Intron sequence directs RNA editing of the glutamate receptor subunit GluR2 coding sequence

JAN EGEJERG*^{†‡}, VALERY KUKKOV*, AND STEPHEN F. HEINEMANN*

*Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA 92037; [†]Molecular Biology, Bioscience, Novo-Nordisk, DK-2880 Bagsværd, Denmark

Contributed by Stephen F. Heinemann, May 19, 1994

ABSTRACT The Ca^{2+} permeability and the rectifying properties of the glutamate receptors assembled from the subunits GluR1–GluR4 depend upon a critical Arg in the GluR2 subunit located in a domain that has been proposed to span the membrane. The GluR2 subunit gene encodes a Gln (CAG) at this position, whereas the mRNA is edited so that it encodes an Arg (CGG) at this position [Sommer, B., Kohler, M., Sprengel, R. & Seeburg, P. H. (1991) *Cell* 67, 11–20]. The editing process is specific since only the GluR2 subunit RNA is edited even though the GluR1, GluR3, and GluR4 RNAs have a similar sequence. We show that this selective RNA editing depends upon a critical intron sequence in the GluR2 gene. This critical intron sequence is sufficient to cause editing of the GluR3 subunit exon in a chimera minigene constructed so that the GluR3 exon is placed upstream to the GluR2 intron sequence. Transfections of a neuronal cell line, N2a, with minigene constructs encoding different fragments of the GluR2 gene demonstrate that the 5' part of the 3' intron is essential for editing. Part of the exon and this critical intron sequence contains an inverted repeat that can fold into a structure consisting of three helical elements. Similar conclusions were reached by Higuchi, M., Single, F. n., Köhler, M., Sommer, B., Sprengel, R. & Seeburg, P. H. [(1993) *Cell* 75, 1361–1370]. These experiments demonstrate that the low Ca^{2+} permeability of the ionotropic non-*N*-methyl-D-aspartate glutamate receptors depends upon RNA editing, which requires a sequence in an intron 3' to the exon.

L-glutamate is the most abundant excitatory neurotransmitter in the mammalian brain. In addition to fast synaptic transmission glutamate is thought to play a role in synaptic plasticity and excitotoxic cell death. These effects are thought to be associated with the Ca^{2+} permeability of glutamate receptors. The ionotropic glutamate receptors can be divided into two pharmacologically distinct classes; the *N*-methyl-D-aspartate (NMDA) receptors and the non-NMDA glutamate receptors. The former exhibit a high Ca^{2+} permeability, and in most neurons studied, the non-NMDA glutamate receptors exhibit a low Ca^{2+} permeability. The cloning of the ionotropic non-NMDA glutamate receptors from rat revealed the existence of at least nine genes encoding subunits that can be grouped into three structurally distinct classes: GluR1–GluR4 (or GluRA–GluRD), GluR5–GluR7, and KA1–KA2 (1). Expression studies have demonstrated that the GluR1–GluR6 subunits and GluR5 or GluR6 coexpressed with the KA2 subunit can generate functional channels permeable to cations. The permeability ratio of Ca^{2+} /monovalent ions and the rectification properties of these channels depend upon which amino acid is located at a critical position in the domain proposed to be transmembrane region (TM) 2 (2–5). When subunits possessing a Gln at this position are coexpressed, the receptors exhibit a high Ca^{2+} /

monovalent permeability ratio and a strong inwardly rectifying current–voltage relationship. If at least one of the subunits in the receptor complex contains an Arg at this position, the Ca^{2+} permeability is almost eliminated and the current–voltage relation is linear. Studies of the genes encoding GluR1–GluR7 demonstrated that all these genes encode a Gln (CAG) at this position (6). However, analysis of the cDNA made from rat brain RNA revealed that >99, 39, or 74% of the GluR2, GluR5, or GluR6 mRNA encodes an Arg (CGG) at this position, respectively (6). Furthermore, GluR6 is edited at two sites in the proposed TM1 where Ile-567 (AUU) and/or Tyr-571 (UAC) are edited to a Val (GUU) and/or a Cys (UGC), respectively (7). The editing in TM1 of GluR6 influences the Ca^{2+} /Cs⁺ permeability ratio of the Gln form but not the permeability ratio for the Arg form (7).

RNA modifications are common for stable RNAs such as tRNAs and rRNA molecules (8). Data on specific posttranscriptional modifications of the bases in nuclear-encoded mRNA have been limited to the transcript of the apolipoprotein B gene (9, 10), the Wilms tumor susceptibility gene *WT1*, a zinc-finger transcriptional regulator (11), and the glutamate receptors genes (6, 7, 12). Apolipoprotein B mRNA editing involves a C → U conversion, which introduces a stop codon. In this case the recognition site for the editing enzyme is limited to the adjacent sequences. Both the nature of the recognition site and the pyrimidine conversion suggest that the apolipoprotein B editing activity is distinct from the purine conversion observed for the glutamate receptor subunits GluR2, GluR5, and GluR6.

METHODS AND MATERIALS

Cell Growth Conditions. The mouse neuroblastoma cell line N2a was cultured in Dulbecco's modified Eagle's medium with Glutamax (GIBCO) containing penicillin (50 units/ml) and streptomycin (50 $\mu\text{g}/\text{ml}$) supplemented with 10% (vol/vol) fetal calf serum. Transfection were performed by the calcium phosphate method (13). Cells were incubated 12–16 h with the DNA precipitate followed by a wash with serum-free medium and PBS prior to harvesting.

cDNA Synthesis. RNA was purified (14). Total RNA (5 μg) was incubated for 1 h with 10 units of RNase-free DNase (Boehringer) and 1 unit of RNasin (United States Biochemical) in 50 μl of 100 mM sodium acetate, pH 5.0/10 mM MgSO_4 to remove residual plasmid DNA. The sample was phenol/chloroform-extracted and precipitated. To check the completeness of the DNase treatment, 50% of the sample was treated with RNase (0.1 mg/ml) for 1 h. cDNA was generated by incubating 10% of each sample (equivalent to 0.25 μg of total RNA) in 10 μl with 10 μM random hexamers (Pharmacia), 0.2 unit of RNasin, 50 unit of Moloney murine leukemia

virus reverse transcriptase (BRL), and all four dNTPs (each at 1 mM) in 10 mM Tris-HCl, pH 8.3/5 mM MgCl₂/50 mM KCl/1 mM dithiothreitol for 10 min at room temperature followed by a 50-min incubation at 37°C. The cDNA was used as source for a PCR amplification by adding 40 µl of PCR buffer (10 mM Tris-HCl, pH 8.3/1 mM MgCl₂/100 mM KCl/0.001% gelatin) containing 50 pmol of the appropriate primers and 2 units AmpliTaq (Perkin-Elmer). The template was amplified for 35–40 cycles. The procedure was repeated if bands were observed in the RNase/DNase-treated sample.

Extension Assay. The amplified fragment was purified from residual nucleotides by electrophoresis on a 1.2% low-melting-temperature agarose gel. The fragment was denatured at 100°C for 5 min in water and cooled on ice. The denatured fragment was mixed with the end-labeled primer [³²P]TTCCTTGGGTGCCTTTA for GluR2 or [³²P]TTCCTTGGGTGCCTTTTCA for GluR3 and buffer (10 mM Tris-HCl, pH 7.8/5 mM MgCl₂/50 mM KCl in 10 µl) and incubated for 15 min at 37°C (see Fig. 1B). The annealed primer was extended by adding 5 µl of extension solution (1 mM dideoxyadenosine 5'-triphosphate, 0.1 mM dCTP, 0.1 mM dGTP and 0.1 mM dTTP, and 1 unit of the Klenow fragment of DNase polymerase I) and incubated for 10 min at 37°C. The reactions were terminated by addition of 10 µl of formamide and heating to 95°C for 3 min. The samples were analyzed on a 15% polyacrylamide gel. The bands were excised from the gel and radioactivity was measured in a scintillation counter. An unedited template was PCR-amplified in parallel as a control to evaluate the "read through." The editing level was calculated as the $[E/(E + U)] - [E_c/(E_c + U_c)]$, where *E* is the edited template, *U* is the unedited template, *c* refers to the bands excised from the control sample. The latter fraction represents the "read through," which is 2–3%.

We observed variations between 24 and 46% in the level of editing for different transfections when primers 1 and 6 were used for the amplification of RNA from cells transfected with the genomic construct. However, <3% variation in editing was observed for the same transfection between different dishes transfected with the same construct. Consequently, all comparisons of different constructs are done at the same transfection.

Generation of Clones. Rat genomic DNA was purified (15) and a part of the GluR2 gene was amplified using the primers 2 and 7 and cloned as a *Bam*HI-*Xho* I fragment into the eukaryotic expression vector JG3.6. The initial 3' deletions were generated by linearizing the clone with *Xho* I and then treated with BAL-31 exonuclease. The reaction was stopped. The DNA was cut with *Bam*HI and fragments of appropriate lengths were selected after electrophoresis and cloned into JG3.6. The shortest clone generated by BAL-31 digestion was used to generate the 3' and 5' mutants. The deleted fragments were generated using restriction sites or by PCR. The chimera between the R3 exon and the R2 intron was generated by overlap extension (16).

RESULTS

Editing of the GluR2 Subunit RNA Is Fast Compared to Splicing and It Occurs Before Splicing. Comparison of the four subunit GluR1–GluR4 sequences around the edited site revealed a high degree of sequence identity, suggesting that elements determining the selectivity of the editing might reside in more distant regions. We isolated a fragment (≈6 kb) containing the exon encoding TM2 and the two flanking introns by a PCR strategy using primers that annealed in the adjacent exons (Fig. 1A and Table 1). The genomic sequence was used to design primers that would amplify distinct splicing intermediates of the GluR2 subunit RNA. cDNA derived from rat cerebellar RNA amplified with primers 3 and 6 should selectively amplify an early state in the processing of RNA

FIG. 1. (A) Structure of the GluR2 gene from T1397 to A1906 (numbers refer to the coding region). The position for edited residue A1820 is indicated by an asterisk. Open or solid bars, exon sequence or vector sequence, respectively; lines, intron sequences. The genomic fragment is flanked by a cytomegalovirus (CMV) promoter and the simian virus 40 small tumor antigen intron. The upstream intron and downstream intron are called A and B, respectively. Numbers indicate locations of the primers used for the amplification (Table 1). (B) Schematic representation of the primer-extension assay. The end-labeled extension primer will be extended by the Klenow fragment of DNA polymerase I to a 21-mer if the cDNA is generated from an unedited template but to a 24-mer if the template is edited. (C) Primer-extension products generated from edited templates (E) or unedited templates (U) were separated by PAGE on a 15% gel. The lowest band is due to residual extension primer. The templates were amplified from rat cerebellar RNA at different states during the splicing (lanes 1–4). Lanes 5 and 6 are extension products from unedited DNA (U p1) or edited DNA (E p1), respectively. Pr indicates the primer set used for the amplification. The intensity of the bands was estimated by measuring the radioactivity in the excised bands in a scintillation counter.

where introns A and B are not yet spliced out (Fig. 1C, lane 1), and amplification with primers 2 and 7 should amplify RNA where both introns are spliced out (Fig. 1C, lane 4). The intermediate splice products where intron B is spliced out but not intron A or visa versa are selectively amplified with primers 3 and 7 or primers 2 and 6 (Fig. 1C, lane 2 or 3), respectively. In agreement with the initial report of the GluR2 editing (6), we observed that the GluR2 mRNA was edited >97% and we found that even the unspliced RNA was edited

Table 1. Oligonucleotides used for the PCR amplification

No.	Sequence
1	GAAATTAATACGACTCACTATAGG
2	CGCGGATCCTTGGGGATGGCAAGTATGGG
3	GCGAATCTTAACCACAATCTGGGTCCTG
4	CCAAGCCCTTCATGAT
5	CTTGGCGAAATATCGCATCC
6	GGCAGCTGGTGACATCTTTATGGTG
7	GCGGATCCTCGAGGAGGAGATTATGATCAGGG
8	TCTAGCATTAGGTGACACTATAG
9	ATAGGTTGGAATCTTAGAGCTTT

Underlined sequences represent restriction sites.

to >97%, indicating that the editing can take place before splicing and that the editing is fast compared to splicing (see *Discussion*).

We confirmed the editing assay by sequencing clones of the amplified products. All clones were edited at A1820 (nucleotides numbered from the initiation codon) but 4 out of 20 clones generated with primers 2 and 7 and 5 of 20 clones amplified with primers 3 and 6 were also edited at another position, A1824. The possibility that the base change at A1824 was due to allelic variation rather than RNA editing was eliminated by the observation that position A1824 was edited in RNA from cells that had been transiently transfected with cloned cDNA (see below).

Editing Requires Intron Sequences. To study the possible role of intron sequences, we cloned the 6-kb genomic fragment from T1397 to A1906 and placed it downstream of a cytomegalovirus promoter. A similar construct without the introns was generated from the GluR2 cDNA clone. Both DNA constructs were transfected into the N2a neuroblastoma cell line and total RNA was purified from the transfected cells and used as template for a reverse transcription-PCR amplification. The primer-extension analysis shows that the genomic transcript is edited to 20% (Fig. 2A, lane 2), whereas the cDNA transcript is edited <3% (Fig. 2A, lane 1), indicating that intron A and/or intron B are necessary and sufficient for editing.

Possible artifacts were eliminated by the experimental design. Contamination with endogenous mouse GluR2 RNA was eliminated by using primers that recognize sequences present only in the transfection vector, primer 1, or present in the rat GluR2 sequence, primer 4. DNA contamination was eliminated by extensive DNase treatment. For the analysis of transfection experiments with constructs containing intron A, the PCR amplification was performed across intron A. In this case, PCR products were isolated corresponding to

transcripts in which intron A had been spliced out, demonstrating that the transfected construct had been transcribed and that the transcript was at least partially spliced in the N2a neuroblastoma cell.

Intron A Is Not Essential for Editing. Deletion analysis was performed to determine which intron sequences are essential for editing. Deletions of intron A were done in combination with full-length intron B. To avoid bias in the estimates of the editing level due to the presence of intron A in the case of the genomic clone, primers 4 and 6 were used for PCR amplification (Fig. 1A). The use of these primers results in the amplification of the transcript whether or not intron A is spliced out. Deletion of the majority of the 5' part of intron A did not change the editing level significantly (from 20 to 17%; Fig. 2A, lane 2 and 3). Further deletion into the exon (187 nt upstream of intron A) reduced the editing to 14%. A mutant with only 11 nt upstream of A1820 showed ≈3% editing, which is the lower limit of our assay. Cloning and sequencing 40 clones from this construct revealed one edited clone.

A Segment of Intron B Is Essential for Editing. To attempt to identify sequence elements in intron B that contribute to the editing of A1820, clones containing various amounts of intron B were generated by BAL-31 exonuclease digestion. Five clones were isolated containing 478 bp, ≈800 bp, ≈1.6 kb, 2.4 kb, or ≈3.0 kb of the 5' part of intron B. None of the transcripts from the deletion clones were edited to a level significantly different from the clone with the full-length intron B (data not shown). Restriction sites in the 478-bp intron B fragment were used to generate further deletions (Fig. 2B). A dramatic change was observed in the editing efficiency when intron B was shortened from the *HincII* site at 380 bp, which gave 51% edited RNA to the *Asp* 718 site at base 278 where the editing was reduced to <3% (Fig. 2B, lane 6).

FIG. 2. Primer-extension analysis (of A1820) of DNA generated by reverse transcription-PCR from mouse neuroblastoma N2a cells transiently transfected with constructs encoding the cDNA or 5' deletions of intron A (A), 3' deletions of intron B (B), or internal deletions in intron B (C). E, edited template; U, unedited template. (A) The cDNA generated from the RNA purified from cells transfected by the clone encoding the cDNA was amplified with the following primers. Lanes: 1, primers 1 and 8; 2, primers 4 and 6; 3 and 4, primers 1 and 6. (B) Lanes: 1, genomic clone; 2, the deletion mutants generated by BAL-31 exonuclease digestion; 3, a *HincII* fragment; 4, a *Asp* 718 fragment; 5, a *Nco* I fragment; 6, a *EcoRI* fragment. The primers 1 and 6 (lanes 1–5) and the primer 1 and 8 (lane 6) were used for the amplification. The deletions were made from the BAL-31-generated clone (lane 2). Numbers refer to the length of intron B. (C) The deletions start either at the splice site (S) or the *EcoRI* site (E) and end at the *Asp* 718 site (Δ SA and Δ EA), the *EcoRI* site (Δ SE), or the *Nco* I site (Δ EN). Results shown in lanes 1–3 and 4–6 originate from different transfection experiments. The primers 1 and 8 were used for all constructs.

A model of the RNA structure of the minimal fragment determined by the deletion analysis shown in Fig. 2 was constructed using Zukers free energy minimization algorithm to predict the RNA structure (17). The fragment contains an inverted repeat structure interrupted by 120 nt that could fold into two hairpins. The inverted repeat created three helical units (I–III) consisting of 22 or 23 bp with a distortion after 15–17 bp, a bulge in helix II, and a mismatch in helix I and helix III (Fig. 3). Unstructured regions are predicted to separate the helical units. We studied the importance of this predicted structure by introducing more deletions. The editing was abolished when the region from the splice site to the *Asp* 718 restriction site was removed (Δ SA) (Fig. 2C, lane 1). Since this mutation interfered with helix I, a shorter deletion, Δ SE, that does not interfere with helix I was tested, and the editing was still eliminated. Deletion of helix III (Δ EN) reduced the editing from 32 to 4%.

A Chimeric RNA Constructed from the GluR3 Exon and the GluR2 Intron B Is Edited. The base pairing potential of the GluR2 intron with the GluR2 exon sequence provides a mechanism for the specificity of editing of only GluR2 despite the high sequence identity of exon sequences in the GluR1–GluR4 genes. This supports a model in which the intron determines the selective editing of GluR2. To test this model, we made a chimera minigene between the GluR3 exon that is normally not edited and the GluR2 intron B to determine whether or not the editing specificity can be transferred to the GluR3 exon by simply placing the GluR2 intron downstream of the GluR3 exon. A fragment containing 172 nt of the GluR3 exon upstream of the splice site and the first 478 bases of the GluR2 intron B was generated by overlap extension. A second minigene chimera was made as a control. The GluR3

exon was combined with the 5' 278 nt of the GluR2 intron B that does not encode the region complementary to the exon sequence.

To eliminate contamination with DNA used for the transfections and to analyze only processed RNA, we used primer 9 (Fig. 1A) as the downstream primer. Primer 9 anneals to both sides of the simian virus 40 small tumor antigen intron in the construct (Fig. 1A) and will, therefore, amplify only cDNA made from RNA in which the intron is spliced out. The editing of the GluR2 construct, which is the same construct used in Fig. 2A, lane 4, was in this experiment 35% whereas the R3 exon–R2 intron chimera minigene was edited to 17% (Fig. 4), indicating that the specificity for editing is determined by the GluR2 intron. The editing of the GluR3 exon was eliminated in the control minigene chimera where the intron–exon potential for base pairing was eliminated (Fig. 4, lane 3).

DISCUSSION

The present study demonstrates that a region in the GluR2 intron B is essential for the editing. The 5' part of intron B and the edited exon can fold into a structure containing three double-stranded elements. The importance of these predicted helix regions is demonstrated by the dramatic changes in editing efficiency when they are altered by mutations. Deletion Δ SA in which helix I is destroyed and deletion Δ EN in which helix III is eliminated both reduce or eliminate the editing, indicating that not only the base pairing in the exon sequence is necessary but also the integrity of the whole inverted repeat is also essential for the editing. Intron A could be removed without severe reduction in the editing effi-

FIG. 3. Putative secondary structure model based on Zuker energy minimization algorithm. Boxes indicate the edited residues A1820 and A1824. The exon–intron border (S) and the restriction sites *Asp* 718 (A), *Eco*RI (E), and *Nco*I (N) are marked. Residues C1812, C1830, and G1839 are all changed to uridines in GluR3. I–III indicate the three similar helical elements.

FIG. 4. Primer extension of a chimera between the R3 exon and the R2 intron. The primers 1 and 9 were used for the extension. GluR2 (lane 1), GluR3 exon–GluR2 intron (478 nt) chimera (lane 2), and GluR3 exon–GluR2 intron (278 nt) chimera (lane 3). E, edited template; U, unedited template.

ciency. However, a reduction in editing was observed when the edited exon was shortened. This indicates that exon sequences upstream of G1809 contribute to the editing substrate or that truncating the sequence causes the RNA to fold into a structure less compatible with editing. Recent observations have provided evidence for the requirement of intron sequences in the editing process and additional evidence for the importance of the base-paired regions (12).

The almost complete editing of unspliced GluR2 RNA from cerebellum (Fig. 1) suggests that the editing of A1820 is fast compared to the splicing. At steady state, the ratio of edited vs. unedited GluR2 RNA measured before splicing (Fig. 1A, lane 1) is equal to the ratio of the rate constants for editing vs. splicing. If the rate of the editing was comparable to the splicing rate, the steady-state level of unedited GluR2 RNA before splicing should have been comparable with the amount of edited RNA before splicing. Our experiment does not rule out the possibility that editing might be required for splicing, but it is unlikely since a higher percentage of unedited GluR2 RNA is present in embryos (4), suggesting that splicing can occur without editing.

The observation that the GluR3 exon–GluR2 intron chimeric minigene transcript is edited demonstrates that the specific GluR2 editing observed in the brain is due to differences in the intron sequence rather than differences in the coding region. The reduction in the editing efficiency observed for the chimera might be due to the few base differences in the exon shown at Fig. 3.

The maximal editing of the GluR2 transcript observed in transfected N2a cells is 50% of the total transcript, which is in contrast to the >99% editing observed in GluR2 mRNA isolated from adult brains. This discrepancy might be an artifact due to saturation of the editing system caused by the high transcriptional activity of the cytomegalovirus promoter in the transfected N2a cells. The fact that editing can occur prior to splicing suggest that the editing machinery is located in the nucleus. We cannot eliminate the possibility that the reduction of the editing frequency in the more truncated constructs is due to the fact that these constructs may not be retained in the nucleus sufficiently long for the editing machinery to work. This possibility was minimized by using primers that amplify spliced products that must have spent enough time in the nucleus to be processed.

Although our experiments show that the edited base is recognized as a guanosine by the reverse transcriptase, the molecular identification of the edited base remains to be determined. It could be a modification of the adenosine, a

deamination to an inosine, or a substitution of the base. A deaminase that recognizes double-stranded RNA helices as substrate and converts base-paired adenosines to inosines has been characterized in *Xenopus* embryos (18, 19) and a number of eukaryotic cells and tissues including neuronal tissue (20). The effect of the base conversion by the deaminase is a destabilization of the helix due to the reduced base-pairing energy of an I-U base pair compared to an A-U pair. The minimal helical length required for the conversion by this deaminase is between 15 and 23 bp (21), but the level of editing depends on the helix length in a cooperative manner so that the maximal level of deaminations is observed for helices >100 bp (21). We speculate that a similar activity is responsible for the GluR2 editing although the inverted repeat we observe for the GluR2 transcript is imperfect and disrupted by nonhelical elements. The non-base-paired regions between the helical units may introduce a structural flexibility that allows proteins that could bind to each helical unit to interact in a cooperative manner and, thereby, increase the efficiency and specificity of the editing. It is also possible that different components are involved in the editing of different glutamate receptor subunits. We have shown in a GC4 oligodendrocyte precursor cell line expressing both the GluR2 and GluR6 genes that GluR2 is edited >95% whereas the three edited sites in GluR6 (5) are edited <5%, indicating that there may be cellular components that differentially regulate the editing of different glutamate receptor subunit genes (22).

We thank Dr. R. Puchalski for providing the neuroblastoma N2a cell line and advice on the transfection procedures and Dr. J. Boulter for the rat genomic DNA. This study was supported by the Danish Medical Research Council (J.E.) and by National Institute of Neurological Disorders and Stroke grants to S.F.H. (NS11549 and NS28709), and The McKnight Foundation (S.F.H.).

- Hollmann, M. & Heinemann, S. (1994) *Annu. Rev. Neurosci.* 17, 31–108.
- Hume, R. I., Dingle, R. & Heinemann, S. (1991) *Science* 253, 1028–1031.
- Verdoorn, T. A., Burnashev, N., Monyer, H., Seeburg, P. H. & Sakmann, B. (1991) *Science* 252, 1715–1718.
- Burnashev, N., Monyer, H., Seeburg, P. H. & Sakmann, B. (1992) *Neuron* 8, 189–198.
- Egebjerg, J. & Heinemann, S. F. (1993) *Proc. Natl. Acad. Sci. USA* 90, 755–759.
- Sommer, B., Köhler, M., Sprengel, R. & Seeburg, P. H. (1991) *Cell* 67, 11–20.
- Köhler, M., Burnashev, N., Sakmann, B. & Seeburg, P. H. (1993) *Neuron* 10, 491–500.
- Björk, G. R., Ericson, J. U., Gustafson, C. E. D., Hagervall, T. G., Jönsson, Y. H. & Wikström, P. M. (1987) *Annu. Rev. Biochem.* 56, 263–287.
- Cattaneo, R. (1991) *Annu. Rev. Genet.* 25, 71–88.
- Chan, I. (1993) *BioEssays* 15, 33–41.
- Sharma, P. M., Bowman, M., Madden, S. L., Rauscher, I. F. J. & Sukumar, S. (1994) *Genes Dev.* 8, 720–731.
- Higuchi, M., Single, F. N., Köhler, M., Sommer, B., Sprengel, R. & Seeburg, P. H. (1993) *Cell* 75, 1361–1370.
- Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* 7, 2745–2752.
- Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
- Boulter, J. & Gardner, P. D. (1990) *Methods Neurosci.* 1, 328–344.
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989) *Gene* 77, 61–68.
- Devereux, J., Haeblerli, P. & Smithies, O. (1984) *Nucleic Acids Res.* 12, 385–387.
- Rebagliati, M. R. & Melton, D. A. (1987) *Cell* 48, 599–605.
- Bass, B. L. & Weintraub, H. (1987) *Cell* 48, 607–613.
- Wagner, R. W., Yoo, C., Wrabetz, L., Kamholz, J., Buchhalter, J., Hassan, N. F., Khalili, K., Kim, S. U., Perussia, B., McMorris, F. A. & Nishikura, K. (1990) *Mol. Cell. Biol.* 10, 5586–5590.
- Nishikura, K., Yoo, C., Kim, U., Murray, J. M., Estes, P. A., Cash, F. E. & Liebhafner, S. A. (1991) *EMBO J.* 10, 3523–3532.
- Puchalski, R. B., Louis, J., Brose, N., Traynelis, S. F., Egebjerg, J., Kukekov, V., Venthold, R., Rogers, S. W., Lin, F., Moran, T., Morrison, J. H. & Heinemann, S. F. (1994) *Neuron* 13, 131–147.