chain reaction. The usefulness of this approach was demonstrated by mapping a cloned human brain sodium channel (α subunit) gene sequence to chromosome 2q22–q23. When DNA from single, dissected chromosome segments 2q21–qter and 2q24–pter were used as templates, a sodium channel-specific 172-base-pair polymerase chain reaction product was obtained. This product was not synthesized when segments 2q21–pter and 2q24–qter were used. Chromosome microdissection–polymerase chain reaction is not only a simple, fast, and accurate method for gene mapping but also may offer significant advantages for other applications, such as cancer cytogenetics and linkage analysis.**

Assignment of human genes or polymorphic DNA markers to specific chromosomal locations is a prerequisite for linkage analysis, location of individual genes, and the definition of their structure and function. In this communication we present a strategy for chromosome mapping that is direct, fast, and simple compared with traditional mapping techniques.

Several methods, including somatic-cell genetics, *in situ* hybridization, and fluorescence-activated cell sorting of metaphase chromosomes, have been widely used to map structural genes and other identifiable DNA sequences onto chromosomes (1, 2). The most commonly used method for identifying the chromosomal locus of a specific human DNA sequence is *in situ* hybridization with radiolabeled probes. This approach is lengthy, labor-intensive, and often difficult to interpret because of cross-hybridization of the probe to related DNA sequences. Further, its mapping precision is limited by a variety of factors, including distortions in banding patterns caused by the preparation of specimens for hybridization and the spatial dispersion of isotopic signals captured on an emulsion overlay. Many of these drawbacks have been obviated by the recent introduction of nonisotopic *in situ* hybridization methods, especially those employing fluorescence visualization (3, 4), but these methods are ill-suited to correlations with conventional Giemsa-banding patterns.

Microdissection of banded chromosomes and enzymatic amplification has been used to clone and sequence selected regions of the human genome (5, 6). When a sequence is known, the reverse of this procedure—i.e., using the polymerase chain reaction (PCR) with microdissected chromosomal segments as templates—could in principle provide a method to localize the known sequence to a particular chromosomal region. Previous studies also have shown that PCR amplification can be used to identify unique DNA sequences from the haploid genome in a single human sperm (7), demonstrating the requisite sensitivity for work at the level of a single chromosomal fragment. Thus, the combination of chromosome microdissection and PCR (CM–PCR) should provide a relatively simple, precise, and direct method for gene mapping. We have tested the method by direct mapping of a voltage-gated sodium channel gene to a single band on human chromosome 2.

The sodium channel is prototypical of many voltage-gated ion channels; it is responsible for the rapid rising phase of the action potential in a variety of excitable cells, including human brain neurons. This important integral membrane protein has been intensively studied in animal models, especially the rat, where it has been characterized electrophysiologically, pharmacologically, and biochemically (8–10). In recent years, cDNA sequences encoding the major functional α subunit from a number of sources, including electric eel and rat, have been cloned. Analysis of these sequences has revealed extensive interspecies homology and sequence conservation (11, 12). It is now clear that at least four homologous subtypes (isoforms) of the sodium channel are expressed in rat brain: types I, II, IIA, and III (12–14). There is very little information currently available concerning the human counterpart of this channel protein (15–17). To address this situation, we devised an approach to clone a human brain sodium channel‖ based upon information accumulated from animal studies.

METHODS

Molecular Cloning and Sequencing of a Human Brain Sodium Channel Gene Fragment. Two oligonucleotide primers (NaCh 6, 5′-AACTCCATGATCTGCCTGTT-3′; and NaCh 7A, 5′-ATGTACATGTTCACCACCAC-3′) were constructed based on the hypothesis that known sodium channel gene sequences exhibiting high interspecies homology and conservation would also be represented in the human sodium channel gene. The final sequence of the primers was chosen from a group of degenerate sequences with the aid of the BIG PROBE computer program, which takes into account species preferences in codon usage (18). NaCh 6 and NaCh 7A were then used to prime PCRs using human genomic DNA as the template. Human genomic DNA was prepared from leukocytes by using standard procedures (19). The PCR mixture

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PCR, polymerase chain reaction; CM–PCR, chromosome microdissection–PCR; RT–PCR, reverse transcriptase–PCR.

¶To whom reprint requests should be addressed at: University of Alabama at Birmingham, 1900 University Boulevard, 513B Tinsley Harrison, University Station, Birmingham, AL 35294.

‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55662).

contained 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP), 100 ng of genomic DNA, and 100 ng of each primer. Immediately before the PCR reaction, 2.5 units of *Thermus aquaticus* (*Taq*) polymerase and 80 μl of mineral oil were added. Thirty cycles of PCR (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) were carried out. The DNA fragment obtained from this reaction was isolated by agarose gel electrophoresis and cloned into bacteriophage M13mp18 and M13mp19 for sequencing by using standard methods (19). Sequence information obtained from this experiment was used to design the nested oligonucleotide primers NaCh 11 (5'-CCAAATTACAACCTCTGCTG-3') and NaCh 12 (5'-GTCCTTCCTATACTACTACA-3') for other studies described below.

Use of PCR on First-Strand cDNA. Reverse transcriptase–PCR (RT–PCR) was used to test for the presence of RNA transcripts corresponding to the gene segment cloned above. Poly(A)$^+$ RNA was isolated from human brain (frontal pole) and T84 cells by the FastTrack method (InVitrogen, San Diego, CA) according to manufacturer's directions. First-strand cDNA was synthesized from 50 ng of each mRNA with 200 units of Moloney murine leukemia virus reverse transcriptase (BRL) using primer NaCh 12 and, as a control, a γ-actin primer (5'-GTACTCCTGCTTGCTAATCCA-3') (20). Second-strand cDNA synthesis and PCR amplification (buffer and conditions as described above) utilized primer NaCh 11 and an upstream γ-actin primer (5'-AAGAGCTACGAGCTGCCCGAT-3') (20–22). The presence or absence of PCR product was evaluated by agarose gel electrophoresis and ethidium bromide staining.

CM–PCR. Giemsa-banded metaphase chromosomes were prepared by standard cytogenetic techniques. Microdissection of chromosomes was performed on a Nikon microscope under ×400 magnification with a long working distance objective. Chromosome dissection was accomplished with borosilicate glass needles pulled to a tip diameter of 0.5 μm or less by using a Kopf model 700 D pipet puller. A micromanipulator (Narishige model MX-1) was used to direct the glass needles to cut the chromosome. Immediately after the cut, the needle was driven downward, under the chromosome, and then moved along its axis to remove and lift the dissected segment from the cutting point to one of the terminals. It is essential to change needles after each cut to prevent possible template contamination in the PCR reaction. The microdissected chromosome fragments were transferred by immersion of the needle tip into 100 μl of PCR reaction mixture [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTP, and 100 ng of primers NaCh 6 and NaCh 7A] in a 500-μl tube. The solution was premixed and filtered by spinning through Centricon 100 to remove any potential template contaminants. *Taq* polymerase (2.5 units) and 80 μl of mineral oil were added before the PCR reaction. After 20 cycles of PCR (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min), 2 μl of the PCR product was added to a reaction mixture containing nested primers NaCh 11 and NaCh 12, and another 30 cycles of amplification were carried out. The use of nested primers was found to increase the specificity of the reactions. The second round of amplification was necessary because the use of only one chromosome in the original PCR reaction will yield $\approx 10^6$ copies of the amplified gene product, an amount that is not enough to detect in an ethidium bromide-stained agarose gel. After second-round amplification, a portion of the product (30 μl) was separated by electrophoresis in 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

Primers NaCh 6 and 7A Identify a Human Sodium Channel Gene Segment. With human genomic DNA as template, PCR

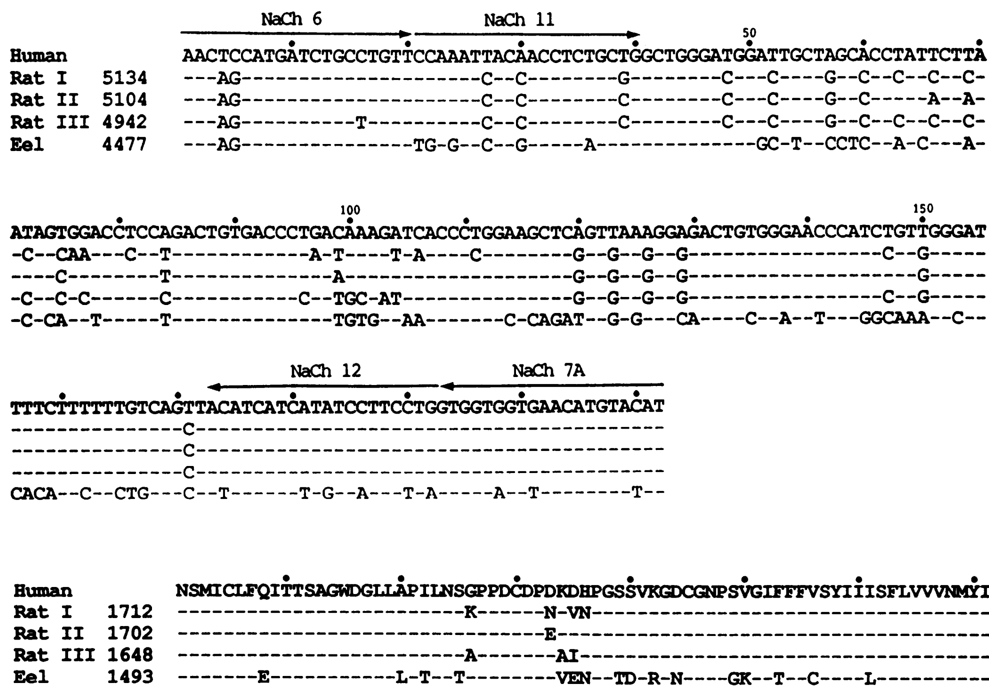

FIG. 1. DNA and amino acid sequence homology of a human brain sodium channel segment with known sodium channels. (*Upper*) Sequence of a region of the human brain α-subunit sodium channel aligned with homologous regions in the rat type I, II, and III and electric eel cDNA. Primers referred to in the text are indicated by lines with arrows marking the 3' end. Oligonucleotides NaCh 6 and NaCh 7A were designed by using the program BIG PROBE (18), so it may or may not represent the true sequences of the gene in these regions. The nested primers NaCh 11 and NaCh 12 were synthesized directly from human sequence. Sequence alignment and data base searching was accomplished with the University of Wisconsin Genetics Computer Group programs (23). Differences between the sequences are indicated by the letter code for the divergent bases. Dashes indicate identical residues. (*Lower*) Deduced amino acid sequences of human and nonhuman sodium channels for the region shown above. Divergent residues are indicated as above. Residues are numbered according to published schemes (11–14).

reactions primed by NaCh 6 and NaCh 7A consistently yielded a single product of the predicted size of 212 base pairs (bp). Sequence analysis of the human-specific 212-bp PCR product reveals that the fragment contains no introns and shares 86%, 91%, 86%, and 68% homology with the type I, II, and III rat brain and electric eel (*Electrophorus electricus*) α-subunit sodium channel genes, respectively (Fig. 1). The homology between the human gene and the rat type II channel is even higher when the deduced amino acid sequences are compared. Only a single difference is found between the two sequences, as depicted in Fig. 1 *Lower*. Comparison of the sequence to the GenBank data base (release 64.0) yielded no significant homologies to other known genes.

The presence of RNA transcripts of this gene in human brain tissue was demonstrated by the use of PCR on first-strand cDNA (RT-PCR) synthesized from various sources (Fig. 2). Primers NaCh 11 and NaCh 12 generated amplification product with human brain mRNA but not with mRNA from T84 cells, a human colonic carcinoma line with well-defined ion currents (24) in which no voltage-gated sodium-mediated excitability has been observed.

Localization of the Gene on Chromosome 2q22–q23 by CM-PCR. In a recent study by Litt *et al.* (17), a putative sodium channel gene was mapped to a broad region of human chromosome 2 (2q21–q33) using a partially characterized genomic DNA probe to screen panels of somatic cell hybrids followed by *in situ* hybridization. Our initial studies were therefore directed to chromosome 2. Fig. 3 shows a metaphase chromosome spread before and after microdissection was performed on its homologous chromosomes 2. As shown in Fig. 4, when chromosome segments 2q21–qter, 2q24–pter, 2q31–pter, and 2q33–pter were used as templates in the CM-PCR protocol, a gene-specific 172-bp PCR product was obtained corresponding to the sequence bracketed by NaCh 11 and NaCh 12. This product was not synthesized when segments 2q21–pter, 2q24–qter, 2q31–qter, and 2q33–qter were used. These results indicate that chromosome segment 2q22–q23 is responsible for the positive PCR results, thereby localizing a human brain sodium channel gene (α subunit) to this region.

DISCUSSION

Compared with the relatively rapid progress of research in recent years on the sodium channel in mammalian animal models (8–10), information concerning this vital mediator of electrical excitability in humans has been slow to appear. Successful cloning of a human sodium channel holds the promise of facilitating the transfer of information gleaned from animal studies to the human case by comparisons of gene and protein structures. Three lines of evidence support our contention that the clone described in this report represents a portion of a human brain voltage-gated sodium channel. The most convincing evidence derives from the extensive sequence homology between this gene and known

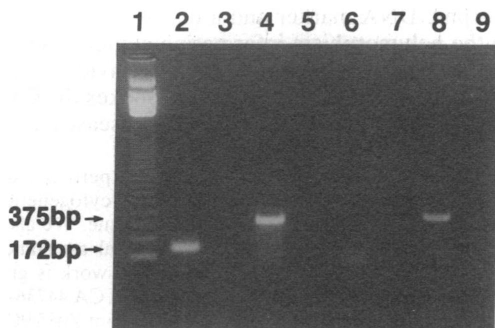

FIG. 2. Expression of sodium channel gene mRNA in human brain. Products from RT-PCR under conditions as noted were analyzed on 1.5% agarose gel stained with ethidium bromide. Lanes: 1, molecular size markers 123-bp ladder (BRL); 2, 172-bp PCR product obtained with frontal pole cDNA and primers NaCh 11 and NaCh 12; 3, negative control, primers NaCh 11 and NaCh 12 without cDNA template; 4, 375-bp PCR product from frontal pole cDNA using actin primers; 5, negative control for actin primers without cDNA template; 6, T84 cDNA with primers NaCh 11 and NaCh 12; 7, control, no template; 8, T84 cDNA with actin primers; 9, control, no template.

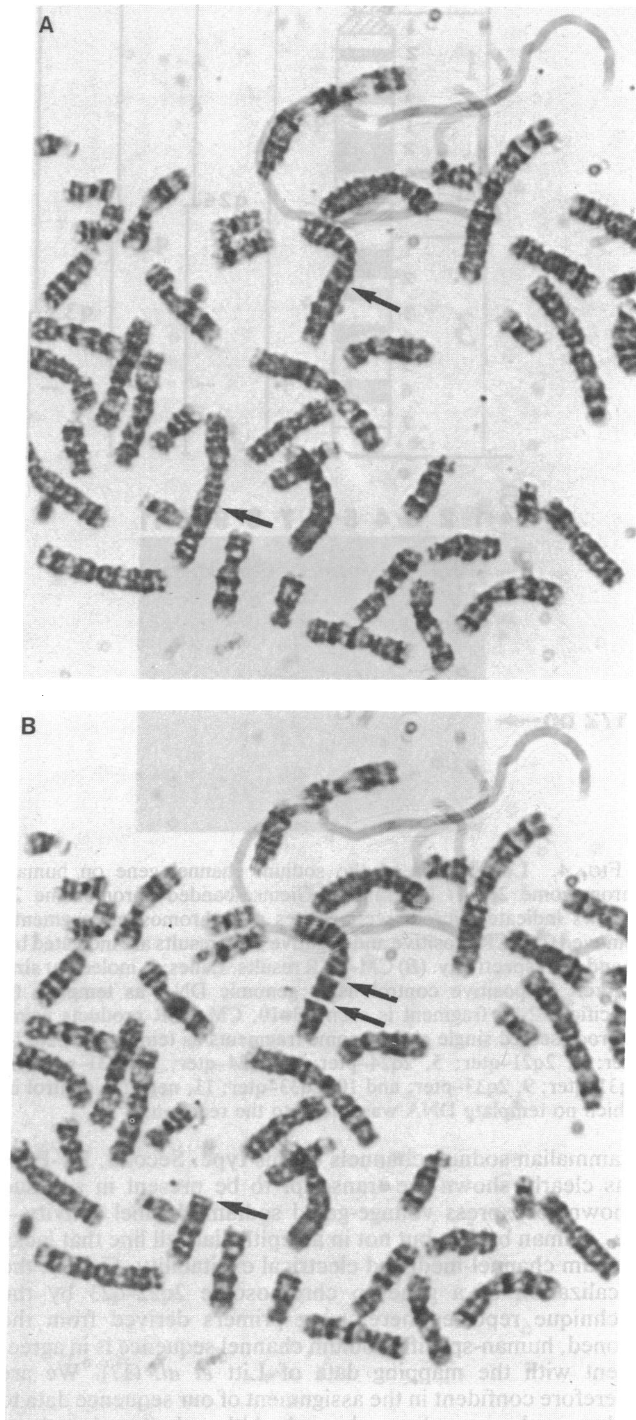

FIG. 3. Chromosome microdissection. (*A*) Metaphase chromosome spread before cutting and removing chromosome fragments. Arrows indicate two homologous chromosomes 2. (*B*) Same chromosome spread after cutting at 2q21 and 2q31 (upper arrows) and after removing segment 2q14–pter (lower arrow).

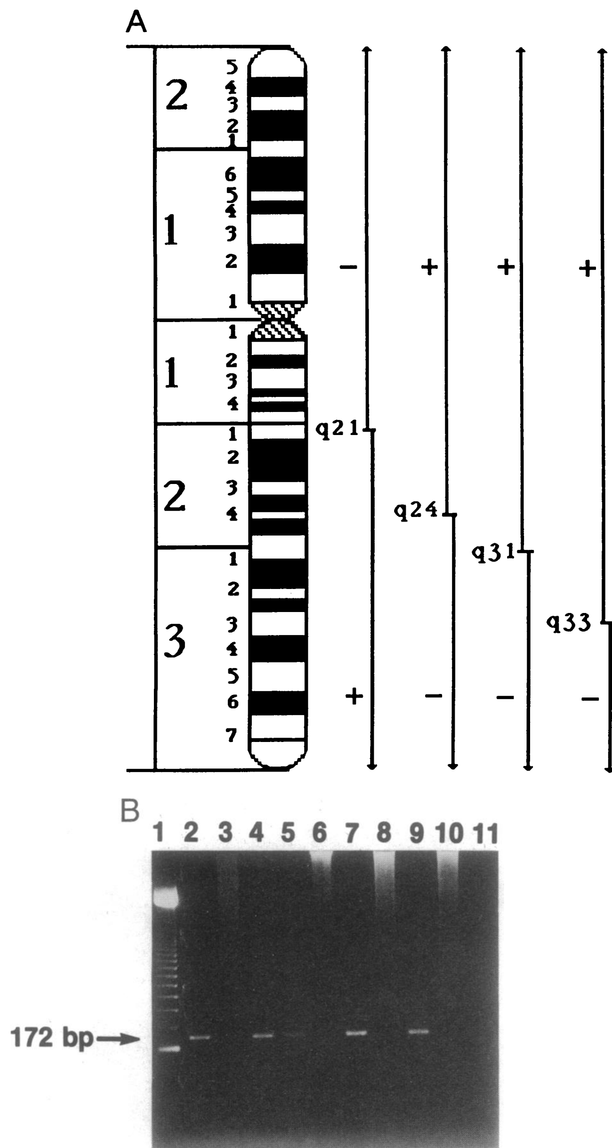

FIG. 4. Localization of the sodium channel gene on human chromosome 2. (*A*) Diagram of Giemsa-banded chromosome 2. Arrows indicate microdissection sites and chromosome segments removed for PCR. Positive and negative PCR results are indicated by + and −, respectively. (*B*) CM-PCR results. Lanes: 1, molecular size marker; 2, positive control using genomic DNA as template (a specific 172-bp fragment is seen); 3–10, CM-PCR products using microdissected single chromosome fragments as template; 3, 2q21–pter; 4, 2q21–qter; 5, 2q24–pter; 6, 2q24–qter; 7, 2q31–pter; 8, 2q31–qter; 9, 2q33–pter; and 10, 2q33–qter; 11, negative control in which no template DNA was added to the reaction.

mammalian sodium channels of this type. Second, RT-PCR has clearly shown the transcript to be present in a tissue known to express voltage-gated sodium channel activity—i.e., human brain—but not in an epithelial cell line that lacks sodium channel-mediated electrical excitability. Finally, the localization of a gene to chromosome 2q22–q23 by the technique reported here using primers derived from the cloned, human-specific sodium channel sequence is in agreement with the mapping data of Litt *et al.* (17). We are therefore confident in the assignment of our sequence data to a human brain sodium channel. Although the cloned sequence shown in Fig. 1 shares a higher homology with the rat subtype II sodium channel gene, it would be premature to relate this sequence to a similar human classification scheme in the absence of data concerning the number and sequences of other possible human subtypes.

The ability to manipulate individual chromosomes and to exploit the remarkable amplification power of the PCR to localize a known sequence on an identified chromosomal segment should provide a useful strategy to complement existing methods for studying genomic structure and organization. The direct application of CM-PCR to gene mapping as demonstrated in this report is but one example that highlights some significant advantages over traditional techniques. (*i*) The process is rapid: analysis of chromosomal segments by CM-PCR requires only 12 hr from microdissection until the PCR product can be identified by gel electrophoresis. (*ii*) The technique uses readily available technology and resources. We have found that archival metaphase spreads, rehydrated with phosphate-buffered saline, provide adequate material for CM-PCR. (*iii*) CM-PCR produces a level of resolution less ambiguous than that often achieved with *in situ* hybridization of isotopically labeled probes. Localization of a gene to a single GTG band on a chromosome provides a resolution of ≈10 megabases.

Other types of genetic studies may also benefit from the CM-PCR strategy because of its ability to study DNA sequences from an individual chromosome or to distinguish between members of a pair of homologous chromosomes on the basis of DNA polymorphisms. In the area of cancer cytogenetic research, CM-PCR techniques could provide a direct approach to the analysis of the abnormalities (deletions, duplications, translocations, and inversions) characteristic of many leukemias and solid tumors (25). Studies dependent upon linkage analysis may also benefit because, using CM-PCR, the linkage phase of two or more linked polymorphic markers can be recognized in any informative individual. For example, if A and B are two polymorphic loci closely linked on a chromosome, and a and b are different alleles, traditional Southern blot analysis cannot distinguish the linkage phase (AB/ab or Ab/aB) of an individual with an AaBb phenotype. But with CM-PCR, the linkage phase of the individual can be easily recognized by using two sets of the locus-specific CM-PCR primers to amplify both loci simultaneously from each of the homologous chromosomes and then identify their allelic states. When the genotype (linkage phase) of informative parents has been defined, recombinations between the loci can be recorded in the next generation, so that the recombination fraction, a measurement of the genetic distance between the loci, can be obtained by a direct counting of the recombinants and nonrecombinants among offspring. Knowing the linkage phase between a polymorphic DNA marker and a disease-related gene can increase the polymorphism information content value of the marker because the probability of any individual being informative is increased (26). This feature makes the CM-PCR approach especially valuable for genetic diseases diagnosis.

We thank Dr. R. J. Bradley for sharing his expertise and equipment for microdissection, Dr. A. J. Carroll for cytogenetic specimens, and Dr. R. E. Powers for human brain tissue. We also thank Messrs. W.-C. Chau and M. Edge for technical assistance. The advice of Dr. W. H. Finley at all stages of this work is gratefully acknowledged. This work was supported by Grant CA 44736-01 from the National Institutes of Health (T.A.R.) and Grant Z085 9C-1 from the Cystic Fibrosis Foundation (T.A.R.).

1. Naylor, S. L., McGill, J. R. & Zabel, B. U. (1987) *Methods Enzymol.* **151,** 279–292.
2. Lebo, R. V. & Bruce, B. D. (1987) *Methods Enzymol.* **151,** 292–313.
3. Lichter, P., Cremer, T., Borden, J., Manuelidis, L. & Ward, D. C. (1988) *Hum. Genet.* **80,** 224–234.
4. Trask, B., Pinkel, D. & Engh, G. (1989) *Genomics* **5,** 710–717.
5. Ludecke, H.-J., Senger, G., Claussen, U. & Horsthemke, B. (1990) *Nature (London)* **338,** 348–350.
6. Johnson, D. H. (1990) *Genomics* **6,** 243–251.

7. Li, H., Gyllensten, U. B., Cui, X., Saiki, R. K., Erlich, H. A. & Arnheim, N. (1988) *Nature (London)* **335**, 414–417.
8. Catterall, W. A. (1980) *Annu. Rev. Pharmacol. Toxicol.* **20**, 15–41.
9. Lazdunski, M. & Renaud, J. F. (1982) *Annu. Rev. Physiol.* **44**, 463–473.
10. Catterall, W. A. (1988) *Science* **242**, 50–61.
11. Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M. A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. & Numa, S. (1984) *Nature (London)* **312**, 121–127.
12. Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. (1986) *Nature (London)* **320**, 188–192.
13. Auld, V. J., Goldin, A. L., Krafte, D. S., Marshall, J., Dunn, J. M., Catterall, W. A., Lester, H. A., Davidson, N. & Dunn, R. J. (1988) *Neuron* **1**, 449–461.
14. Kayano, T., Noda, M., Flockerzi, V., Takahashi, H. & Numa, S. (1988) *FEBS Lett.* **228**, 187–194.
15. de Rycker, C., Grandfils, C., Bettendorff, L. & Schoffeniels, E. (1989) *J. Neurochem.* **52**, 349–353.
16. Tomaselli, G. F., Marban, E. & Yellen, G. (1989) *Clin Invest.* **83**, 1724–1732.
17. Litt, M., Luty, J., Kwak, M., Allen, L., Magenis, R. E. & Mandel, G. (1989) *Genomics* **5**, 204–208.
18. Dubnick, M., Lewis, L. K. & Mount, D. W. (1988) *Nucleic Acids Res.* **16**, 1703–1714.
19. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
20. Erba, H. P., Gunning, P. & Kedes, L. (1986) *Nucleic Acids Res.* **14**, 5275–5294.
21. Frohman, M. A., Dush, M. K. & Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8998–9002.
22. Forhman, M. A. (1990) in *PCR Protocols*, eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Academic, New York), pp. 28–38.
23. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–392.
24. Dharmsathaphorn, K., McRoberts, J. A., Mandel, K. G., Tisdale, L. D. & Masui, H. (1984) *Am. J. Physiol.* **246**, G204–G208.
25. Heerema, N. A., Palmer, C. G. & Baehner, R. L. (1985) *Cancer Genet. Cytogenet.* **17**, 165–179.
26. Thompson, J. S. & Thompson, M. W. (1986) *Genetics in Medicine* (Saunders, Philadelphia), 4th Ed., pp. 193–209.