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Deamination of mammalian glutamate receptor RNA by *Xenopus* dsRNA adenosine deaminase: Similarities to in vivo RNA editing

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ABSTRACT

Double-stranded RNA (dsRNA) adenosine deaminase (dsRAD) converts adenosines to inosines within dsRNA. A great deal of evidence suggests that dsRAD or a related enzyme edits mammalian glutamate receptor mRNA in vivo. Here we map the deamination sites that occur in a truncated glutamate receptor-B (gluR-B) mRNA after incubation with pure *Xenopus* dsRAD. We find remarkable similarities, as well as distinct differences, between the observed deamination sites and the sites reported to be edited within RNAs isolated from mammalian brain. For example, although deamination at the biologically relevant Q/R editing site occurs, it occurs much less frequently than editing at this site in vivo. We hypothesize that the similarities between the deamination and editing patterns exist because the deamination specificity that is intrinsic to dsRAD is involved in selecting editing sites in vivo. We propose that the observed differences are due to the absence of accessory factors that play indirect roles in vivo, such as binding to and occluding certain sites from dsRAD, or promoting the RNA structure required for correct and efficient editing. The work reported here also suggests that dsRAD is capable of much more selectivity than previously thought; a minimal number of deamination sites (average ≤5) were found in each gluR-B RNA. We speculate that the observed selectivity is due to the various structural elements (mismatches, bulges, loops) that periodically interrupt the base paired region required for editing.

Keywords: double-stranded RNA; inosine

INTRODUCTION

Double-stranded RNA (dsRNA) adenosine deaminase (dsRAD) converts adenosines to inosines within dsRNA (reviewed in Bass, 1993; Kim & Nishikura, 1993). The enzyme was discovered in 1987, rather serendipitously, when a synthetic dsRNA was injected into *Xenopus* embryos (Bass & Weintraub, 1987; Rebagliati & Melton, 1987). dsRAD has now been detected in every metazoan cell tested and purified to homogeneity from *Xenopus* eggs (Hough & Bass, 1994), calf thymus (O'Connell & Keller, 1994), and bovine liver (Kim et al., 1994a). In addition, several mammalian cDNAs have been cloned (Kim et al., 1994b; O'Connell et al., 1995; Patterson & Samuel, 1995). Despite this progress, until recently, dsRAD has been an enzyme in search of a function.

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Because biological substrates have been elusive, in vitro studies have been conducted using dsRNAs of artificial sequences. Such studies show that, although dsRAD will bind to dsRNA of any sequence, as well as deaminate multiple adenosines within a single RNA, it does not choose adenosines for deamination randomly (Polson & Bass, 1994). For example, dsRAD has a 5′ nearest-neighbor preference (A = U > C > G) and disfavors adenosines that are close to 3′ termini. These determinants of deamination specificity have been called *preferences*. With certain substrates, dsRAD also exhibits *selectivity*, that is, with these substrates, only a subset of the adenosines in preferred context are modified. dsRAD decreases the thermodynamic stability of a duplex when AU base pairs become the less stable IU pair. Thus, it has been hypothesized that *selectivity* occurs because the reaction stops after a minimal number of modifications make the dsRNA too single-stranded in character to be bound by dsRAD (Polson & Bass, 1994). In support of this idea, dsRAD is more

selective on shorter, less stable, duplexes (Polson & Bass, 1994).

In 1991, an RNA editing event was reported to occur on certain mammalian glutamate receptor (gluR) mRNAs that could be explained by the action of dsRAD (Sommer et al., 1991). The observed RNA editing occurred at sites of genomic adenosines, which, after editing, appeared as guanosines in cDNAs. Because inosine prefers to pair with cytidine, inosines produced by dsRAD appear as guanosines in cDNAs. Subsequent studies have shown that multiple editing sites often occur within a single gluR mRNA (Higuchi et al., 1993; Köhler et al., 1993), as expected if dsRAD is responsible for the editing. Not all editing events on gluR mRNAs appear to be functionally relevant, but some produce codon changes that clearly alter the properties of the ion channels assembled from the gluR subunits. For example, an editing event found within gluR mRNAs encoding subunits B, 5, and 6 converts a glutamine codon to an arginine codon (Q/R site) and results in ion channels with altered calcium permeability (Sommer et al., 1991; Higuchi et al., 1993). Another editing event converts an arginine to a glycine (R/G site) within mRNAs for subunits B, C, and D, and changes the kinetic properties of the ion channels (Lomeli et al., 1994).

In further support of the idea that dsRAD edits gluR mRNAs, editing at both the Q/R site (Higuchi et al., 1993; Egebjerg et al., 1994) and the R/G site (Lomeli et al., 1994) has been shown to require base pairing between exon sequences surrounding the editing site and the downstream intron. The unspliced RNAs are predicted to form structures that are largely double-stranded, but sometimes interrupted by loops, mismatches, or bulges (e.g., see Figs. 2, 3).

Despite the fact that dsRAD clearly exhibits *preferences* and *selectivity*, we and others postulated that further deamination specificity would be required to produce a precise biological event, and that this additional specificity would be provided by accessory factors in vivo (Bass, 1992; Higuchi et al., 1993). Thus, it was surprising to find that the editing sites that occur in vivo on gluR-B mRNA are entirely consistent with the deamination specificity observed in vitro on artificial substrates (Polson & Bass, 1994). In addition, mutations in gluR-B mRNA alter editing in ways predicted by dsRAD's specificity (Polson & Bass, 1994). For example, mutation of the C, which naturally occurs as the 5′ nearest neighbor of the Q/R site, to a G, reduces editing significantly (Higuchi et al., 1993).

Several laboratories have shown that in vitro-transcribed gluR-B mRNAs can be correctly edited in crude extracts from mammalian cells and, further, that this in vitro editing involves an adenosine to inosine conversion (reviewed in Bass, 1995; see Melcher et al., 1995; Rueter et al., 1995; Yang et al., 1995). Taken together, the existing data strongly suggest that dsRAD, or a highly related enzyme, is the enzyme responsible for gluR mRNA editing. A more controversial issue is whether dsRAD can edit gluR mRNA alone or requires accessory factors, and further, what the role of such putative accessory factors might be.

We have mapped sites of deamination that occur on a truncated gluR-B mRNA after incubation with purified *Xenopus* dsRAD. We find remarkable similarities, as well as distinct differences, between the observed deamination sites and editing sites reported to occur within RNAs isolated from mammalian brain (Higuchi et al., 1993; Rueter et al., 1995). We hypothesize that the similarities exist because the deamination specificity that is intrinsic to dsRAD functions in the selection of editing sites in vivo, in much the same way that it allows deamination of only certain adenosines in vitro. We propose that the observed differences are due to the absence of accessory factors that play indirect roles in vivo, such as binding to and occluding certain sites from dsRAD, or promoting the RNA structure required for correct editing by acting as RNA chaperones.

RESULTS

As discussed above, several laboratories have demonstrated that gluR mRNAs can be edited accurately in vitro using crude extracts from mammalian cells (Melcher et al., 1995; Rueter et al., 1995; Yang et al., 1995). Extracts from non-mammalian organisms have not been tested, nor has the editing been demonstrated to occur with a purified protein. To explore these issues, we incubated a truncated glutamate receptor-B RNA with a pure preparation of dsRAD from *Xenopus* eggs (Fig. 1A). The RNA was transcribed in the presence of α-^{32}P-ATP, so that adenosine to inosine conversion could be monitored by thin layer chromatography (TLC) of mononucleotides derived from the RNA products. The 643-nt RNA (gluR-B590) contained 50 nt of vector sequence as well as a region of mouse gluR-B mRNA predicted to form the base paired structure required for Q/R site editing; specifically, 210 nt of exon 11 followed by 383 nt of the downstream intron.

As shown in Figure 1B, adenosine to inosine conversion was detected in the gluR-B590 transcript after incubation with the purified preparation of dsRAD. Deamination of gluR-B590 could be competed with a dsRNA of a different sequence, but not a single-stranded RNA (ssRNA). The latter is consistent with modification by dsRAD, which binds to dsRNA of any sequence but not ssRNAs (Bass & Weintraub, 1987; Wagner & Nishikura, 1988).

In vitro studies show that dsRAD will modify dsRNA of many different sequences (Polson & Bass, 1994 and references therein). Thus, it seemed possible that deamination of the gluR-B590 sequence was fortuitous and unrelated to the RNA editing observed in vivo. To explore this possibility, we cloned and then se-

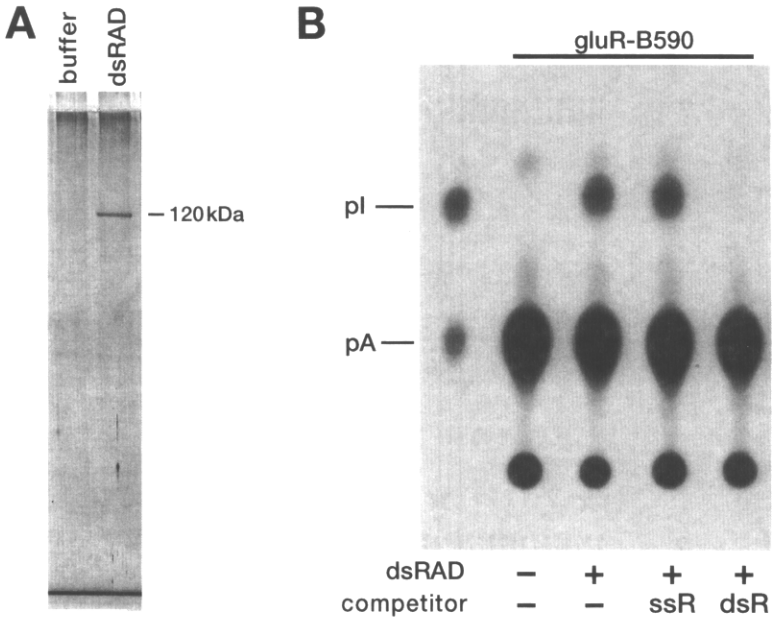

FIGURE 1. Purified *Xenopus* dsRAD can deaminate gluR-B mRNA in vitro. A: The Xenopus dsRAD preparation was analyzed by 8–12% SDS-PAGE to determine its purity. Lanes were loaded with 100 μL of 1× (final) gel sample buffer containing either 75 μL dsRAD in TBS (dsRAD) or 75 μL TBS only (buffer). As shown, only a single band is visible by silver staining after artifact bands are eliminated by comparison to the buffer-only lane. Buffers, electrophoresis conditions, and staining conditions were as described (Hough & Bass, 1994). B: ^{32}P-A labeled gluR-B590 (10 fmol) was incubated with or without 10 μL of the dsRAD preparation shown in A and, as indicated, 10 fmol of an unlabeled double-stranded RNA (dsR) or single-stranded RNA (ssR) competitor. After 1 h at 25 °C, RNA was deproteinized, digested to mononucleotides, and separated by TLC (see the Materials and methods). Unlabeled lanes show ^{32}P-AMP and ^{32}P-IMP markers. Under these incubation conditions, gluR-B590, in the absence of competitor, showed 2.6% deamination ($n = 3$, SD = 0.2%; $[I/(A + I + \text{origin})] \times 100$; quantified as described in Saccomanno and Bass [1994]).

quenced PCR products of cDNAs made from three populations of RNA modified in vitro and compared the observed deamination sites with the editing sites published for endogenous RNAs of rat. Two of the RNA populations were modified by purified dsRAD (reactions 1 and 2). For comparison, the third population of RNA was modified in vitro by incubating with crude extracts of *Xenopus* oocyte nuclei. The latter samples gave TLC results that were similar to those shown in Figure 1B. That is, nuclear extracts deaminated gluR-B590, and deamination could be competed with a second molecule of dsRNA (data not shown). Vector sequences that were unique to the in vitro transcribed RNAs were included in the cloned sequences for samples modified with pure dsRAD so that we could verify that the observed deamination patterns were derived from RNAs modified in vitro. Sequencing primers were chosen to encompass a ~100-bp region surrounding the Q/R site (see Figs. 2, 3).

For the samples modified with pure dsRAD, 35 of 36 cDNAs sequenced from reaction 1 showed A to G changes, whereas all 66 cDNAs sequenced from reaction 2 showed A to G changes. Thirty-three cDNAs corresponding to RNAs modified with oocyte nuclear extracts were sequenced and, of these, 29 clones showed A to G changes. As shown in Table 1, the number of A to G changes found in each cDNA was minimal. For the region of gluR-B590 mRNA analyzed (see Figs. 2, 3), which contained 52 adenosines, samples modified with nuclear extract showed an average of 1.8 A to G changes per cDNA, and reactions 1 and 2 showed 2.6 and 5.2 changes, respectively. Note that the amount of dsRAD used to modify the various RNA populations, in all cases, was an amount sufficient to modify 40–50% of the adenosines in a dsRNA consisting of 800 contiguous base pairs (chloramphenicol acetyltransferase or CAT duplex, see the Materials and methods; data not shown and Hough & Bass, 1994). The latter emphasizes the high degree of selectivity observed with the gluR-B590 substrate. The number of A to G changes found in each cDNA was reminiscent of the cDNAs made from endogenous rat gluR-B mRNAs, which have an average of 3.6 A to G changes per cDNA (SD = 1.2; R. Emeson, pers. comm.). Furthermore, the average number of A to G changes per cDNA correlated with the number of deamination sites predicted by TLC to be in the entire gluR-B590 transcript, which contains 161 adenosines, suggesting that few deaminations occurred outside of the base paired structure shown in Figures 2 and 3, and also known to be important for editing (Higuchi et al., 1993; Egebjerg et al., 1994).

In contrast to cDNA sequences reported for endogenous rat gluR-B RNAs, where 99% of the molecules show A to G changes at the Q/R site (Rueter et al., 1995), or mouse, where 100% of the cDNAs sequenced have an A to G change at the Q/R site (Higuchi et al., 1993), only 3 of the total 135 cDNAs sequenced exhibited an A to G change at the Q/R site. However, the deamination patterns observed in these three molecules were quite similar to editing patterns observed in vivo (Fig. 2). One of the molecules deaminated at the Q/R site was found in the population modified with nuclear extract and the other two in reaction 2 derived from samples treated with pure dsRAD. Each molecule found to be deaminated at the Q/R site had a minimal number of additional A to G changes, with the majority at or near sites previously designated as editing hot spots within endogenous RNAs (Higuchi et al., 1993; Rueter et al., 1995). There were no obvious differences between the single clone derived from the population

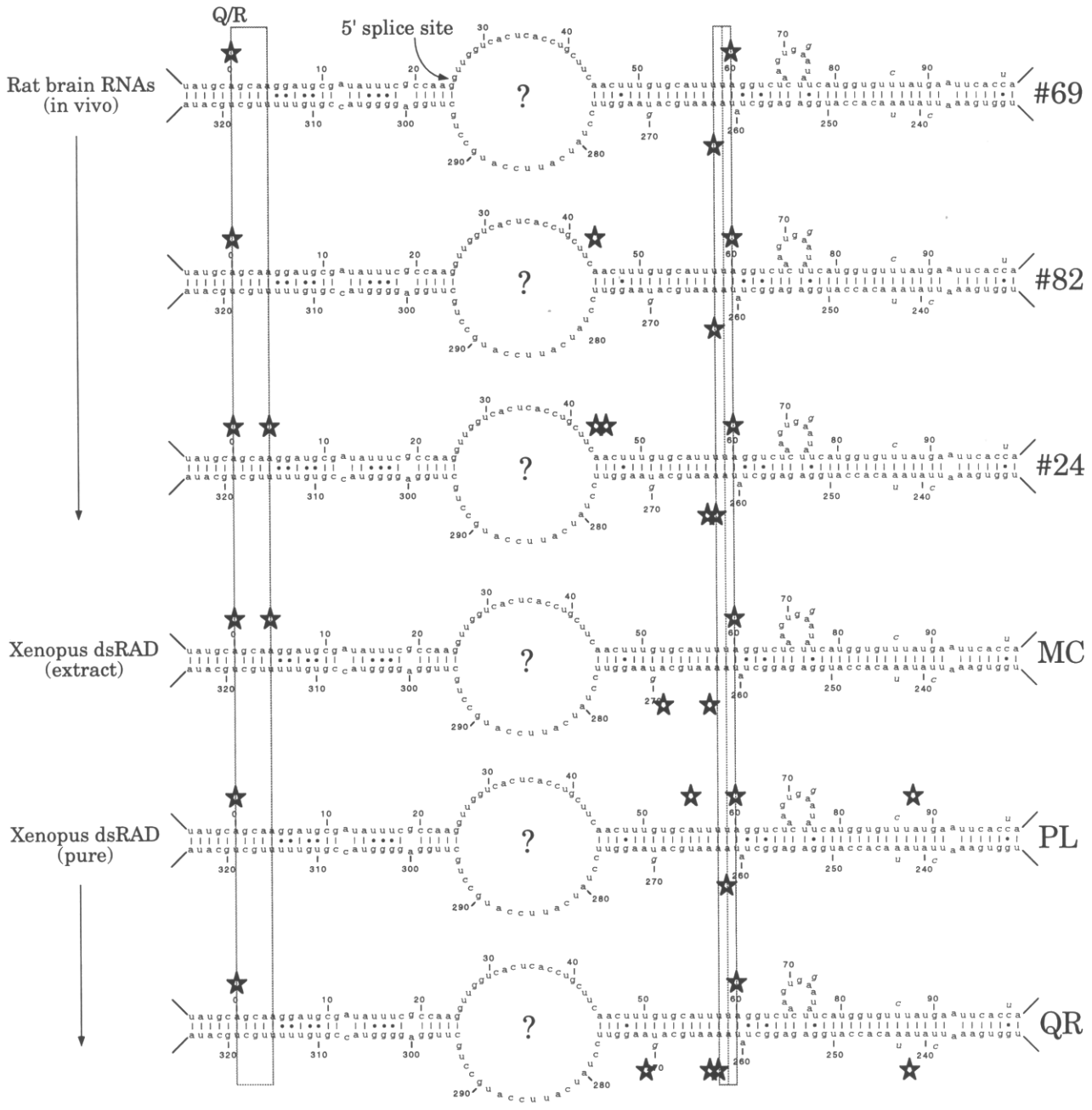

FIGURE 2. The Q/R site of gluR-B mRNA can be deaminated in vitro with *Xenopus* dsRAD. A base paired structure encompassing the Q/R site of rat gluR-B mRNA is shown with stars indicating deamination or editing sites inferred by cDNA sequencing; numbering scheme assumes Q/R site = 0. Italics above or below the sequence show nucleotides of the mouse gene that differ from the rat. Hot spots determined previously (Rueter et al., 1995) are shown as lines passing through the relevant adenosines. The top three duplexes show editing patterns found in cDNAs prepared from endogenous rat mRNA (#69, #82, #24; Rueter et al., 1995, and C. Burns & R. Emeson, pers. comm.). The bottom three duplexes show deamination sites found in cDNAs derived from RNA deaminated in vitro with *Xenopus* nuclear extract (MC) or pure dsRAD (PL, QR). In vitro reactions contained 20 fmol of gluR-B590 RNA, nuclear extract, or pure dsRAD, and were incubated as described (see the Materials and methods). The base paired structure was modified from Bass (1995) and only helices supported by compensatory mutagenesis data are shown (Higuchi et al., 1993; Rueter et al., 1995; Yang et al., 1995; as cited in Bass, 1995); little is known about the structure of nucleotides surrounding the question mark. As indicated, the 5′ splice site is between nt 24 and 25.

modified with the crude extract and the two molecules that derived from the reaction with pure dsRAD.

The deamination patterns found in each of the three populations of in vitro-modified RNAs as a whole, along with the editing patterns mapped within endogenous rat RNAs (Rueter et al., 1995), are summarized in Figure 3. To aid in visualization of similarities and differences between the editing and deamination pat-

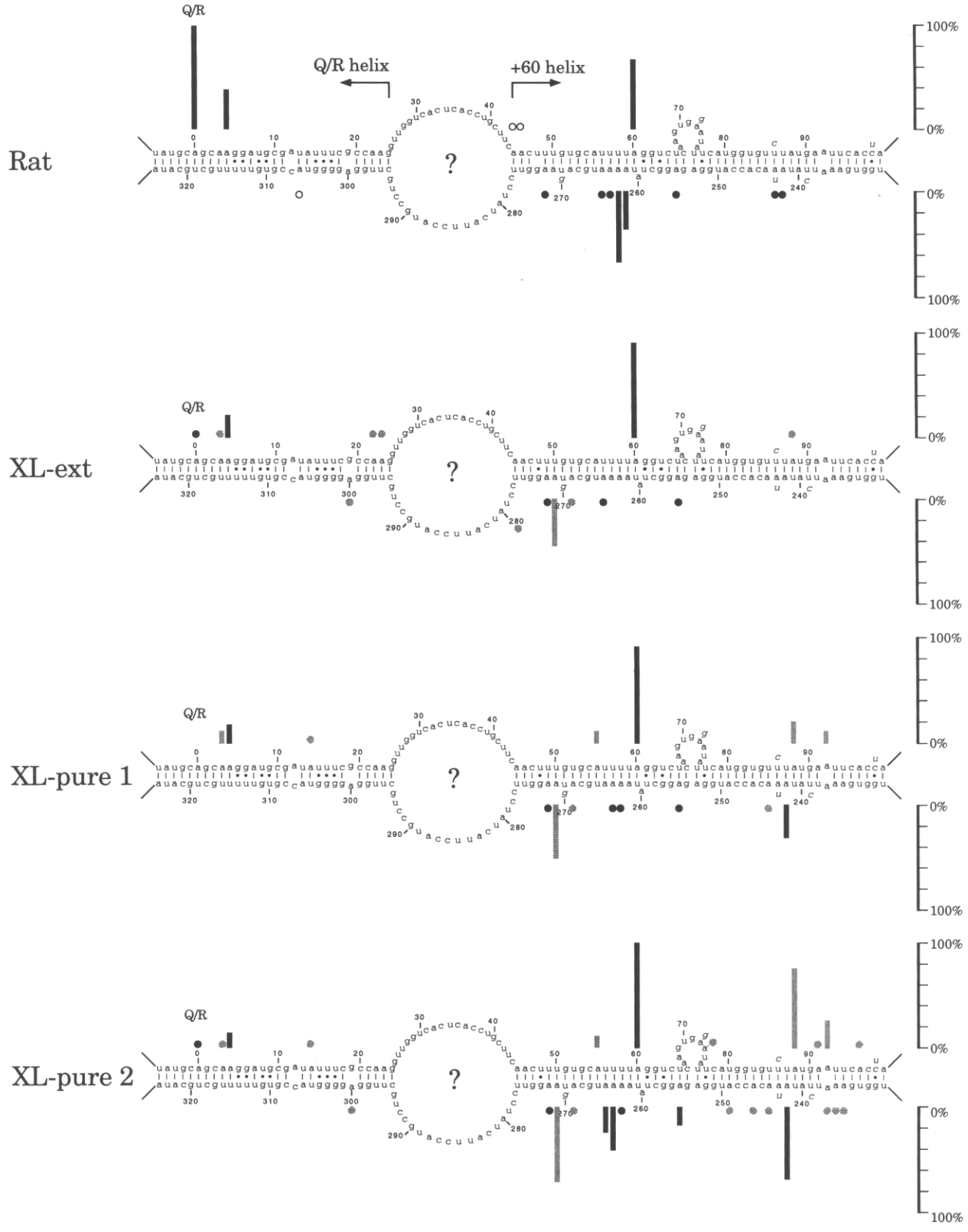

FIGURE 3. Deamination patterns exhibited by the entire sequenced population of in vitro modified RNAs are compared and contrasted with the editing patterns found in populations of endogenous rat RNAs. Data for the rat RNA is based on sequencing 100 cDNAs and is described in Rueter et al. (1995). Data for RNAs modified in vitro with nuclear extracts (XL-ext) and the two populations of RNAs modified with pure dsRAD (XL-pure 1 and 2) are described in the text. Adenosines that appeared as both deamination and editing sites (black symbols), those found only as editing sites (open symbols), or those found only as deamination sites (gray symbols) are indicated. The percent of the population that contained an A to G change at a particular site is represented by bar height, and sites edited in ≤10% of the population are indicated with small circles. In vitro reactions and other notations are as in Figure 2.

TABLE 1. Summary of TLC and sequencing data.

Sample	Percent deamination[a]	Inosines per RNA[b]	A to G changes per cDNA[c]	Total clones sequenced
Nuclear extract	–[d]	–[d]	1.8 ± 1.3	33
Pure-1	1.9%	3.1	2.6 ± 1.4	36
Pure-2	3.4%	5.5	5.2 ± 1.4	66

[a] Values are from TLC assays and quantification was as described (Saccomanno & Bass, 1994) with percent deamination = $[I/(A + I + \text{origin})] \times 100$.

[b] Values were inferred from the observed percent deamination and the total number of adenosines in gluR-B590 (161).

[c] Averages with standard deviation (±) are given as determined using n = all clones sequenced.

[d] Because of high background, possibly due to contaminating nucleic acids in the oocyte nuclear extracts, these TLC assays were not considered quantifiable.

terns, Figure 3 shows adenosines that appeared as both deamination and editing sites (black filled symbols), those found only as editing sites (open symbols), and those that appeared only as deamination sites (gray symbols). As expected from previous studies, deamination and editing sites are not random, and certain adenosines (hot spots) appear as guanosines in many of the cDNAs sequenced (black and gray bars). In addition to hot spots, both deamination and editing patterns exhibit a number of minor sites, found in ≤10% of the population (small circles).

In endogenous rat gluR-B RNAs, 5 of the 52 adenosines within the base paired region shown in Figure 3 are editing hot spots. Editing hot spots are clustered in two regions, one near the Q/R site within the Q/R helix (see Fig. 3 for labeling of helices) and the other within a run of AU base pairs in the +60 helix that includes the adenosine at +60. Notably, deamination hot spots were also observed in these two regions. However, there were two obvious differences between deamination patterns and editing patterns. First, the prevalence of a particular deamination site within a given in vitro-modified population (as shown by bar height) often differed from its prevalence as an editing site within the endogenous population. Second, deamination hot spots were observed that were not represented as editing hot spots. For example, in the Q/R helix, deamination hot spots were clustered in the same region as the editing sites, but, in one of the populations (XL-pure 1), an additional hot spot was observed at +3. In addition, as discussed, the Q/R site, which is an editing hot spot in vivo, was a minor deamination site in all of the in vitro-modified populations. Similarly, within the +60 helix, the adenosine at +60, which is an editing hot spot, was also a deamination hot spot in all three of the in vitro-modified populations. However, other adenosines within this cluster that are editing hot spots, in general, if modified at all, were only minor deamination sites. Taking the three in vitro-modified populations as a whole, four additional hot spots were observed within the +60 helix that were not found as editing sites in vivo. Finally, the deamination pattern observed with the *Xenopus* oocyte nuclear extract, for the most part, was a subset of those found with purified *Xenopus* dsRAD. In particular, the extract-treated sample lacked the hot spots at nt 55, 88, 92, and 242 that were present in both samples modified with pure dsRAD.

DISCUSSION

Here we show that adenosines within in vitro-synthesized gluR-B transcripts can be deaminated by purified *Xenopus* dsRAD as well as extracts prepared from *Xenopus* oocyte nuclei. The sites of deamination found within the population of RNAs modified in vitro show obvious similarities, as well as distinct differences, when compared to the editing sites found within gluR-B mRNAs isolated from mammalian brain (Higuchi et al., 1993; Rueter et al., 1995). Only a few of the molecules deaminated in vitro show deamination at the biologically relevant Q/R editing site, but, given our current understanding of the in vivo editing patterns, these few molecules are essentially indistinguishable from molecules edited in vivo. Like gluR-B mRNAs isolated from mouse or rat brain, which show multiple editing sites in a single molecule, molecules deaminated at the Q/R site in vitro have several additional deamination sites in nearby regions, the majority of which are at or near sites that are editing hot spots in vivo (Fig. 2).

Differences between in vivo editing patterns and in vitro deamination patterns were most apparent during analyses of populations, rather than individual clones (Fig. 3). The two most notable differences were the additional hot spots found within in vitro-modified populations compared to endogenous RNAs (gray bars), and the scarcity of deamination at the Q/R site in vitro, which is the most prevalent editing site in vivo. Accepting for a moment that dsRAD is the enzyme involved in gluR-B editing in vivo, how can the differences be explained? One possibility is that *Xenopus* dsRAD has slight differences from the mammalian enzyme that result in a lower efficiency of Q/R site editing and additional hot spots. In this regard, a recent report states that the purified mammalian enzyme cannot by itself edit gluR-B mRNA (unpubl. data as cited in Melcher et al., 1995). However, the latter conclusion was based on primer extension across the Q/R site only. Thus, if deamination at the Q/R site is inefficient with the purified mammalian enzyme, as observed with the purified *Xenopus* enzyme, a minor amount of deamination may have been overlooked. Regardless, at present we cannot rule out the possibility that at least some of the observed differences are due to differences between

the mammalian and *Xenopus* enzymes. A related issue is the fact that in Figure 2 and 3 we are comparing in vitro editing of a mouse sequence with the editing pattern determined for endogenous rat RNAs. However, a sequence analysis of 38 cDNAs (from position −61 to 405) made from murine brain RNA shows editing hot spots at the Q/R site, 4, 60, 262, 263, and 264, as well as minor sites scattered in surrounding regions (Higuchi et al., 1993), suggesting differences are not due to the comparison of heterologous sequences.

Another obvious possibility for the observed differences is that dsRAD requires accessory factors to give the editing patterns observed within endogenous RNAs. Because the editing sites found within endogenous RNAs are consistent with the deamination specificity intrinsic to dsRAD (Polson & Bass, 1994), and the in vitro- and in vivo-edited molecules (see Fig. 2) look quite similar, we propose that putative accessory factors do not act directly on dsRAD to alter its deamination specificity, but serve other, more indirect, roles. For example, the additional hot spots observed in vitro may not be deaminated in vivo because they are bound by other proteins which block, or compete for, binding by dsRAD. Evidence that deamination sites can be protected from dsRAD by protein binding has been reported (Saccomanno & Bass, 1994) and, in fact, some of the editing sites in gluR-B mRNA are very close to the 5′ splice site, and certainly bound by components of the splicing machinery some of the time.

Accessory factors with indirect roles can also be invoked to explain the difference in the efficiency of Q/R site editing. Our favorite explanation for why Q/R site editing is rare in our in vitro reaction is that the RNA structure required for editing the Q/R site is also rare in our population of in vitro-synthesized gluR-B RNAs. Thus, putative accessory factors may act as RNA chaperones (Herschlag, 1995) to promote the formation of the structure needed for accurate editing by dsRAD. For the studies reported here, conditions for reacting gluR-B mRNA were chosen to maximize inosine production rather than to favor correct folding of the RNA. In future studies, we will aim for the latter in hopes that the accuracy and efficiency of Q/R site editing will increase, perhaps even in the absence of RNA chaperones. In this regard, by optimizing the reaction with respect to deamination at the Q/R site, rather than total inosine, we have recently increased the efficiency of deamination at the Q/R site to 4.2% ($n = 3$, SD = 0.7%; M. Öhman & B. Bass, unpubl. data).

It is important to point out that editing of gluR-B mRNA in mammalian nuclear extracts has been reported to occur with very efficient Q/R site editing, and to yield editing patterns that are essentially indistinguishable from those found within endogenous RNAs (Melcher et al., 1995; Rueter et al., 1995; Yang et al., 1995). In contrast, the deamination patterns we observed in RNAs modified with *Xenopus* nuclear extracts, like those treated with the pure *Xenopus* enzyme, showed additional hot spots and inefficient editing at the Q/R site. Thus, if dsRAD requires accessory factors to produce the editing patterns observed in vivo, such factors must be present in mammalian nuclear extracts, but missing, or incomplete, in *Xenopus* nuclear extracts.

There are some minor differences between the deamination pattern observed with the *Xenopus* nuclear extract and the purified *Xenopus* protein, and certainly these may be due to proteins other than dsRAD that are present in the nuclear extract. However, another explanation for these minor differences is that the RNAs treated with the nuclear extracts were simply modified to a lower extent. In fact, the average number of A to G changes found per cDNA is consistent with this idea (see Table 1). Previous studies have shown that, with low amounts of dsRAD or low incubation times, RNA intermediates can be detected along the pathway to complete reaction (Polson & Bass, 1994). Early intermediates typically show the most preferred deamination sites and less preferred sites appear only near reaction completion. In this light, the RNAs modified with the crude extract may represent early intermediates and reflect a lower amount of dsRAD in the crude extract or the presence of other proteins that competed for the dsRNA substrate in the in vitro reaction. Interestingly, an analogous situation exists for gluR-B RNAs edited in vitro with mammalian extracts (Melcher et al., 1995; Rueter et al., 1995). These RNAs are edited at fewer sites overall, but the observed editing sites are a subset of those found in vivo (as discussed in Bass, 1995).

Finally, the differences between the deamination and editing patterns may exist simply because dsRAD is not the enzyme that edits gluR mRNA in vivo. Given the similarities between the deamination and editing sites, especially when individual clones are compared, at its extreme this explanation seems unlikely. A related, but more feasible explanation is that gluR mRNA is not edited by dsRAD in vivo, but by a related deaminase with similar deamination specificity; at present, we cannot rule out this possibility.

Regardless of whether dsRAD is responsible for editing gluR mRNA in vivo, the studies reported here demonstrate that dsRAD, in the absence of other factors, is capable of remarkable selectivity. Using amounts of dsRAD sufficient to modify 40–50% of the adenosines in our standard CAT duplex (Hough & Bass, 1994; see the Materials and methods), TLC analyses showed only 1–3% of the 161 adenosines in the gluR-B590 transcript were deaminated. cDNA sequencing indicated most of the inosines were in regions immediately surrounding the Q/R site so that, at most, 5 adenosines, or ~10%, of the 52 adenosines in the region shown in Figures 2 and 3 were deaminated. This high degree of selectivity contrasts with observations made using long, completely base paired dsRNAs, which show up

to 50% deamination (Bass & Weintraub, 1988; Wagner et al., 1989), and is even greater than recent studies that showed a maximum of ~25% deamination with shorter duplexes of 36 base pairs (Polson & Bass, 1994). The work described in this paper on gluR-B mRNA, as well as a forthcoming manuscript on the editing of hepatitis delta virus antigenome (Polson et al., 1995), suggests dsRAD may be very selective with regard to its biological substrates. The predicted structures for both candidate biological substrates contain double-stranded regions that are not completely base paired, but periodically interrupted by mismatches, bulges, and internal loops. The observed selectivity may be due to these structural elements, which were not present in the artificial sequences studied to date. Certainly, we still have much to learn with regard to dsRAD's potential for selectivity.

MATERIALS AND METHODS

RNA transcriptions

GluR-B RNA was transcribed from the plasmid pcDNA-590A (kindly provided by P. Sklar), which was constructed by inserting a *Bgl* II–*Hin*c II fragment of the mouse gluR-B gene into pcDNA (Invitrogen) digested with *Bam*H I and *Eco*R V. pcDNA-590A was linearized with *Not* I for synthesis of gluR-B590. Transcription reactions (100 μL) contained 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 1 U/μL RNasin, 100 μM ATP, 366 μM GTP, 500 μM CTP and UTP, 500 μM GpppG, 0.25 mCi α-^{32}P-ATP, ~3 μg DNA, and 150 U T7 RNA polymerase. Transcription was at 37 °C for 45 min followed by the addition of 100 U additional polymerase and 30 min additional incubation. Samples were treated with DNase, extracted with phenol and chloroform, then precipitated with ethanol. RNAs were electrophoresed with 1× TBE on a 6% polyacrylamide gel containing 8 M urea, and the appropriate band was excised and eluted after visualization by UV shadowing.

In vitro deamination reactions

GluR-B RNA was deaminated by incubating with *Xenopus laevis* oocyte nuclear extract or dsRAD purified from *X. laevis* eggs. Nuclear extracts contained the contents of 0.5 nuclei/μL and were prepared by dissecting nuclei as described (Saccomanno & Bass, 1994) except nuclei were diluted with an equal volume of TGKED. Twenty femtomoles of gluR-B590 were incubated with nuclear extract for 1.5 h at 25 °C in 12.5 mM Tris, pH 7.8, 250 μM DTT, 5 mM EDTA, 1.6 U/μL RNasin, and 5 nuclei equivalents extract/3 fmoles RNA. Final volumes were twice the volume of extract added. Reactions were stopped by making the reaction 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.5% SDS, and 200 μg/mL proteinase K (in addition to ingredients already present), followed by incubation at 37 °C for 30 min. Samples were then extracted with phenol, chloroform, and precipitated with ethanol. At this point, an aliquot of RNA was electrophoresed on a 6% polyacrylamide, 8 M urea, 1× TBE gel to verify that the RNA was intact.

For deamination with pure dsRAD, the enzyme was purified from *Xenopus* eggs as described (Hough & Bass, 1994) except the procedure was stopped after the AF-Blue-650M column, because, as described, additional chromatographic steps did not result in further purification. The specific activity, measured and defined as in Hough and Bass (1994), was ~ 0.6 pmol inosine per μL dsRAD per hour using CAT duplex as a substrate (see below). Deamination was in standard assay buffer as described (Hough & Bass, 1994), except RNasin was reduced to 200 U/mL, and 50 μg/mL glycogen replaced the salmon sperm DNA and torula RNA. Reactions (100 μL) contained 1 μL of dsRAD for every femtomole of gluR-B590 RNA and were incubated for 1 h at 25 °C. Note that this ratio of enzyme to substrate is similar to that we use typically to react our standard CAT duplex (1 μL dsRAD:1 fmol CAT dsRNA). Of course, the amount of enzyme per mole of adenosine differs slightly because 1 fmol of CAT duplex contains ~ 0.4 pmol of adenosine, and 1 fmol of gluR-B590 contains ~0.2 pmol of adenosine. Reactions were stopped and RNA purified as for reactions with nuclear extracts. In all cases, competitions were with sense or antisense RNAs derived from the CAT gene or the two complementary RNAs hybridized to form a duplex. CAT RNAs were prepared as described (Bass & Weintraub, 1987), except the RNAs were not capped.

Deamination analyses

Adenosine to inosine conversion was monitored directly by digestion of RNAs with P1 nuclease followed by separation of the mononucleotides by TLC as described (Saccomanno & Bass, 1994). Deamination sites were mapped by sequencing cDNAs made from deaminated RNAs. cDNA was synthesized with AMV reverse transcriptase; deoxyoligonucleotide PS130 was used to prime cDNA synthesis of nuclear extract-treated RNAs and SHTAG2 for RNAs treated with pure dsRAD (see below for sequences). Mock incubations in which RNA or dsRAD were omitted gave no gluR-B mRNA products or no modified products, respectively. cDNAs were amplified with PCR according to standard protocols (Ausubel et al., 1987), using oligonucleotide primers PS4 and PS130 for extract-modified RNAs, and SHTAG1 or SHTAG2 for pure dsRAD treated RNAs. PCR products were cloned using the TA Cloning Kit (Invitrogen) or the pGem-T Vector System (Promega) according to manufacturer's specifications. For nuclear extract-modified RNAs, two colonies were picked simply on the basis of containing an insert (identified by blue/white screening). All others were selected by screening colonies with a radiolabeled probe complementary to gluR-B sequences (PS127, see below) using standard nucleic acid hybridization protocols (Ausubel et al., 1987; Sambrook et al., 1989). Although the clones identified by blue/white screening were not modified at the site of PS127 hybridization, we cannot rule out the possibility that we selected against cDNAs containing A to G changes in this region. For samples modified with pure dsRAD, colonies were selected if they contained inserts (blue/white screening) and also hybridized to radiolabeled deoxyoligonucleotide SH130; in most cases, both selection

methods identified the same colonies and, because SH130 hybridizes to a region that does not seem important for editing (Yang et al., 1995), selection artifacts were considered unlikely. Plasmid was isolated from overnight cultures of colonies using a QIAprep Spin Plasmid Kit (Qiagen) and sequenced using a Sequenase v. 2.0 kit (USB) according to manufacturer's specifications. cDNA clones derived from nuclear extract-modified RNAs were sequenced in their entirety using primers corresponding to the Sp6 and T7 promoter sequences in the vector, and ddA, ddT, ddC, and ddG. cDNAs derived from pure dsRAD samples were sequenced using primers PS4 and SH130 and, usually, only with ddT and ddC so as to identify sites of A to G changes. Q/R site-edited molecules of the pure dsRAD sample were also sequenced using vector primers to verify the presence of "tag" sequences that indicated derivation from in vitro-transcribed gluR-B RNA. For 15,016 nt analyzed from the extract-modified samples, 75 A to G changes and 19 other changes were observed; the latter are most likely due to errors introduced by reverse transcriptase or Taq polymerase, with a combined error rate of 0.13%.

Deoxyoligonucleotides

Deoxyoligonucleotide sequences used to prime cDNA synthesis and in sequencing are listed 5′ to 3′. Primers identical to the RNA sequences are denoted as sense, and those complementary are denoted as antisense. Numbering scheme assumes Q/R site = 0. Note that nucleotides in small letters were for cloning purposes (PS130) or correspond to vector sequence present in gluR-B590 (SHTAG1 and SHTAG2) and do not refer to gluR-B sequence. PS4 = CAAAGTAGTGAAT CAACTAATGAA (sense; −71 to −47); PS127 = GGTGTATA TGATTTCACCAGG (antisense; 228–248); PS130 = cgtctagag tTGACCCTGTAGGAAAAATCTAACCTC (antisense; 383–408); SH130 = GGAAAAATCTAACCTCGCCCA (antisense; 378–398); SHTAG1 = gagctcgGATCTGGATGTG (sense; −185 to −174); SHTAG2 = gccagtgtgatggatGACC (antisense; 404-407).

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