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Q/R site editing in kainate receptor GluR5 and GluR6 pre-mRNAs requires distant intronic sequences

(nuclear RNA editing/site-selective adenosine deamination/recombinant double-stranded RNA adenosine deaminase)

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ABSTRACT **RNA editing by adenosine deamination in brain-expressed pre-mRNAs for glutamate receptor (GluR) subunits alters gene-specified codons for functionally critical positions, such as the channel's Q/R site. We show by transcript analysis of minigenes transiently expressed in PC-12 cells that, in contrast to GluR-B pre-mRNA, where the two editing sites (Q/R and R/G) require base pairing with nearby intronic editing site complementary sequences (ECSs), editing in GluR5 and GluR6 pre-mRNAs recruits an ECS located as far as 1900 nucleotides distal to the Q/R site. The exon–intron duplex structure of the GluR5 and GluR6 pre-mRNAs appears to be a substrate of double-stranded RNA-specific adenosine deaminase. This enzyme when co-expressed in HEK 293 cells preferentially targets the adenosine of the Q/R site and of an unpaired position in the ECS which is highly edited in brain.**

RNA editing of mammalian nuclear transcripts has been recognized as a new genetic mechanism for changing gene-specified codons and hence protein structure and function (1). There are currently two major known editing mechanisms which introduce site-selective base modifications in mammalian mRNAs. One is cytidine deamination, originally discovered in the intestinal apolipoprotein B (apoB) mRNA (2, 3), and the other is the deamination of adenosines (4), found in transcripts for glutamate receptor (GluR) channels (5–7) mediating rapid excitatory neurotransmission in the mammalian brain (8).

Studies on site-selective adenosine conversion for two different editing sites in GluR transcripts have delineated a strict requirement for a short double-stranded RNA (dsRNA) structure formed in the pre-mRNA of the exonic editing site and an editing site complementary sequence (ECS) located downstream in the adjacent intron (7, 9). Thus, RNA editing in GluR pre-mRNAs is a nuclear event and occurs prior to splicing. The adenosine of two editing sites was shown to undergo selective adenosine-to-inosine conversion *in vitro* if the GluR pre-mRNAs contained the required sequence determinants for dsRNA formation (10–12), predicting the involvement of a dsRNA-specific adenosine deaminase. As of today, the only candidate enzyme for the adenosine modification in GluR pre-mRNAs is dsRAD (13), also termed DRADA (14), a ubiquitously expressed nuclear protein (15) that converts a high proportion of adenosines in synthetic dsRNAs into inosines. Though this enzyme lacks site selectivity on extended dsRNAs, it displays a sequence-dependent preference for particular adenosines when incubated with short synthetic dsRNAs (16). The cDNA for this 140-kDa enzyme has been cloned recently from humans and rats (17, 18), but the physiological role of DRADA and the cellular RNAs targeted by this enzyme have remained unknown.

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As of today, a total of eight positions in subunits of pharmacologically distinct GluRs at which adenosine conversion leads to an amino acid change have been characterized (5–7). These positions, named after the amino acids that can occupy them, are the Q/R site (Q, gene; R, mRNA) in subunits GluR-B, GluR5, and GluR6, the R/G site in GluR-B, -C, and -D, and the I/V and Y/C sites in GluR6. Each residue change has functional consequences for the glutamate-gated ion channel, including the permeability to Ca^{2+} (8) and kinetic aspects of channel gating (7). Our knowledge concerning the structural determinants for GluR pre-mRNA editing derives solely from an analysis in α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor transcripts, where the ECS element resides in the downstream intron, in close vicinity to the exonic editing site, either around 50 nucleotides (R/G site in GluR-B, -C, and -D) or 310 nucleotides (Q/R site in GluR-B) from the editing site (9). Furthermore, in the predicted exon–intron structures for GluR-B pre-mRNA, the Q/R site adenosine is base-paired but the adenosine of the R/G site is mismatched; this is true also in GluR-C and -D pre-mRNAs. To explore the natural variation in structural determinants for GluR pre-mRNA editing we have now investigated the Q/R site in the kainate receptor subunits GluR5 and GluR6, edited in brain to 40% and 80%, respectively (6, 19, 20). GluR5 and GluR6 are distantly related to AMPA receptor subunits GluR-A to -D, from which they differ in functional properties, expression characteristics, and in sequence and exon–intron organization of the nuclear genes (5, 8).

MATERIALS AND METHODS

Analysis of GluR5 and GluR6 Gene Fragments. A 6.2-kb *Hin*dIII–*Sal*I fragment containing the GluR5 M2 and M3 exons and a 6.1-kb *Bgl*II–*Bam*HI fragment containing the corresponding GluR6 exons were isolated from a mouse genomic library (5) with exon-specific oligonucleotide probes. Both gene fragments were sequenced in their entirety† on a 373A sequencer (Applied Biosystems). Sequences were aligned with the Inherit Analysis 1.2 software (Applied Biosystems). Parameters were match, 10; mismatch, −5; gap, 10 + 10 times length.

Prediction of Secondary RNA Structures. Secondary structures were generated with the FOLD program (21, 22) of the UWGCG software package for the GluR5 (positions −206 to 294 and 1544 to 2059) and GluR6 (positions −293 to 107 and 1777 to 2277) pre-mRNA sequences.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; dsRNA, double-stranded RNA; dsRAD or DRADA, dsRNA adenosine deaminase; ECS, editing site complementary sequence; GluR, glutamate receptor; RT, reverse transcription.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U31443 and U31444).

GluR Minigenes and Mutant Constructs. For GluR6, the 6.1-kb *Bgl*II–*Bam*HI fragment (positions −1192 to 4879 relative to position 1 for the adenosine in the Q/R site) was inserted into the mammalian expression vector pRK5 (23). This construct was designated minigene 1. Other constructs were minigene 3 (positions −360 to 2431), minigene 4 (positions −360 to 2234), minigene 5 (positions −533 to 2160), minigene 6 (positions −1192 to 1497), minigene 4ΔH (positions −1192 to 2234, positions 295 to 953 deleted), minigene 3ΔM (positions −1192 to 2431, positions 805 to 1746 deleted), minigene 3ΔPK (positions −533 to 2431, positions 1497 to 2118 deleted), minigene 3ΔD (positions −533 to 2431, positions 1814 to 2076 deleted), minigene 3ΔK2 (positions −1192 to 2431, positions 1964 to 2154 deleted), minigene 3ΔH1 (positions −533 to 2431, positions 295 to 1739 deleted), minigene 3ΔStM (positions −1192 to 2431, positions 154 to 1746 deleted), minigene 3ΔBM (positions −1192 to 2431, positions 9 to 1746 deleted). Deletions were introduced by endogenous or PCR-generated restriction sites. Mutants M3–M11 were constructed in minigene 3 by oligonucleotide-directed mutagenesis: M3 (positions 1801 to 1805 deleted; percent Q/R site editing ± SD in PC-12 cells, 7.1% ± 0.6%), M5 (positions 1864 to 1869 deleted; 0%), M6 (GGG insertion after position 1863; 0.3% ± 0.4%), M7 (GGG insertion after position 1866; 0%), M8 (GGG insertion after position 1858; 0%), M9 (GGG insertion after position 1838; 3.2% ± 0.8%); M7res (CCC insertion after position −3 in construct M7; 17.1% ± 0.3%), M7off (CCC insertion after position −4 in construct M7; 0%), M8res (CCC insertion after position 4 in construct M8; 19.6% ± 1.2%), M10 (substitution of CTG for positions 1861 to 1864; 0.2% ± 0.2%), M11 (substitution of CCG for positions 1861 to 1864; 20.4% ± 2.2%). For GluR5, a 5.2-kb *Eco*RI–*Sal*I fragment was inserted into pRK7 (minigene 51, positions −363 to 4859; 2.3% ± 1.4%). Other GluR5 constructs tested were minigene 53 (positions −363 to 2315; 2.9% ± 0.5%) and minigene 56 (positions −885 to 1722; 0%).

Cell Transfections. Transfection of PC-12 cells was as described (9). For HEK 293 cells, minigene plasmids (2 μg each) were used to transfect (24) a half-confluent 14-cm culture dish of HEK 293 cells (American Type Culture Collection CRL 1573) in the presence or absence of DRADA vectors (10 μg, see below).

Analysis of GluR5 and GluR6 Sequences from Transfected Cells and Brain. Reverse transcription (RT)-PCR amplification of RNA from transfected cells reverse transcribed with random primers (9) was performed with primer combinations rsp23/O3gPCR2 and rsp23/O3gRTBam1 (GluR6 Q/R site), rsp23/O3gK2 (GluR6 Q/R site plus ECS), and rsp23/O1gPCR2i (GluR5 Q/R site) with Vent$_R$ DNA polymerase (Biolabs) and 0.5 μl of RT reaction mixture in 50 μl total volume. PCR-generated DNA fragments were gel-isolated (Jetsorb, Genomed), and cloned in phage M13 vectors. Oligonucleotides for hybridization analysis were O3gR (specific for R-form), O3gQ (specific for Q-form), O3ghoted (specific

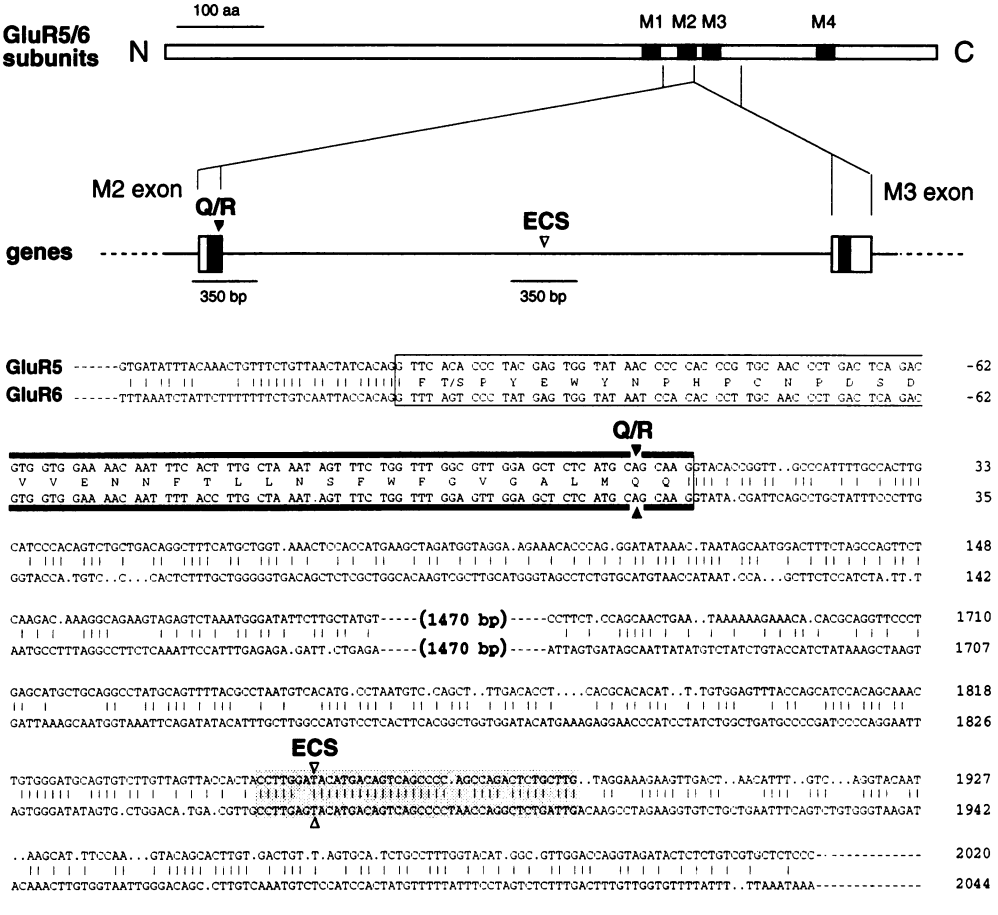

FIG. 1. Homologous but sequence-diverged introns in the GluR5 and GluR6 genes contain a conserved sequence element around their midpoint. The mature GluR5 and GluR6 subunits of kainate-preferring GluR channels (8) are schematically shown on top from N to C terminus (scale bar, 100 amino acids), including four hydrophobic regions (M1 to M4, black boxes) for membrane insertion. The genomic organization around the M2 and M3 exons is depicted below; the exonic editing site (Q/R) and the ECS element in the middle of the intron are indicated by arrowheads. An alignment of the numbered nucleotide sequences (position 1 is the adenosine in the Q/R site) in the 350-bp regions underlined in the exon–intron map is presented. The coding sequence for the M2 exon is boxed and the coding region for the channel segment M2 (25) containing the Q/R site is outlined in bold. The intron alignment is interrupted for 1470 nucleotides and continues around the intronic midpoint with the conserved ECS element (shaded). GenBank accession numbers for the entire GluR5 and GluR6 intron sequences are U31444 and U31443, respectively.

for position 1864, edited form) or O3V(RQ) (specific for position −4, edited form), and O1gR or O1gQ. More than 500 plaques from at least two transfections were analyzed for each construct. For the determination of additional editing sites in GluR6 pre-mRNA (see Table 1), PCR fragments derived from HEK 293 cells cotransfected with DRADA vector and minigenes 4 or 3ΔH1 were inserted into a phage M13 vector and their sequences were analyzed (see Fig. 3, top strand: 80 clones R-form, 40 clones Q-form; bottom strand: 40 clones R-form, 40 clones Q-form). Brain cDNA from two mice (3 weeks and 6 months of age) was PCR amplified with primers O3PCR1/O3gRTBam1 (GluR6 Q/R site), and O3g8/O3gK2 (GluR6 ECS). Cloned PCR fragments were analyzed by hybridization for editing at the Q/R site, M/V site, and ECS element and were sequenced for determination of additional editing sites (Fig. 3, top strand: 16 clones R-form, 16 clones Q-form; bottom strand: 16 clones ECS edited, 16 clones ECS unedited). The intron containing sequence from the M2 exon to ECS could not be amplified to evaluate Q/R site and ECS editing in the same sequences.

DRADA Constructs. Cloned full-length cDNA for rat DRADA (18) engineered to encode an N-terminal FLAG epitope and six histidine residues at the C terminus was inserted into the mammalian expression vector pRK (23). A DRADA mutant (SQAD) inactivated in the deaminase domain was constructed by substituting amino acid residues CHAE (amino acid positions 855 to 858) for SQAD and PCG (positions 911 to 913) for QSA. The size of the 293 cell-expressed DRADA forms was analyzed by Western blotting and activity was quantified by a dsRNA conversion assay (18).

Oligonucleotides Used in This Study. Vector primer was rsp23 (9); GluR6 oligonucleotides were O3gPCR2, O3PCR1, O3gQ, and O3gR (5); O3gRTBam1 was 5′-GACACGGATCCACACAACGGATCAAATTGAGACAGGAAACAGG-3′ (antisense, positions 398–356); O3g8 was 5′-GAGTGGAGTAGTGTAGTCTAC-3′ (sense, positions 1443–1463); O3gPCRK2 was 5′-GGCGGTACCAATTACCACAAG-3′ (antisense, positions 1968–1948); O3V(RQ) was 5′-TTGCYGCACGAGAGCTCC-3′ (antisense, positions 5 to −13); O3ghoted was 5′-GACTGTCATGCACTCAAGGCAA-3′ (antisense, positions 1874–1853); GluR5 oligonucleotides were O1gQ and O1gR (5); and O1gPCR2i was 5′-CCGGTACCGAGAACTGGCTAGAAAGTCC-3′ (antisense, positions 158–131). Mismatches to the gene sequence are underlined.

RESULTS

The ECS Element for Q/R Site Editing of GluR5 and GluR6 Pre-mRNAs. To determine the intronic ECS element essential for Q/R site editing of kainate receptor GluR5/6 pre-mRNAs, we isolated from a murine genomic library segments of the GluR5 and GluR6 genes, comprising the M2 and M3 exons and the intervening and adjoining intronic sequences (5). DNA sequencing revealed that the homologous introns downstream of the M2 exon had widely diverged in the GluR5 and GluR6 genes. We determined the entire intron sequences, 4450 nucleotides for GluR5 and 3525 for GluR6, and the alignment (Fig. 1) identified a short nucleotide sequence conserved in both introns, located ≈1850 nucleotides distal to the 5′ splice sites and complementary to the editing site in the M2 exon.

We sought functional evidence for the location of the ECS and employed an assay for Q/R site editing of GluR6 pre-mRNA in PC-12 cells. These cells do not express the endogenous GluR6 gene but produce GluR-B mRNA, edited to >99% in its Q/R site and to 26% in its R/G site, and also edit transcripts from transfected GluR-B minigenes (7, 9). A series of GluR6 minigenes was constructed (Fig. 2) containing the M2 exon and all, or part, of the downstream intron placed under the transcriptional control of a human cytomegalovirus promoter/enhancer. These minigenes were transiently expressed in PC-12 cells, and GluR6-specific transcripts were analyzed for the edited and unedited versions of the Q/R site. Results indicated the presence of two critical intron regions (lightly shaded in Fig. 2), one around the 5′ splice donor and the other in the middle of the intron. The intron could be progressively deleted from its 3′ end without loss of Q/R site editing (minigenes 1, 3, 4, 5). Editing was lost upon deleting more than the distal half of the intron (minigene 6), indicating the loss of a functionally critical element. Editing-competent minigenes with 3′ shortened introns were used for placing successive, overlapping deletions in the 5′ half of the intron. These remained editing competent (minigenes 4ΔH, 3ΔM) until the 5′ deletions removed the middle of the intron (minigenes 3ΔPK, 3ΔD), substantiating the presence of an important element in this region. A deletion (minigene 3ΔK2) placed 3′ to this critical region yielded edited GluR6 transcripts, which permitted us to locate within 210 nucleotides the intronic sequence essential for Q/R site editing (Fig. 2, lightly shaded area). Notably, this small segment contains the only significant sequence stretch shared by the homologous GluR5 and GluR6 introns (Fig. 1). The functional assays further identified as indispensable for GluR6 Q/R site editing part or all of the 150 nucleotides immediately downstream of the intronic 5′ donor (shaded in Fig. 2). The deletion mutants 3ΔStM and 3ΔH1 were editing competent but 3ΔBM was not. GluR5 constructs analogous in length to GluR6 minigenes 1 and 3 also generated Q/R site-edited GluR5 transcripts in PC-12 cells, and editing competence was lost when deletions removed the intronic midpoint (GluR5 construct analogous to minigene 6), indicating that the critical ECS element is located in the same region as in the GluR6 gene.

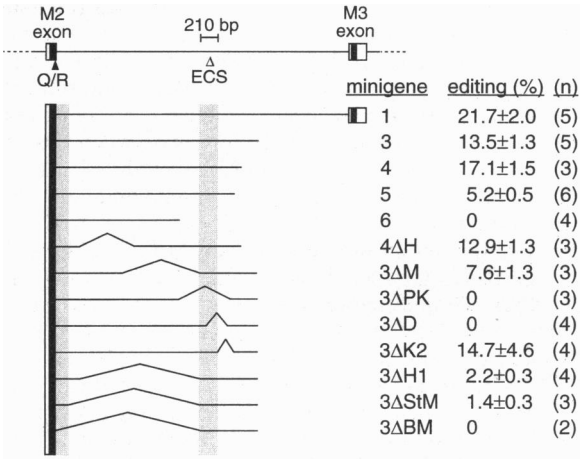

FIG. 2. Identification of critical intron regions for Q/R site editing by deletion mapping of GluR6 minigenes. A series of GluR6 minigenes is depicted below a genomic map of GluR6 comprising the M2 exon (positions −113 to 6), the downstream intron (positions 7 to 3527) and the M3 exon (positions 3528 to 3745). These minigenes (see *Materials and Methods*) were tested for Q/R site editing of GluR6 transcripts after transient expression in PC-12 cells. The editing efficiencies ± SDs are listed in percent. Editing-incompetent minigenes showed zero editing. The number of independent transfections for each construct is given in parentheses. The two critical intronic regions delineated by deletions are lightly shaded and comprise positions 7 to 154 (proximal intron region) and 1746 to 1964 (intronic midpoint).

Mutations in the Predicted Exon–Intron dsRNA Structure for GluR6 Q/R Site Editing. In the dsRNA structures essential for Q/R site editing of the GluR5 and GluR6 pre-mRNAs, predicted by the program RNAFOLD (21), the functionally identified intronic sequences pair with the exonic editing site. The relevant section of these structures comprising the region around the Q/R site (Fig. 3) shows that the Q/R site codon CAG with its to-be-edited adenosine occurs in both pre-mRNAs entirely mismatched opposite a four-nucleotide in-

tronic sequence, in contrast to both the Q/R and R/G site in GluR-B pre-mRNA (7, 9). Furthermore, the proximal intron sequence participates in the formation of an extended base-paired segment, which might explain the strict requirement for the proximal intron sequence as revealed by deletion mapping (see above). The pronounced differences between the proximal intron sequences in the GluR5 and GluR6 genes are compensated by differences in the complementary downstream sequences.

We investigated by mutational analysis the validity of the predicted dsRNA structure around the Q/R site of GluR6 pre-mRNA. The editing-competent minigene 3 was mutated to carry small deletions or insertions in the distant intronic ECS element (Fig. 3). A deletion of six nucleotides (M5) obliterated GluR6 Q/R site editing in transfected PC-12 cells, whereas a five-nucleotide deletion placed further upstream (M3; Fig. 3) reduced editing levels by approximately 50%. Insertions of three consecutive G residues in the intronic ECS region drastically reduced (M6) or abrogated (M7, M8) Q/R site editing of GluR6, whereas a triple-G insertion placed ≈25 nucleotides further upstream (M9) had a much lesser effect. Importantly, both intronic mutants M7 and M8 characterized by loss of editing could be functionally rescued by inserting C residues in the complementary exonic sequence (M7res, M8res). Both rescue mutants were edited to higher levels than the wild-type sequence, possibly reflecting an improved stability of the dsRNA around the editing site. The triple-C insertions had to be placed with positional precision, as no functional rescue was observed for an insertion offset by a single residue relative to the triple-G intronic mutation (M7off). These results attest to the predictive value of the dsRNA structure around the Q/R site and strongly indicate the involvement of the identified ECS element in the editing of this site.

DRADA Catalyzes Q/R Site Editing in GluR6 Pre-mRNA. HEK 293 cells when transfected with GluR6 minigenes (e.g., minigene 4; Fig. 2) produce GluR6 transcripts edited to low levels (0.5%). Editing increased dramatically (>30-fold), to

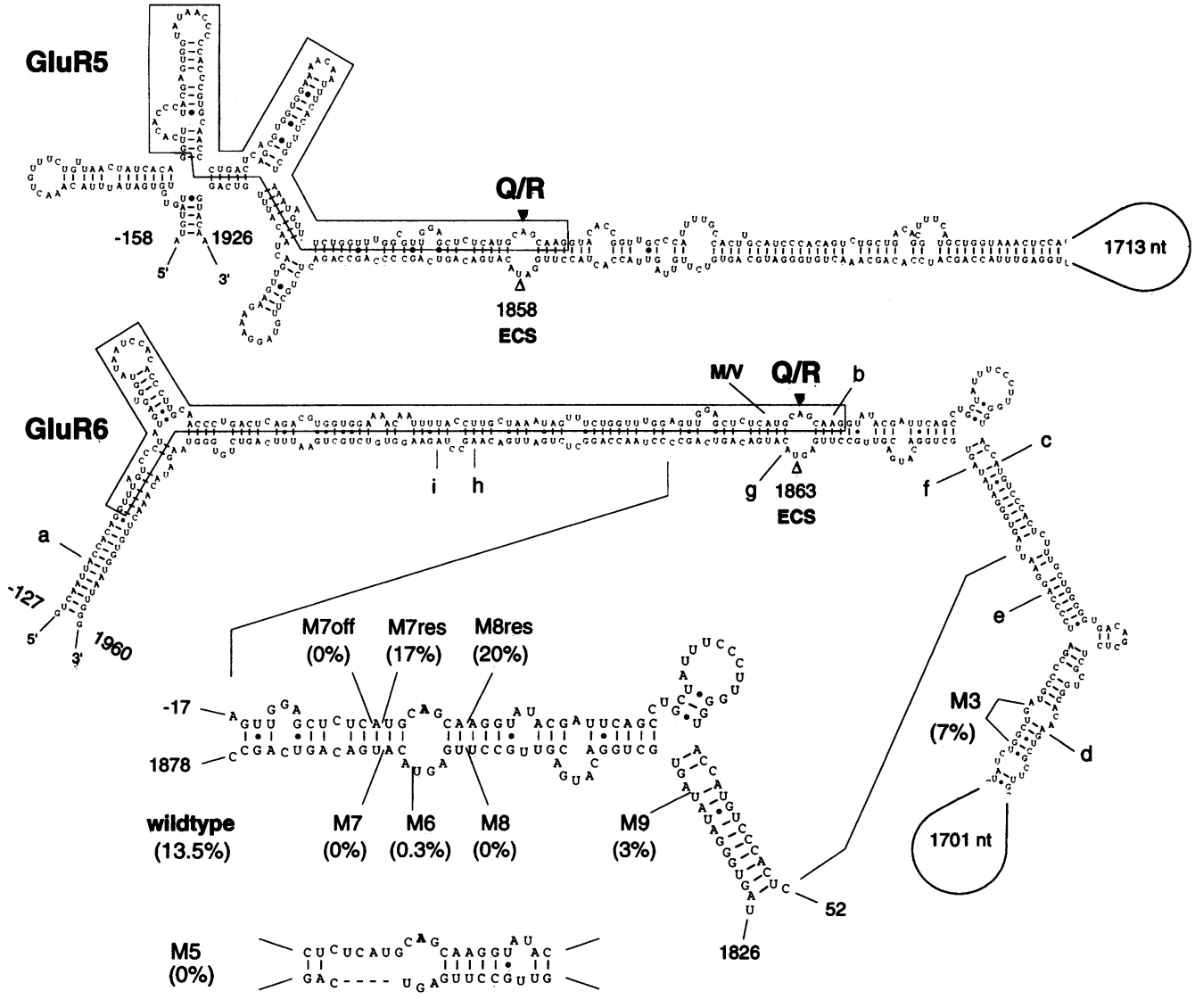

FIG. 3. Predicted exon–intron dsRNAs around the Q/R editing site of GluR5 and GluR6 pre-mRNAs. Structures presented are segments of those predicted by the RNAFOLD program (21). The intronic nucleotides omitted from presentation are indicated in the loops. The exonic sequences in the secondary structures are boxed, and nucleotide positions are in reference to the Q/R site adenosine (filled arrowhead, position 1). The ECS nucleotide opposite the Q/R site adenosine is indicated by an open arrowhead. The adenosine located four nucleotides upstream of the Q/R site in GluR6 and labeled M/V is edited to a low extent in brain-derived GluR6 sequences. Lowercase letters a to i in the GluR6 structure point to adenosines edited by recombinant DRADA (see also Table 1). The GluR6 structure in minigene 3 (wild type) was probed by deletions (M3, M5) and insertions (M6, M7, M8, M7res, M7off, M8res, M9). These are indicated in the large dsRNA structure or in the expanded structure centered on the internal loop for the Q/R site, together with extent of editing in PC-12 cells.

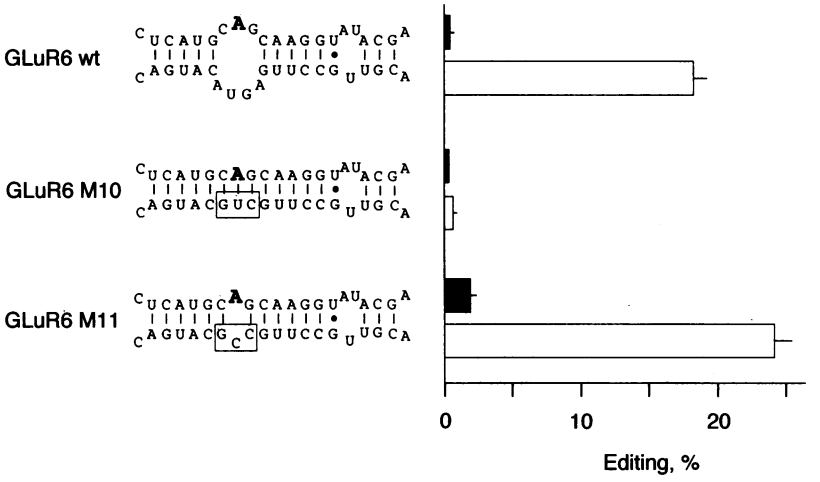

FIG. 4. Cotransfection of DRADA potentiates GluR6 Q/R site editing in HEK 293 cells. GluR6 minigene 4 (wt) and two mutants (M10 and M11) were transfected into HEK 293 cells. Transfections were in the presence (open bars) or absence (filled bars) of a mammalian expression vector for the full-length version of rat DRADA. The extent of Q/R site editing is depicted by bar graphs (mean ± SD, $n = 3$).

levels seen in PC-12 cells, when HEK 293 cells were cotransfected with an expression vector for the full-length version of rat DRADA (Fig. 4). Editing remained low (0.5%) when HEK 293 cells were cotransfected with a vector for a DRADA mutant incapacitated in its deaminase domain. Furthermore, the intronic ECS mutants M10 and M11, which mimic the base pairing in GluR-B pre-mRNA of the Q/R site (fully paired) and the R/G site (A–C mismatch), yielded similar editing levels in PC-12 and recombinant DRADA-expressing 293 cells (Fig. 4). Mutant M11 was edited to a higher extent than the wild-type sequence, whereas mutant M10 was not edited. In 293 cells not coexpressing recombinant DRADA, M11 was edited to considerably higher levels (≈4%) than the wild-type sequence, indicating that endogenous DRADA levels in 293 cells, but not in PC-12 cells, may be too low for the efficient editing of the wild-type configuration of GluR6 pre-mRNA. Indeed, when assayed in nuclear extracts with synthetic dsRNA as a substrate (18), HEK 293 cells expressing recombinant DRADA (but not the DRADA mutant) displayed an averaged DRADA activity similar to that of PC-12 cells, approximately 10-fold higher than untransfected 293 cells (not shown). Together, these results indicate that DRADA edits the Q/R site in GluR6 pre-mRNA in both PC-12 and recombinant DRADA-expressing 293 cells.

DNA sequencing of cloned RT-PCR products from HEK 293 cell assays showed that adenosines in positions other than the Q/R site, preferentially intronic adenosines, were converted by DRADA at different frequencies. This is reminiscent of the situation encountered in GluR-B intron 11 sequences derived from brain or GluR-B-expressing cells (9, 10). Targeted adenosines were found more frequently in the Q/R site-edited version (R-form) than in the unedited form (Q-form). Table 1 lists adenosines in the dsRNA (Fig. 3) targeted in more than 10% of the R- and Q-forms. These occurred in loops and bulges, at the ends of short dsRNA structures, and in sequence environments compatible with previously established selectivity rules (16). Attesting to positional preferences of DRADA on GluR6 pre-mRNA, the Q/R site and another site constituted the most frequently targeted adenosines. This other editing site (position 1864), part of the ECS and positioned opposite the Q/R site, was targeted in >50% of all clones, rendering it the most edited adenosine in GluR6 sequences by recombinant DRADA. The adenosines of the Q/R and ECS sites were edited independently, as evidenced from cloned sequences in which either adenosine occurred edited alone. The ECS adenosine at position 1864 is also highly edited (70% of all brain-derived clones) in mouse brain (80% R-forms), and correspondence to positions edited *in vivo* was also observed for other targeted adenosines in the GluR6 sequence (Table 1). A coding position (−4) was found edited in 5% of the Q/R site edited brain-derived GluR6 clones and, similarly rarely, in the 293 cell-derived clones. Editing at this

Table 1. Adenosines targeted in GluR6 pre-mRNA by recombinant DRADA

Position	Site	Sequence	Structure	% in R-forms	% in Q-forms
−120	a	UAC	ds	25	<10
1	Q/R*	CAG	int. loop	100	0
5	b*	AAG	ds	10	10
42	c*	CAU	5′ds	40	<10
84	d*	AAG	5′ds	15	<10
1820	e*	CAG	ds	15	0
1837	f*	UAG	int. loop	40	10
1864	g*	UAC	int. loop	80	40
1901	h	AAG	ds	20	10
1906	i*	UAG	ds	20	<10

Adenosines appearing as guanosines in more than 10% of the GluR6 sequences cloned after cellular editing by recombinant DRADA are listed by nucleotide position, site designation (see Fig. 3), immediate sequence environment (5′ to 3′, edited adenosines shown in boldface), structural prediction (int. loop, internal loop; 5′ds, at the 5′ end of a double-stranded segment), and occurrence in Q/R site edited (R) or unedited (Q) forms. Positions at which edited adenosines were also observed in GluR6 pre-mRNA from brain are indicated (*) (site a was not sequenced from brain material).

site results in a codon change from methionine to valine (M/V; Fig. 3). These results demonstrate that recombinant DRADA converts adenosines site selectively in GluR6 pre-mRNA.

DISCUSSION

dsRNAs formed by exonic and intronic sequences are essential determinants for site-selective adenosine deamination across distantly related GluR genes, and they may also direct RNA editing in pre-mRNAs for other genes. As shown here for GluR5 and GluR6, the distance between the editing site and its complementary intronic ECS element can be large (>1500 nucleotides), compatible with long-range interactions in RNA characteristic of other posttranscriptional processes (26). Notably, a comparison of dsRNA structures for the different editing sites in GluR pre-mRNAs reveals that the critical editing-targeted adenosine occurs in distinct structural environments. The adenosine of the GluR-B Q/R site is positioned off center in a 17-bp RNA duplex (9); the R/G site adenosine in GluR-B, -C, and -D is mismatched [or non-Watson–Crick paired (27)] to a cytosine residue in a predicted ≈30-bp imperfect RNA stem-loop structure (7); and the Q/R site adenosine in GluR5 and GluR6 pre-mRNA occurs in an asymmetric internal loop of three exonic and four intronic nucleotides. These different local structures for the editing sites in exon–intron dsRNAs predict that the to-be-edited adenosines have distinct intrinsic accessibilities for the nuclear enzyme(s) catalyzing the site-selective RNA editing.

We investigated the activity of recombinantly expressed DRADA (4) on GluR6 pre-mRNA. This was facilitated by our observation that GluR6 minigenes edited in PC-12 cells can also be edited in HEK 293 cells upon recombinant coexpression of DRADA, whereas 293 cells not expressing recombinant DRADA edit GluR6 pre-mRNA very poorly. Indeed, PC-12 cells contain higher DRADA levels than 293 cells, and this may explain the different editing efficiency of GluR6 pre-mRNA in these cells. Editing in cotransfected 293 cells appeared to be directly mediated by the enzymatic activity of DRADA, as shown by failure of editing with a DRADA mutant incapacitated in deaminase function. We observed that GluR6 ECS mutant M10, not edited in PC-12 cells, was also not edited in recombinant DRADA-expressing 293 cells. Furthermore, in these cells, the ECS mutant M11 yielded higher editing than the wild-type sequence, again as in editing-competent PC-12 cells. Collectively, these results indicate that DRADA recognizes the exon–intron dsRNA of GluR6 pre-mRNA as a substrate for Q/R site editing, in both 293 and PC-12 cells. Since DRADA is expressed in all cells (15), including central neurons (18), GluR5 and GluR6 pre-mRNAs may be natural substrates for this enzyme. Our experiments do not permit us to decide whether DRADA can perform the Q/R site editing of GluR5/6 by itself or if it requires cofactors. Attempts at using an *in vitro* system (10–12) to investigate this issue were not met with success, possibly due to the inefficient formation of the required dsRNA structures by the large-sized synthetic GluR6 pre-mRNA.

The recombinant expression of DRADA in HEK 293 cells generated higher than 10-fold enzyme activity relative to endogenous levels. Screening cloned GluR6 sequences from 293 cells for evidence of DRADA-mediated adenosine conversion yielded a pattern with several adenosine positions affected, each to different extents. A comparison of this pattern with that seen in brain-derived GluR6 sequences showed that the adenosine of the Q/R site was preferentially targeted, together with an intronic adenosine predicted to occupy an ECS position opposite of the Q/R site. This latter site is also prominently edited in GluR6 pre-mRNA from brain. Furthermore, most additional edited adenosines were found in the intron, with exonic GluR6 sequences showing virtually no changes. An exception is position +5, which is the wobble position of the adjacent glutamine codon, also found in brain-derived GluR6 sequences. These data are largely compatible with the view that DRADA may mediate GluR6 editing *in vivo*. The failure of DRADA to edit a Q/R site configuration in the GluR6 mutant M10 in which the to-be-edited adenosine is base paired as it is in GluR-B might indicate that Q/R site editing in GluR-B and GluR6 pre-mRNAs operates by different mechanisms.

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