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16. The VLPs were isolated as described (*13*) with a sucrose step gradient. The VLP protein from 1 liter of cells (~330 μg) was resuspended in 40 μl of buffer [13.5 mM KCl, 9 mM Hepes (pH 7.8), 4.5 mM $MgCl_2$, and 10% glycerol] and kept on ice until used. A standard integration reaction consisted of 2 μl of resuspended VLP protein, 400 ng of pDLC374 (0.075 pmol), 20 mM Hepes (pH 7.8), 65 mM KCl, 1 mM dithiothreitol, 5 μl of BR500 transcription extract (at a final concentration of 1.8 to 2.0 mg/ml), and 10 mM $MgCl_2$ in a 50-μl reaction volume. If $MnCl_2$ replaced $MgCl_2$ in the reaction, integration was severely decreased (*21*). Per reaction, the VLPs provided 0.18 to 0.34 pmol of IN and 0.03 to 0.07 pmol of replicated Ty3 DNA (determined by comparison with standards). Reaction components were assembled on ice then incubated at 30°C for 30 min. Reactions were stopped by addition of proteinase K to a final concentration of 0.2 mg/ml, SDS to 0.2%, and EDTA (pH 8.0) to a concentration twice that of $MgCl_2$ in the integration reaction, then incubated at 37°C for 30 min. When necessary, $MgCl_2$ was added with the stop buffer to equalize the concentration of $MgCl_2$ in different samples.
17. The DNA from each integration reaction was isolated and quantitated by fluorometric analysis with a Mini TKO 100 DNA Fluorometer (Hoeffer Scientific). The PCR conditions for amplification of *HIS3* were similar to those described previously (*14*) except that the reactions with primer set 284-285 included 0.05 mM deoxynucleotide triphosphates (dNTPs) and 0.1 ng of template DNA and 18 cycles were run. The PCR conditions for detection of integration were similar to those described previously (*14*) except that the reactions with primer set 278-279 included 0.5 mM dNTPs, 3.5 mM $MgCl_2$, and 1 to 10 ng of template DNA (depending on the linear range, determined for each set of integration reactions). Primer 278 is complementary to nucleotides 444 to 418 of Ty3 on the transcribed strand. Primer 279 is complementary to the modified *SUP2* $tRNA^{Tyr}$ gene at nucleotides +25 to −5 on the transcribed strand (+1 corresponds to the transcription start site) and its 3′-most two nucleotides are complementary to Ty3 nucleotides 1 and 2 on the minus strand. The 278-279 PCR detected as little as $1 \times 10^{-5}$ ng of pTM42 in the presence of 2 ng of pDLC374. The PCR products were analyzed by native polyacrylamide gel electrophoresis on 8% gels and visualized by transillumination with ultraviolet light after staining with ethidium bromide. Photographs were taken with Polaroid film (type 55), and the negatives were used for the figures presented here. For 284-285 PCRs, the negative was scanned with an LKB Ultroscan II Laser Densitometer (Pharmacia) to determine the band intensity for quantitation before the 278-279 PCR.
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# Glutamate Receptor RNA Editing in Vitro by Enzymatic Conversion of Adenosine to Inosine

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**RNA encoding the B subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of ionotropic glutamate receptor (GluR-B) undergoes a posttranscriptional modification in which a genomically encoded adenosine is represented as a guanosine in the GluR-B complementary DNA. In vitro editing of GluR-B RNA transcripts with HeLa cell nuclear extracts was found to result from an activity that converts adenosine to inosine in regions of double-stranded RNA by enzymatic base modification. This activity is consistent with that of a double-stranded RNA-specific adenosine deaminase previously described in *Xenopus* oocytes and widely distributed in mammalian tissues.**

The editing of mRNA transcripts is an important mechanism for augmenting the flexibility of eukaryotic gene expression (*1*). The A→G conversion seen in mRNAs encoding glutamatergic ion channel subunits exemplifies the functional relevance of this posttranscriptional modification (*2*). Glutamate receptors of the AMPA subtype are generated by the assembly of GluR-A, -B, -C, and -D subunits into homo- and heteromeric channels (*3*). A single positively

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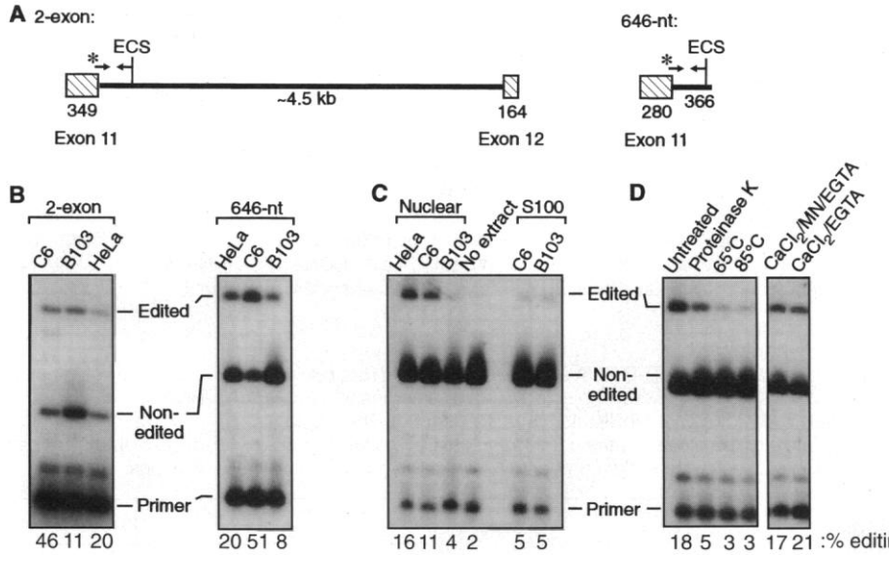


**Fig. 1.** In vivo and in vitro editing of GluR-B transcripts. (**A**) Schematic of GluR-B transcription units indicating the Q/R site (asterisk) and inverted repeat (arrows). (**B**) Primer-extension analyses of RNA from cells permanently transfected with GluR-B transcription units. (**C**) Primer-extension analyses of the 646-nt in vitro reaction products with nuclear and S100 extracts from the indicated cells. (**D**) Primer-extension analyses of in vitro reaction products generated in HeLa cell nuclear extracts pretreated as indicated. MN, micrococcal nuclease. The numbers at the base of each gel indicate the percent of A→G conversion (editing efficiency).

charged Arg residue within the second hydrophobic domain (TMII) of the GluR-B subunit is responsible for the linear current-voltage relation and low divalent cation permeability demonstrated by the AMPA receptor (*4*). This critical Arg is produced by a posttranscriptional modification that is represented by conversion of a genomically encoded Gln codon (CAG) to an Arg codon (CGG) in the GluR-B complementary DNA (cDNA) (*2*). Intron 11, immediately downstream of the edited position (Q/R site), is essential for accurate and efficient RNA editing (*5*). This intron contains 10 nucleotides (nt) that are complementary to the exonic sequence immediately surrounding the Q/R site. It has been proposed that this editing site complementary sequence (ECS), as well as an imperfect inverted repeat, contributes to the formation of an RNA duplex within the GluR-B primary RNA transcript (pre-mRNA) that is critical for editing (*5*).

To characterize the molecular events involved in GluR-B RNA editing, we developed a model system with transfected cell lines that exhibit RNA processing patterns analogous to those observed in vivo. Human epithelial (HeLa), rat glioma (C6), and rat neuronal (B103) cell lines were assessed for their ability to edit a permanently transfected GluR-B transcription unit extending from exon 11 through exon 12 (2-exon; Fig. 1A) (*6*, *7*). All three cell lines were capable of editing RNAs derived from this exogenous GluR-B minigene (Fig. 1B). Experiments with a 646-nt GluR-B transcription unit extending from –256 to +390 relative to the Q/R site demonstrated that this RNA substrate contains sufficient regulatory information for efficient editing (Fig. 1B) (*5*).

We developed an in vitro editing system with extracts from C6, HeLa, and B103 cells (*8*) and the 646-nt RNA substrate (*9*). The reaction products were amplified by the RNA template–specific polymerase chain reaction (RS-PCR) and analyzed by primer extension (*10*). The HeLa cell nuclear extracts showed 16 ± 3% A→G conversion ($n$ = 4), whereas the C6 and B103 nuclear and cytoplasmic extracts exhibited lower editing efficiencies (Fig. 1C). Pretreatment of HeLa nuclear extracts with proteinase K or incubation at high temperatures abrogated editing, suggesting that a protein or proteins are required, whereas pretreatment with micrococcal nuclease had no effect (Fig. 1D) (*11*).

To assess the specificity of editing, we compared the editing patterns of various GluR-B RNA substrates in different editing systems (Table 1). Rat brain pre-mRNA transcripts showed extensive editing at the Q/R site (99%), and additional A→G replacements were identified at intronic "hot spots" (positions +60 and +262 through +264) which are predicted to reside in roughly equivalent positions on opposite sides of the RNA duplex (*5*). Editing within the exon at +4 was also seen in 38 of 100 cDNAs, altering the wobble position of a

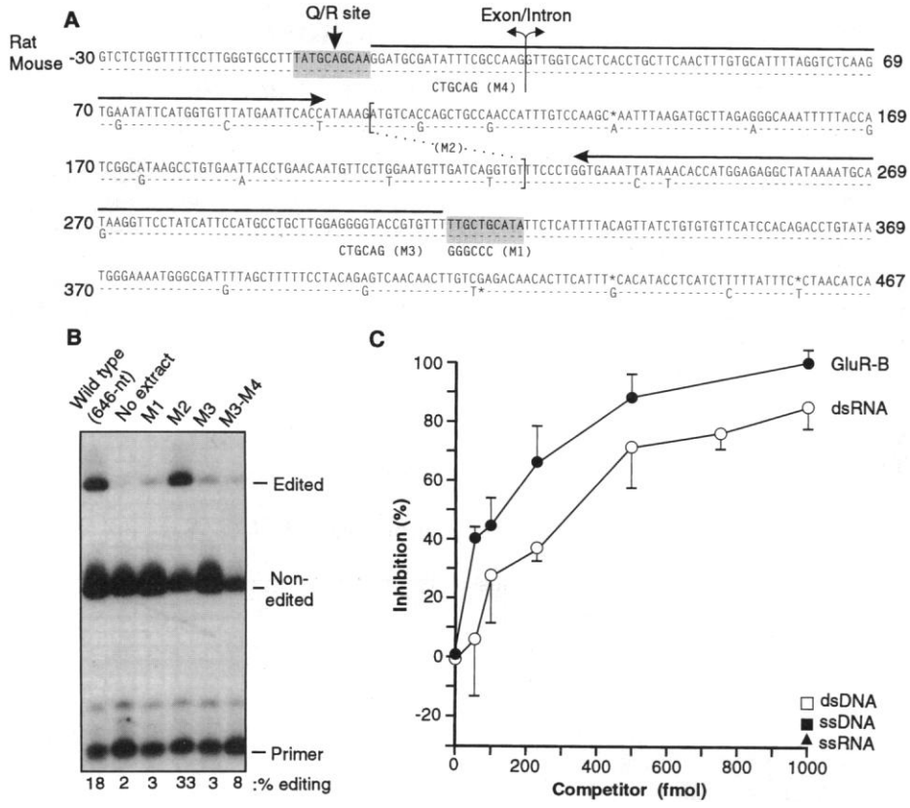


**Fig. 2.** Dependence of in vitro RNA editing activity on a double-stranded RNA (dsRNA) substrate. (**A**) Nucleotide sequence alignment of rat and mouse GluR-B genes in the proximal region of intron 11; gaps (asterisks) have been introduced for alignment purposes and dashes represent sequence identity. The imperfect inverted repeat is shown with overlying arrows. The complementary regions surrounding the Q/R site and the ECS are indicated with gray boxes. (**B**) Editing analyses of in vitro reaction products with mutant GluR-B RNA substrates. (**C**) Effect of single- and double-stranded nucleic acid competitors on the inhibition of in vitro GluR-B editing activity. The percentage of editing in the absence of competitor was 16.6 ± 4.4% ($n$ = 7) (not shown); GluR-B RNA (●), dsRNA (○), dsDNA (□), ssDNA (■), and ssRNA (▲). Error bars represent the standard error from at least three independent experiments.

**Table 1.** Quantitation of GluR-B RNA editing patterns by direct nucleotide sequence analysis. RNAs were subjected to reverse transcription–PCR amplification, subcloned into pBKSII⁻, and sequenced. The nucleotide position (relative to the Q/R site), the percentage of cDNA isolates containing an A→G replacement, and the number of individually sequenced cDNA isolates are indicated. Intronic sites demonstrating <2% editing from all RNA sources have been omitted. The error rate for Taq polymerase misincorporation was estimated to be 0.06% (17 errors per 30,000 bases sequenced), on the basis of sequence discrepancies other than A→G conversions.

| Editing system | GluR-B RNA substrate | No. of cDNAs sequenced | Editing (%) at nucleotide position | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 4 | 45 | 46 | 60 | 242 | 243 | 255 | 262 | 263 | 264 | 265 | 272 | 306 |
| Rat brain | Endogenous | 100 | 99 | 38 | 8 | 3 | 67 | 3 | 4 | 7 | 36 | 67 | 10 | 2 | 2 | 1 |
| C6 cells | Endogenous | 55 | 36 | 2 | 0 | 0 | 29 | 2 | 5 | 0 | 2 | 4 | 2 | 0 | 0 | 2 |
| C6 cells | 2-exon | 50 | 46 | 2 | 0 | 0 | 2 | 0 | 0 | 2 | 8 | 6 | 2 | 2 | 0 | 0 |
| HeLa cells | 646-nt | 100 | 24 | 3 | 0 | 0 | 7 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 0 | 0 |
| HeLa extract | 646-nt | 100 | 26 | 0 | 0 | 0 | 13 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |

Gln codon from CAA to CAG. Endogenous GluR-B transcripts in C6 cells, transfection-derived transcripts from C6 and HeLa cells, and in vitro reaction products exhibited the greatest amount of editing at the Q/R site; the extent of editing at other sites was markedly reduced in these systems (Table 1).

DNA sequence comparisons between the rat and mouse GluR-B genes have indicated extensive conservation of nucleotide sequence in the proximal region of intron 11 (Fig. 2A). To examine the cis-active elements required for in vitro editing, we introduced a series of mutations (M1 to M4) into highly conserved regions surrounding the Q/R site in the 646-nt GluR-B RNA substrate (Fig. 2, A and B) (*12*). Insertion of an Apa I restriction site into the ECS (M1) abolished editing at the Q/R site (Fig. 2B) as previously observed (*5*). Deletion of 120 nt between the inverted repeat sequences (M2; Fig. 2A, brackets) increased editing efficiency. Disruption of the RNA duplex by introduction of a Pst I restriction site within the second half of the inverted repeat (+301 to +306; M3) reduced editing to background levels (3 ± 1%), whereas introduction of a second Pst I site (+13 to +18; M3-M4) to restore the duplex slightly restored editing efficiency (8 ± 1%). These observations suggest that an RNA duplex structure is critical for editing activity.

We further characterized the sequence requirements for in vitro editing of GluR-B RNA by addition of single-stranded (ss) and double-stranded (ds) nucleic acid competitors. An RNA substrate containing 646 nt of the GluR-B sequence and a 3′ extension, derived from the pRC/CMV (cytomegalovirus) expression plasmid, was used to differentiate between the editing of the RNA substrate and the competitor GluR-B–derived sequences (*13*). Wild-type GluR-B RNA competitor (646 nt) inhibited editing in a concentration-dependent manner (Fig. 2C); a dsRNA of unrelated sequence also inhibited editing, although with decreased potency. In contrast, dsDNA, ssDNA, and ssRNA had no effect on editing.

The competition experiments suggested that the editing of GluR-B transcripts is dependent on a dsRNA substrate with little sequence specificity. This property is similar to the activity of a dsRNA-specific adenosine deaminase that acts on dsRNA but not ssRNA, ssDNA, or dsDNA (*14*). This enzyme (dsRAD or DRADA) converts adenosine to inosine (I) by hydrolytic deamination of adenosine in regions of dsRNA (*1*, *14*). This modified nucleotide could serve as a template for the incorporation of cytosine by reverse transcriptase and produce a codon that alters the coding potential of GluR-B mRNA from Gln to Arg (*1*, *5*). To investigate this possibility, we used thin-layer chromatography (TLC) to analyze the in vitro reaction products generated from an RNA substrate uniformly labeled with [α-$^{32}$P]adenosine 5′-triphosphate (ATP). The reaction products included a radiolabeled species that comigrated with inosine 5′-monophosphate (IMP) upon digestion with nuclease P1 (Fig. 3A) (*15*, *16*). RNA incubated in the absence of nuclear extract produced a markedly reduced IMP signal. These results suggest that the editing of GluR-B transcripts involves the production of inosine rather than guanosine during the course of the reaction, altering a genomically encoded Gln codon (CAG) to an Arg codon (CIG) in the GluR-B mRNA. Quantitative phosphorimager analysis of the radiolabeled species migrating at the positions of adenosine 5′-monophosphate (AMP) and IMP demonstrated an editing efficiency of 18%, in good agreement with the 14% editing determined by primer extension analyses. Nucleotide sequence analyses of the in vitro reaction product revealed editing solely at the Q/R site and position +60 (Table 1). Although a low level of IMP formation was observed in the absence of nuclear extract (Fig. 3A), sequence analyses of 50 cDNAs generated from this reaction did not reveal the presence of edited nucleotides. Because there are no known polymerases that incorporate nucleotides into a polynucleotide chain without a 5′-phosphate group, these results also suggest that the editing of GluR-B transcripts is not mediated by a mechanism of nucleoside excision and replacement in which the phosphodiester backbone of the RNA is cleaved and then esterified.

To exclude the possibility that IMP is produced by transglycosylation (base exchange) (*17*), we used an RNA uniformly labeled with [2,8-$^{3}$H]ATP as the substrate in the in vitro editing reaction (*15*, *16*). The radiolabeled reaction products from the nuclease P1 digest migrated with the internal standards for AMP and IMP (Fig. 3B). The generation of purine ring–labeled [2,8-$^{3}$H]IMP in this assay demonstrates that the processing of GluR-B transcripts does not involve base exchange, but rather an enzymatic base modification converting adenosine to inosine.

We have demonstrated that the editing of GluR-B RNA transcripts is dependent on a dsRNA-specific activity that converts adenosine to inosine by enzymatic base modification. The apparent nonselectivity of dsRAD compared with the efficiency of GluR-B editing at the Q/R site in rat brain (>99%) suggests that if a dsRAD-like activity is responsible for GluR-B editing, additional specificity factors may be involved (*5*). More recent studies of adenosine preference by dsRAD have indicated a high selectivity for specific adenosine residues within a short RNA duplex (*18*), alleviating the requirement for additional specificity factors. In addition to modifications of GluR-B at the Q/R site, RNA editing is responsible for A→G (I) conversions in RNAs encoding other AMPA and kainate receptor subunits (*2*), suggesting that a dsRAD-like activity may be responsible for the posttranscriptional modification of mRNAs encoding multiple glutamate receptor subunits.

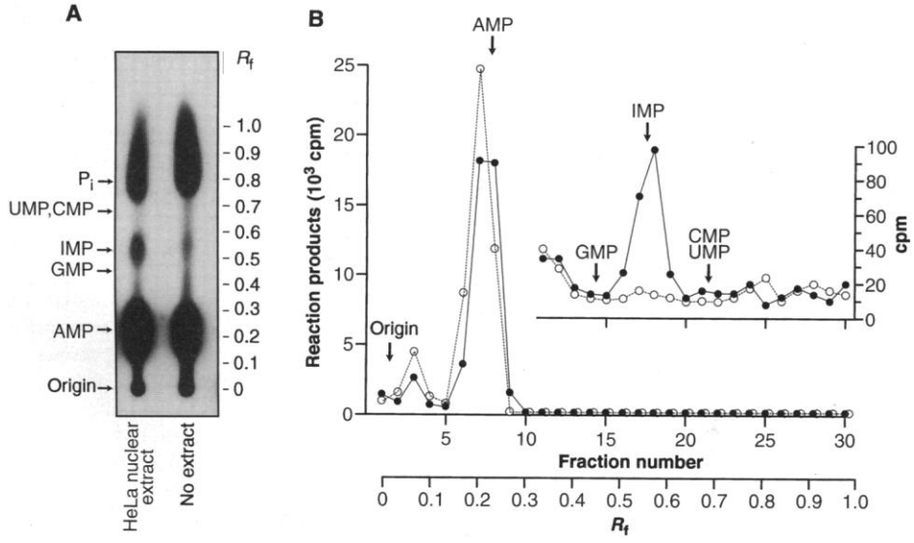


**Fig. 3.** Association of in vitro editing with an enzymatic adenosine-to-inosine conversion. (**A**) TLC analysis of an [α-$^{32}$P]ATP–labeled RNA substrate incubated in vitro. The migration positions of 5′-nucleoside monophosphate standards and their relative mobility ($R_f$) are indicated. $P_i$, inorganic phosphate. (**B**) TLC analysis of a [2,8-$^{3}$H]ATP–labeled RNA substrate incubated in vitro. Recovery of radioactivity for samples with (●) and without extract (○) was 94 and 98%, respectively. The inset contains an expanded *y* axis (counts per minute).

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6. We established permanently transfected polyclonal cell lines by using calcium phosphate coprecipitation with a eukaryotic expression vector (pRC/CMV; Invitrogen) containing a 5-kb (2-exon) or 646–base pair (bp) GluR-B transcription unit. Total RNA was prepared by the guanidinium isothiocyanate method (*7*). Total RNA (2.5 μg) was reverse transcribed with an intronic antisense primer (+390 to +407 relative to the Q/R site); PCR amplification of cDNA was achieved with the initial cDNA synthesis primer and an expression vector–specific primer (5′-CTGGCTTATCGAAATTAATACGAC-3′). The product was purified on a 0.8 or 2% agarose gel and 2 pmol was annealed to a $\gamma$-$^{32}$P–labeled 17-nt oligomer antisense primer corresponding to positions +3 to +19. The primer was extended in the presence of 40 mM tris-HCl (pH 7.5), 50 mM NaCl, 20 mM $MgCl_2$, 3.6 mM $MnCl_2$, 200 μM each of deoxyadenosine 5′-triphosphate, deoxyguanosine 5′-triphosphate, and deoxycytidine 5′-triphosphate, and 1200 μM dideoxythymidine 5′-triphosphate by using 1.6 U of Sequenase (United States Biochemicals) for 10 min at 37°C. Primers annealed to nonedited and edited sequences were extended to the first or second upstream A, generating products of 20 and 24 nt, respectively; the resulting products were separated on a 20% acrylamide–7 M urea gel. Editing was quantified with a Molecular Dynamics phosphorimager.
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9. For synthesis of the in vitro RNA substrate, a 646-bp DNA fragment corresponding to positions −256 to +390 was cloned into the transcription vector pBKSII$^-$ (Stratagene) and linearized with Bam HI. RNA labeled with [$\alpha$-$^{32}$P]uridine 5′-triphosphate (UTP) was transcribed (specific activity = 2.2 x $10^6$ cpm/mmol) with T3 RNA polymerase. The reaction was treated with 10 U of ribonuclease-free deoxyribonuclease (Promega) at 37°C for 40 min, extracted with phenol-chloroform (1:1), and precipitated with ethanol.
10. In vitro products were amplified by RS-PCR [A. R. Shuldiner, R. Perfetti, J. Roth, in *PCR Protocols: Current Methods and Applications*, B. A. White, Ed. (Humana, Totowa, NJ, 1993), pp. 169–176]. The cDNA was synthesized with an antisense oligonucleotide containing a 5′-terminal extension (5′-AATCCGGATTGCCCGGAACGTCTTGGCGAAATATCGCATCCTTGC-3′). The cDNA products were selectively amplified with an antisense PCR primer unique to the RNA-derived cDNA extension sequence. Hybridization and extension of a [$\gamma$$^{32}$P]-labeled 15-nt oligomer sense primer (−18 to −4) in the presence of dideoxy-ATP generated products of 19 and 22 nt for nonedited and edited transcripts, respectively.
11. Protease sensitivity and thermolability studies were performed by preincubation of HeLa cell nuclear extracts (50 μg) with 1 μg of proteinase K for 20 min at 37°C or by preincubation at elevated temperature (65°C and 85°C) for 10 min, respectively. Micrococcal nuclease sensitivity was determined by pretreatment of extracts with $CaCl_2$ (0.75 mM final concentration) and 13.5 U of micrococcal nuclease (Pharmacia) for 10 min at room temperature. Micrococcal nuclease was inactivated by the addition of EGTA (2.5 mM final concentration).
12. Construction of mutant GluR-B RNA substrates was performed by oligonucleotide-directed mutagenesis in pBKSII$^-$ (Stratagene) as previously described (*7*).
13. In competition studies, cDNA was synthesized with an oligonucleotide complementary to the pRC/CMV-derived 3′ extension of the GluR-B RNA substrate. The ssDNA competitor (81 nt) was an oligonucleotide derived from the neomycin resistance gene (pRC/CMV); the dsDNA competitor (178 bp) was obtained by PCR amplification of pBKSII$^-$ polylinker. The ssRNA competitors were transcribed in the presence of [$\alpha$-$^{32}$P]UTP (specific activity = 2.2 x $10^6$ cpm/mmol) from pBKSII$^-$ linearized with Xba I (93 nt) or Xho I (97 nt). The dsRNA competitor was produced by annealing the ssRNAs in 80% formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl, and .1.25 mM EDTA for 14 to 16 hours at 25°C. Residual ssRNA was removed by ribonuclease A digestion (25 μg/ml) (*7*). The dsRNA was purified on an 8% nondenaturing acrylamide gel and eluted by incubation in 1 M ammonium acetate, 10 mM tris-HCl (pH 7.6), and 1 mM EDTA for 2 hours at 4°C.
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15. The 646-nt RNA substrate was transcribed in the presence of [$\alpha$-$^{32}$P]ATP (800 Ci/mmol) or [2,8-$^3$H]ATP (30 Ci/mmol) (RNA specific activity, 4.9 x $10^{10}$ and 9.6 x $10^9$ cpm/mmol, respectively). After the standard in vitro reaction, one-half of the product was used to quantitate GluR-B RNA editing by the RS-PCR primer extension assay, and the remainder of the reaction was resuspended in 50 mM sodium acetate (pH 5.3), digested with nuclease P1 at 37°C for 2 hours, and spotted onto a cellulose TLC plate (Sigmacell Type 100). Chromatography proceeded for ~6 hours in 0.1 M $NaH_2PO_4$ (pH 6.8), ammonium sulfate, and *n*-propanol (100:60:2, v/w/v) as described (*16*). The migration positions of nucleoside monophosphate standards were determined by ultraviolet light absorption, and the migration positions of the [$^{32}$P]-labeled products were detected by autoradiography and quantitated with a Molecular Dynamics phosphorimager. For detection of [$^3$H]-labeled reaction products, 0.5-cm cellulose fractions from the TLC plate were counted by liquid scintillation spectrometry.
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# Involvement of CRAF1, a Relative of TRAF, in CD40 Signaling


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CD40 is a receptor on the surface of B lymphocytes, the activation of which leads to B cell survival, growth, and differentiation. A yeast two-hybrid screen identified a gene, *CRAF1*, encoding a protein that interacts directly with the CD40 cytoplasmic tail through a region of similarity to the tumor necrosis factor–α (TNF-α) receptor–associated factors. Overexpression of a truncated *CRAF1* gene inhibited CD40-mediated up-regulation of CD23. A region of *CRAF1* was similar to the TNF-α receptor–associated factors TRAF1 and TRAF2 and so defined a shared TRAF-C domain that was necessary and sufficient for CD40 binding and homodimerization. The CRAF1 sequence also predicted a long amphipathic helix, a pattern of five zinc fingers, and a zinc ring finger. It is likely that other members of the TNF receptor superfamily use *CRAF*-related proteins in their signal transduction processes.


CD40 (*1*) is a receptor on B cells that interacts with the helper T cell surface protein CD40L (CD40 ligand, also known as T-BAM, gp39, or TRAP) (*2–4*). CD40L is found particularly on lymphoid follicle CD4$^+$ T lymphocytes, where it delivers a contact-dependent signal that stimulates B cell survival, growth, and differentiation (*2–4*). Signaling through CD40 rescues B cells from apoptosis induced by Fas (CD95) or by cross-linking of the immunoglobulin M (IgM) complex (*5*); it also induces B cells to differentiate and to undergo Ig isotype switching (*3*) and to express CD80 (B7 or BB-1) (*6*). The crucial role of CD40L-CD40 interaction is illustrated by humans with defects in CD40L, who manifest a serious immune deficiency syndrome, the X-linked hyper-IgM syndrome (HIGMX-1), characterized by an absence of IgG, IgA, and IgE, elevated IgM, and no lymphoid


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