17. R. S. Hansen, N. A. Ellis, S. M. Gartler, *ibid.* **8**, 4692 (1988).
18. J. Singer-Sam *et al.*, in *Nucleic Acid Methylation*, G. Clawson, D. Willis, A. Weissbach, P. A. Jones, Eds. (Liss, New York, in press); this is volume 128 of *UCLA Symposia on Molecular and Cellular Biology, New Series*.
19. G. P. Pfeifer, S. D. Steigerwald, P. R. Mueller, B. Wold, A. D. Riggs, unpublished results.
20. B. H. Pheiffer and S. B. Zimmerman, *Nucleic Acids Res.* **11**, 7853 (1983).
21. J. R. Rusche and P. Howard-Flanders, *ibid.* **13**, 1997 (1985).
22. G. M. Church and S. Kieffer-Higgens, *Science* **240**, 185 (1988).
23. D. M. Woodcock, P. J. Crowther, W. P. Diver, *Biochem. Biophys. Res. Commun.* **145**, 888 (1987).
24. D. Simon *et al.*, *Nature* **304**, 275 (1983); G. P. Pfeifer, E. Spiess, S. Grünwald, T. L. J. Boehm, D. Drahovsky, *EMBO J.* **4**, 2879 (1985).
25. Supported by National Institute of Aging grant AG08196 (A.D.R.), NIH grants RR07003 and GM355262BW (B.W.), and a fellowship from the Deutsche Forschungsgemeinschaft (Pf212/1-1) (G.P.P.).

11 July 1989; accepted 26 September 1989

Isolation of Human Transcribed Sequences from Human-Rodent Somatic Cell Hybrids

PU LIU, RANDY LEGERSKI, MICHAEL J. SICILIANO*

A method was developed for selectively isolating genes from localized regions of the human genome that are contained in interspecific hybrid cells. Complementary human DNA was prepared from a human-rodent somatic cell hybrid that contained less than 1% human DNA, by using consensus 5′ intron splice sequences as primers. These primers would select immature, unspliced messenger RNA (still retaining species-specific repeat sequences) as templates. Screening a derived complementary DNA library for human repeat sequences resulted in the isolation of human clones at the anticipated frequency with characteristics expected of exons of transcribed human genes—single copy sequences that hybridized to discrete bands on Northern (RNA) blots.

HUMAN-RODENT SOMATIC CELL hybrids are often highly reduced for human genomic content, with the human fraction representing <1% of the total DNA. Regions of the human genome may be isolated by screening DNA libraries made from such hybrids for human-specific repetitive sequences. Whereas this has been an important step in reverse genetic approaches to the cloning of biomedically important genes, subsequent identification of the genes themselves has proven problematic. Preparation of cDNA libraries from mature mRNA produced by such cells, though direct, is not helpful because the repeat sequences that are needed to detect the low percentage of clones containing human sequences are primarily located in introns (which are removed during RNA message maturation) (*1*).

We have developed a strategy using immature transcripts (hnRNA) as templates for cDNA synthesis. Many such cDNAs would contain exon coding sequences as well as introns with human repeat sequences. To enrich for cDNA transcripts of hnRNA in a total RNA population, we began cDNA synthesis with consensus 5′ splice sequences as primer. Such a primer will initiate cDNA synthesis at the 5′ end of an intron (present in hnRNA but not mature mRNA). Complementary DNA synthesis will proceed copying the adjacent 5′ exon and then into the next 5′ intron, which may contain a repeat sequence. Cloning of this cDNA population results in a library in which human repetitive sequences are present in clones representing structural gene transcripts.

We used a human–Chinese hamster ovary (CHO) cell hybrid (20XP3542-1-4) containing the region of human chromosome 19q that carries markers closely linked to the myotonic dystrophy gene (*DM*) (*2*). The human DNA content in the hybrid was determined to be approximately 25 Mb (<1% of total genomic) by slot blot analysis (*3*). Chromosomal mapping of human genomic DNA clones obtained from cosmid libraries made with the hybrid DNA indicates approximately one-half of the human DNA to be from the *DM* region of human chromosome 19q, with much of the remainder DNA from a portion of chromosome 17 (*4*). A cDNA library was constructed from this line by established procedures (*5*) modified according to the strategy outlined above. To selectively use hnRNA as template, as opposed to mature mRNA, and to enrich for coding sequences, we used hexamers complementary to the 5′ splice site as primers. Because of sequence divergence of the 5′ splice site, four hexamers were used that represent the majority of the 5′ splice

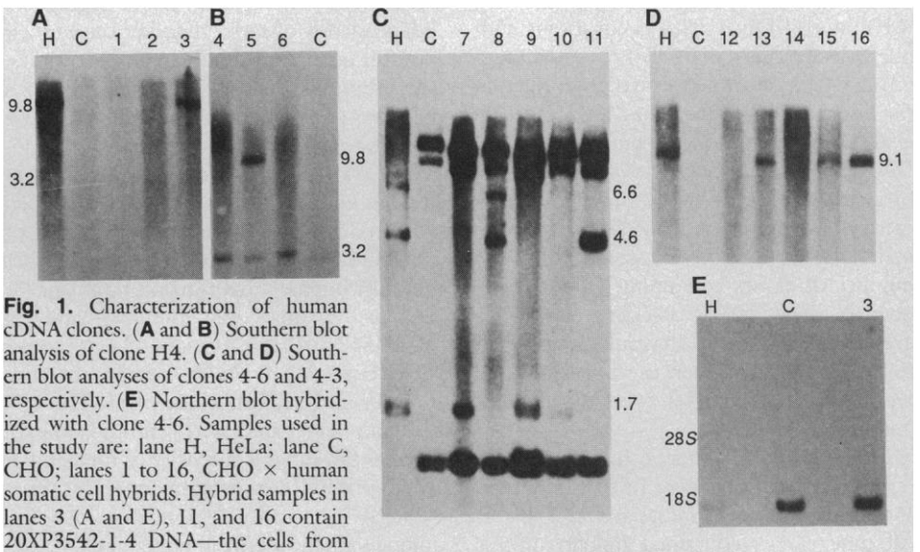

Fig. 1. Characterization of human cDNA clones. (**A** and **B**) Southern blot analysis of clone H4. (**C** and **D**) Southern blot analyses of clones 4-6 and 4-3, respectively. (**E**) Northern blot hybridized with clone 4-6. Samples used in the study are: lane H, HeLa; lane C, CHO; lanes 1 to 16, CHO × human somatic cell hybrids. Hybrid samples in lanes 3 (A and E), 11, and 16 contain 20XP3542-1-4 DNA—the cells from which the cDNA library was made. Putative human cDNA-containing plasmid clones were identified by screening the library with total human DNA. The plasmid DNA was purified, digested with Pst 1, and separated by agarose gel electrophoresis. Insert bands were excised and DNA was isolated from agarose by the squeeze-freeze method (*12*). Insert DNA was labeled with a random prime labeling kit (Amersham). For both Southern and Northern blots, probe DNAs were hybridized with a molar excess of sheared human placental DNA to block hybridization by the repetitive region of the probe (*13*). The Southern blot hybridization was performed according to Amasino (*14*). For the blot shown in (B), the last two washes were done at 60°C, instead of the usual 65°C, to enhance the cross hybridization to CHO DNA. Gel separation of poly(A)$^+$ RNA, Northern blot transfer, and filter hybridization were done by standard procedures (*15*).

Department of Molecular Genetics, The University of Texas, M. D. Anderson Cancer Center, Houston, TX 77030.

*To whom correspondence should be addressed.

Table 1. Characterization of 5′ splice site–primed cDNA library clones isolated after hybridization to total human DNA. Human chromosomal assignment was done by concordant segregation analysis on hybrid clone panels characterized for and informative for every human chromosome (*2*). U, unknown human chromosomal assignment.

Clone	Insert size (kb)	Cross hybridize with CHO DNA	Hybridization with mature RNA from 20XP3542-1-4	Human chromosomal assignment
H2	1.20	−	−	17
H4	1.90	+	+	17
1-1	0.70	−	−	19
1-2	0.80	−	−	17
2-1	1.20	−	−	19
2-2	2.30	−	−	17
4-3	0.55	−	+	19
4-4	0.50	−	+	17
4-6	0.50	+	+	19, 6, U

site sequences (*6*): CTTACC, 50%; CTCACC, 30%; CCTACC, 10%; and CCCACC, 5%. The relative fraction of each hexamer used in the primer mixture is indicated by the percentage, which is roughly proportional to the frequency of its occurrence in sequenced genes.

Approximately 800,000 clones of the library were plated and screened with labeled human total genomic DNA. Approximately 400 positive signals were detected. Thirty positive samples were randomly selected and subjected to a second round of screening, and 20 independent human-positive clones were identified. Therefore, we would predict that approximately 275 human clones (2/3 of 400) were obtained from a library of 800,000 clones (for a frequency of 0.03%) derived from a hybrid cell line containing <1% human DNA. This is consistent with our observation that clones containing CHO repetitive sequences were identified in the library at a frequency of 3% (1% of 3% is 0.03%). Digestion of purified plasmid DNA from these clones with Pst I followed by agarose-gel separation of fragments indicated an average insert DNA size of approximately 1 kb. The insert size affected the number of clones containing repetitive sequences since <25% of clones having an average insert size of 1 kb would be expected to have repetitive sequences [on average one repeat sequence occurs in every 3 to 4 kb of human DNA (*7*)].

Nine of the 20 putative human cDNA-containing plasmids were further analyzed, and the results are summarized in Table 1. All nine clones contained human unique sequences, in addition to repetitive sequences, since they hybridized to specific Hind III fragments in HeLa DNA after Southern (DNA) analysis at high stringency (for example, clone H4 in Fig. 1A). When tested against a characterized hybrid clone panel (*2*), the unique sequences identified by all nine clones segregated concordantly with the human chromosome (17 or 19) segments present in the hybrid from which the library was derived (Table 1). At a slightly lower stringency of hybridization, two clones (H4 and 4-6) hybridized to CHO fragments. Since a full complement of CHO chromosomes are present in all members of the hybrid clone panel, those bands were also present in all hybrids (for example, Fig. 1, B and C). Such cross hybridization is indicative of evolutionarily conserved coding sequences (*8*).

Clone 4-6 identified three CHO Hind III fragments as well as hybridizing to the expected repetitive background in HeLa DNA and three human genomic fragments at 6.6, 4.6, and 1.7 kb (Fig. 1C). The three CHO bands were present in DNA from all hybrid cells, whereas the human bands segregated independently of each other, indicating their location on different human chromosomes. This suggests that clone 4-6 recognizes members of a gene family or a gene that has homologous sequences (perhaps pseudogenes) scattered on other chromosomes in the genome. Its origin from a transcribed portion of the human component of 20XP3542-1-4 is supported by the fact that the most intensely hybridizing fragment (at 4.6 kb) is the only human band in 20XP3542-1-4 (Fig. 1C, lane 11) and segregated concordantly with human chromosome 19 in the hybrid clone panel. The 1.7-kb band segregated with chromosome 6, whereas the variable intensity of the band at 6.6 kb precluded its assignment at this time. Clone 4-3 is typical of one that identifies unique sequences only in human DNA (9.1-kb band in Fig. 1D). It also identified a fragment that segregated with human chromosome 19 (Table 1).

As shown on the Northern (RNA) blot in panel E, clone 4-6 identifies transcribed sequences of approximately the same molecular size in HeLa (weak), CHO, and 20XP3542-1-4 cells. The similar level of intensity of hybridization in the hybrid and CHO cells compared to HeLa is not unexpected considering that the hybrid and CHO cells have different expression characteristics than HeLa cells. Similar patterns of positive Northern blot hybridization were obtained with clones 4-3, H4, and 4-4 when tested against polyadenylated [poly(A)$^+$] RNA extracted from 20XP3542-1-4. Although these results might suggest that the clones are CHO cDNAs isolated because of some level of homology between human and hamster repeat sequences, the Southern blots show them to be of definite human origin. Evidence for this is that prehybridization of probes with total human DNA failed to completely block repetitive background signal in samples containing human chromosomes (HeLa and hybrid) and detected no repetitive signal in CHO controls even though there was no attempt to block putative hamster repeat sequences in the probe. It is also improbable that clones of CHO origin would hybridize to the 25 Mb of human genomic DNA (representing only 1/300 of the human genome) present in 20XP3542-1-4 as did all nine clones studied (for example, samples 3, 11, and 16 representing clones H4, 4-6, and 4-3, respectively, in Fig. 1).

Therefore, all nine clones originated from human chromosomal regions known to be present in the hybrid from which the library was made, and four of them represent regions found in mature transcripts. The fact that five of the clones did not hybridize to Northern blots might be due to the short length of a putative exon or to random priming from introns during cDNA synthesis. However, since average intron length is at least eight times that of exons (*9*), our observation that four of nine of the unique sequence clones detect mature transcripts supports enrichment for cDNA synthesis initiating at 5′ splice junctions in hnRNA.

This technically simple but highly efficient method of isolating species-specific transcribed sequences from somatic cell hybrids should be useful in producing probes for identifying mature transcripts of genes represented in appropriate libraries. Since a computer bank search indicates that approximately half of all 5′ splice sites are identical to the sequences used as primer for first-strand cDNA synthesis, and since estimates indicate that genes with introns likely contain at least one repetitive element (*10*), the expectation is that a segment of most expressed genes can be obtained with this procedure. At present the approach is limited to the isolation of probes for genes actually transcribed in the cultured hybrid cell. Human genes on the chromosomal segment isolated in the hybrid cell that are

not normally expressed in that cell type [for example, the muscle form of creatine kinase, a gene present in the hybrid cell line used here (*2*)] would not be expected to be so expressed. However, this procedure, combined with somatic cell genetic technologies in which chromosomes in one cell type are moved into cells with different expressional characteristics (*11*) should extend its usefulness to other genes in the chromosomal region being studied.

REFERENCES AND NOTES

1. M. F. Singer, *Int. Rev. Cytology* **76**, 67 (1982); C. W. Schmid and W. R. Jelinek, *Science* **216**, 1065 (1982).
2. R. L. Stallings *et al.*, *Am. J. Hum. Genet.* **43**, 144 (1988).
3. P. Liu, R. Legerski, M. J. Siciliano, unpublished data.
4. K. Johnson, personal communication; B. Wieringa, personal communication.
5. RNA was extracted [R. C. Strohman *et al.*, *Cell* **10**, 265 (1977)] and poly(A)$^+$ RNA was purified on oligo(dT) cellulose [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. Four different hexamers complementary to the 5′ splice sites of introns were used as primers for cDNA synthesis. First- and second-strand cDNAs were sythesized as described [U. Gubler and B. J. Hoffman, *Gene* **2**, 263 (1983)]. The double-stranded cDNA was treated with mung bean nuclease to remove hairpin loops at the 3′ ends of the first strands (required since the major fraction of the cDNA was found to contain hairpin loops), and cloned into the Pst I site of pBR322 through homopolymeric tailing [U. Gubler, *Methods Enzymol.* **152**, 330 (1987); W. H. Eschenfeldt, R. S. Puskas, S. L. Berger, *ibid.*, p. 337]. The plasmid cDNA library was transferred into bacteria MC1061 through electroporation with a Bio-Rad Gene Pulser (E. C. Bottger, *Biotechniques* **6**, 878 (1988)]. The efficiency of the library construction was 1×10^6 independent colonies per microgram of RNA.
6. R. A. Padgett, P. J. Grabowski, M. M. Konarska, S. Seiler, P. A. Sharp, *Annu. Rev. Biochem.* **55**, 1119 (1986).
7. H. R. Hwu, J. W. Roberts, E. H. Davidson, R. J. Britten, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3875 (1986); R. K. Moyzis *et al.*, *Genomics* **4**, 273 (1989).
8. S. H. Friend *et al.*, *Nature* **323**, 643 (1986); A. P. Monaco *et al.*, *ibid.*, p. 646; D. C. Page *et al.*, *Cell* **51**, 1091 (1987).
9. J. D. Hawkins, *Nucleic Acids Res.* **16**, 9893 (1988).
10. H. D. Robertson and E. Dickson, *Mol. Cell. Biol.* **4**, 310 (1984).
11. A. M. Killary and R. E. K. Fournier, *Cell* **38**, 523 (1984).
12. R. W. J. Thuring, J. P. M. Sanders, P. Borst, *Anal. Biochem.* **66**, 213 (1975).
13. P. G. Sealey, P. A. Whittaker, E. M. Southern, *Nucleic Acids Res.* **13**, 1905 (1985).
14. R. M. Amasino, *Anal. Biochem.* **152**, 304 (1986).
15. J. G. Williams and P. J. Mason, in *Nucleic Acid Hybridization, A Practical Approach*, B. D. Hames and S. J. Higgins, Eds. (IRL Press, Washington, DC, 1985), pp. 139–143.

16. We thank G. F. Saunders for his reading of the manuscript. Supported in part by NIH grant GM19436, an award from the Muscular Dystrophy Association and Piton Foundation, a gift from K. D. Muller, and a Rosalie B. Hite Fellowship.

17 July 1989; accepted 8 September 1989

Tonic Activation of NMDA Receptors by Ambient Glutamate Enhances Excitability of Neurons

P. SAH, S. HESTRIN, R. A. NICOLL

Voltage clamp recordings and noise analysis from pyramidal cells in hippocampal slices indicate that *N*-methyl-D-aspartate (NMDA) receptors are tonically active. On the basis of the known concentration of glutamate in the extracellular fluid, this tonic action is likely caused by the ambient glutamate level. NMDA receptors are voltage-sensitive, thus background activation of these receptors imparts a regenerative electrical property to pyramidal cells, which facilitates the coupling between dendritic excitatory synaptic input and somatic action potential discharge in these neurons.

IT IS GENERALLY ACCEPTED THAT THE excitability of neurons is controlled by two processes: voltage-gated conductances intrinsic to the neuron and extrinsic influences imposed by neurotransmitters (*1*). In most cases the neurotransmitter is released phasically by depolarization of the presynaptic terminal and is quickly removed from the extracellular space by either rapid uptake or enzymatic degradation. However, the neutrotransmitter glutamate is present in cerebrospinal and extracellular fluid at concentrations from 1 to 50 μ*M* (*2*); this is well within the concentration range known to activate the NMDA subtype of glutamate receptor in cultured hippocampal neurons (*3*), suggesting that the NMDA receptor might be tonically active. However, the concentration of glutamate in close proximity to the NMDA receptor is not known. Furthermore, thus far, the activation of NMDA receptors in the absence of synaptic stimulation has not been reported. Using both conventional recording techniques in 400-μm-thick slices and whole-cell recording techniques in thin slices of hippocampus, we tested the possibility that endogenous resting levels of glutamate exert a tonic action on hippocampal pyramidal cells and also examined what role such an action might have on the functioning of these cells.

Standard procedures were used to prepare and maintain 400-μm-thick slices of hippocampus (*4*). The techniques for whole-cell recording from thin slices were similar to those used by Edwards *et al.* (*5*). Hippocampal slices were held down by a net constructed of nylon filaments and a piece of flattened platinum wire on the stage of an upright microscope and were visualized with Nomarski optics. The CA1 pyramidal cells were voltage-clamped in the whole-cell configuration with a patch clamp amplifier (List Electronics, Darmstadt, West Germany). All drugs were added directly to the bath. Data were collected on-line with a PDP 11 computer. For noise analysis, data were filtered at 1 kHz and digitized as 2048-point samples at 2 kHz. All Lorentzians were fit to the data with a nonlinear least squares algorithm.

We first used the selective NMDA antagonist DL-2-amino-5-phosphonovalerate (APV) (*6*) to look for an APV-sensitive current in pyramidal cells with conventional thick slices. Tetrodotoxin was added to the superfusing medium to block Na^+ currents, and the recording microelectrode was filled with 3*M* CsCl to block K^+ currents. Using single-electrode voltage clamp at −35 mV, we added APV (50 μ*M*), which resulted in a net outward current (Fig. 1A) ($n = 6$). In

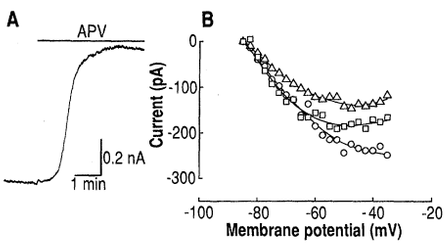

Fig. 1. APV-induced outward currents. (**A**) Membrane current was induced by the application of 50 μ*M* DL-APV in the bath, as indicated by the bar. The holding potential was −35 mV. (**B**) Current-voltage (*I-V*) relations obtained by subtracting the membrane current generated by a slow ramp of membrane potential (4 mV/s) in the absence of APV from that obtained in the presence of APV. The *I-V* curves have been shifted along the current axis to set the APV-sensitive current to 0 pA at −85 mV. Each type of symbol represents data from a different cell. Since the cells were filled with Cs^+ ions it is quite likely, due to space clamp problems, that the membrane potential at sites remote from the soma were less than at the soma.

P. Sah, Department of Pharmacology, University of California, San Francisco, San Francisco, CA 94143.
S. Hestrin, Department of Physiology, University of California, San Francisco, San Francisco, CA 94143.
R. A. Nicoll, Departments of Pharmacology and Physiology, University of California, San Francisco, San Francisco, CA 94143.