Biochemistry

 Volume 36, Number 28 July 15, 1997

Accelerated Publications

Detection of Inosine in Messenger RNA by Inosine-Specific Cleavage†

Daniel P. Morse and Brenda L. Bass*

Department of Biochemistry & Howard Hughes Medical Institute, 50 North Medical Drive, University of Utah, Salt Lake City, Utah 84132

Received April 24, 1997; Revised Manuscript Received May 28, 1997⊗

ABSTRACT: Double-stranded RNA adenosine deaminases catalyze the conversion of adenosine to inosine within double-stranded RNA. A few candidate biological substrates for these enzymes have been discovered by noticing discrepancies between genomic and cDNA sequences. Toward the goal of finding a systematic approach to identify new deaminase substrates, we developed a method to cleave RNA specifically after inosine and an amplification strategy to identify the cleavage sites. We tested our method on a candidate substrate, the messenger RNA for glutamate receptor subunit B (GluR-B). We detected cleavage of the endogenous GluR-B message from rat brain at two known RNA editing sites, thus providing the first direct evidence for the presence of inosine at these sites. The described method will facilitate the mapping of inosines within RNA and, most importantly, will provide a way to identify new deaminase substrates.

Double-stranded RNA[1] (dsRNA) adenosine deaminases convert adenosine (A) to inosine (I) within dsRNA, or RNA that is largely double-stranded [reviewed in Bass (1997)]. So far, two distinct dsRNA adenosine deaminases have been discovered: dsRAD, sometimes called DRADA or ADAR1 (Kim et al., 1994; O'Connell et al., 1995; Patterson & Samuel, 1995; Hough & Bass, 1997), and RED1, sometimes called DRADA2 or ADAR2 (Melcher et al., 1996; Lai et al., 1997; O'Connell et al., 1997). In addition, several cDNAs have been identified that encode proteins that are clearly related to these enzymes [as cited in Kim et al. (1994), Patterson and Samuel (1995), and Hough and Bass (1997)]. Although the latter have not been proven to have deaminase activity, it seems possible there is an entire family of adenosine deaminases that act on RNA (ADARs).

ADAR activity was first observed more than a decade ago (Bass & Weintraub, 1987, 1988; Rebagliati & Melton, 1987), and since that time, it has been detected in every metazoan assayed. However, surprisingly, there are no proven natural substrates for ADARs, although a few good candidates have been identified [reviewed in Bass (1997)]. The best studied candidate substrates are RNAs that undergo RNA editing [reviewed in Simpson and Emeson (1996)], a type of post-transcriptional processing that changes the encoded sequence of an RNA. ADARs are proposed to edit RNAs by adenosine deamination. Thus an A to I modification has been proposed to explain codon changes that occur during editing of several mammalian glutamate receptor (GluR) mRNAs (Sommer et al., 1991; Lomeli et al., 1994; Seeburg, 1996), mammalian serotonin receptor mRNAs (Burns et al., 1997), and the antigenomic RNA of hepatitis delta virus

†This work was supported by funds to B.L.B. from the National Institute of General Medical Sciences (GM 44073, NIH) and the David and Lucile Packard Foundation. B.L.B. is an HHMI assistant investigator. D.P.M. was supported by an NIH training grant (CA 09602) and a postdoctoral fellowship from the American Cancer Society (PF 3891). Oligonucleotides were synthesized by the HHMI oligonucleotide synthesis facility at the University of Utah, supported in part by the Department of Energy (#DE-FG03-94ER61817).

* To whom correspondence should be addressed. Tel: (801) 581-4884. FAX: (801) 581-5379. E-mail: bass@msscc.med.utah.edu.

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: A, adenosine; ADAR, adenosine deaminase that acts on RNA; cDNA, complementary DNA; dsRNA, double-stranded RNA; G, glycine; GluR, glutamate receptor; HDV, hepatitis delta virus; I, inosine; mRNA, messenger RNA; PCR, polymerase chain reaction; Q, glutamine; R, arginine; U, units.

S0006-2960(97)00960-4 CCC: $14.00

(HDV; Polson et al., 1996). In each case, RNA editing is proposed to play an important role in the regulation of gene expression. For example, RNA editing of GluR-B mRNA alters a CAG glutamine codon to an arginine codon, presumably a CIG triplet (Q/R editing site); the resulting amino acid change alters the calcium permeability of the ion channels assembled from the GluR subunits. At another site in the GluR-B mRNA, an AGA arginine codon is changed to a glycine codon, presumably an IGA triplet (R/G editing site); proteins translated from these RNAs result in ion channels with altered kinetic properties.

So far, all putative ADAR substrates have been discovered fortuitously by noticing discrepancies between genomic and cDNA sequences. For example, the genomically encoded adenosines at the Q/R and R/G editing sites of GluR-B mRNA appear as guanosines (G) in cDNAs. Since inosine, like guanosine, prefers to pair with cytidine, such A to G transitions are consistent with the presence of inosine in an mRNA. Of course, such a transition is also consistent with an RNA editing event involving an A to G change, and in fact, the enzymatic synthesis of GMP during *de novo* purine biosynthesis proceeds through an IMP intermediate [reviewed in Zalkin and Dixon (1992)]. In order to obtain definitive proof that inosine was present within endogenous RNAs, we sought to develop a method to specifically cleave RNA at inosine. We reasoned that if endogenous RNAs do contain inosine, and such a method could be developed, it would provide a powerful technique for the discovery of additional ADAR substrates.

MATERIALS AND METHODS

Preparation of RNAs. Synthetic GluR-B RNAs with a single I at the Q/R site (or an A as a control) were constructed by ligating two half-molecules. DNA templates for in-vitro transcription of the half-molecules were generated by amplification of the desired sequences from a rat GluR-B cDNA. PCR primers: (5′-half template) TAA TAC GAC TCA CTA TAG GGA GTG TTT TCC TTT CTT GA and GCA TAA AGG CAC CCA AGG AAA; (3′-half template) TAA TAC GAC TCA CTA TAG CAG GGA TGC GAT ATT TCG CC and TGC TTA GAC AGA TCC TCA GCA CTT TCG A. The 5′-half template contained a T7 promoter followed by the 219 nucleotides immediately upstream of the Q/R site. The 3′-half template contained a T7 promoter followed by the 166 nucleotides immediately downstream of the Q/R site. The Q/R site nucleotide was incorporated by initiating transcription of the 3′-half molecules with either IpG or ApG (USB) by using a 5:1 molar ratio of dinucleotide to GTP. IpG was synthesized on an Applied Biosystems 394 DNA/RNA synthesizer. The 3′-half molecules were 5′ end labeled using T4 polynucleotide kinase (USB) and [γ-^{32}P]ATP, gel purified, and ligated to the 5′-half as described (Moore & Sharp, 1992) except the reactions (20 μL) contained 75 pmol of 3′-half; 100 pmol of 5′-half; 80 pmol of bridging oligodeoxynucleotide; 50 mM Tris-HCl, pH 7.6; 10 mM $MgCl_2$; 1 mM ATP; 1 mM DTT; 5% PEG-8000; and 10 units of T4 DNA ligase (BRL). The bridging oligodeoxynucleotide was GCG AAA TAT CGC ATC CCT GC**C** GCA TAA AGG CAC CCA AGG A or GCG AAA TAT CGC ATC CCT GC**T** GCA TAA AGG CAC CCA AGG A. Gel-purified ligation products were quantified by counting aliquots in a liquid scintillation counter.

For experiments requiring endogenous RNA, rat brains were obtained from Pel-Freez and rat brain polyA^+ RNA was purified according to manufacturer's specifications (Invitrogen Fast Track 2.0). Total *Caenorhabditis elegans* RNA used as carrier (see below) was purified with guanidine thiocyanate using standard procedures.

Cleavage of Glyoxalated RNA with Ribonuclease T1. For treatment of synthetic RNA, glyoxal reactions (100 μL) contained 5 μg of total *C. elegans* RNA as carrier; 0.5 fmol of ^{32}P-labeled synthetic GluR-B RNA; 10 mM sodium phosphate, pH 7; 50% DMSO; and 0.6% deionized glyoxal. After 45 min at 37 °C, 100 μL of 1 M sodium borate, pH 7.5, was added, and the reaction products were precipitated with 500 μL of ethanol and dissolved in 15 μL of 10 mM Tris/1 M sodium borate, pH 7.5 (at 37 °C). The high borate concentration was found to be necessary for optimal protection of guanosines from RNase T1 cleavage at pH 7.5. The glyoxalated RNA was allowed to dissolve at room temperature for 20 min because glyoxalated RNA has reduced solubility, and due to the requisite high borate concentration, there was a large salt pellet that took time to dissolve. 1 μL of an RNase T1 (BRL) solution (concentrations as indicated) was then added, and the mixture was incubated at 37 °C for various times as indicated. Reactions were stopped by adding proteinase K to 300 ng/μL and incubating at 37 °C for an additional 20 min. The reaction was diluted to 100 μL with water and extracted once with phenol and once with phenol/chloroform. The reaction was diluted to 400 μL, and the products were ethanol precipitated. Cleavage of synthetic RNA was monitored by electrophoresis on a 6% polyacrylamide, 8 M urea gel (see Figure 2). RNA lacking inosine produced no specific cleavage products, so the level of nonspecific cleavage was estimated by the loss of full-length RNA.

Inosine-specific cleavage of endogenous RNA was performed similarly except carrier and synthetic RNA were excluded and 5 μg of rat brain polyA^+ RNA was added instead. Rat brain polyA^+ RNA (+, Figure 5) was incubated at 37 °C for 30 min with 400 units of RNase T1.

Amplification of RNAs Cleaved 3′ of Inosine Residues. The entire sample of synthetic GluR-B RNA cleaved after inosine with 400 units of RNase T1 for 30 min as above was treated as follows (see Figure 3). Phosphate removal (1 h, 37 °C): 50 μL reaction containing 20 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 2 units of shrimp alkaline phosphatase (USB); and 60 units of T4 polynucleotide kinase (USB). Glyoxal removal (3 h, 60 °C): 100 μL reaction containing 10 mM sodium phosphate, pH 7, and 50% DMSO. Anchor ligation was as in Tessier et al. (1986) with minor modifications (2 h, room temperature): 50 μL reaction containing 40 pmol of anchor oligonucleotide; 50 mM Tris-HCl, pH 8; 10 mM $MgCl_2$; 10 μg/mL BSA; 12.5% PEG 8000; 1 mM hexamine cobalt chloride; 20 μM ATP; and 10 units of T4 RNA ligase (BRL). Reverse transcription (2 h, 50 °C): 50 μL reaction containing 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 1 mM DTT; 0.5 mM dNTPs; 80 pmol of primer; and 400 units of MLV reverse transcriptase (BRL). PCR (37 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 30 s): 25 μL reaction containing 1 μL of cDNA; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin; 10 pmol of each primer; 20 μM dNTPs; 5 μCi of [α-^{32}P]dCTP, and 1.25 units of Taq DNA polymerase (Perkin-Elmer). 10 μL of the PCR was

loaded onto a 6% polyacrylamide, 8 M urea gel. With the exception of the reverse transcription reaction and PCR, following each reaction, RNA was diluted to 400 μL and precipitated with ethanol. In addition, the phosphate removal reaction was extracted twice with phenol/chloroform prior to precipitation. Anchor oligonucleotide: pAAC TGT GCT GGA TTG ACT GGC TAC GCC TGA CGA TCA. The two adenosines at the 5′ terminus of the anchor were ribonucleotides, the next 33 residues were deoxyribonucleotides, and the 3′ terminus was cordycepin (3′-deoxyadenosine). Ribonucleotides were incorporated at the 5′ end in an attempt to improve ligation efficiency, but no improvement was observed. The 5′ terminal phosphate was included because it is required for the RNA ligase reaction. Cordycepin was included at the 3′ end to prevent multimerization of the anchor during ligation. Reverse transcription primer: GCG TAG CCA GTC AAT CCA GCA CA. PCR primers: CCA GTC AAT CCA GCA CAG TTC NC (discriminating anchor primer, DAP; see text) and GGA GTG TTT TCC TTT CTT GAT CCT TTA (GluR-B-specific primer). Preliminary experiments using DAPs with different numbers of extra 3′ nucleotides (complementarity extending 0−5 nucleotides 5′ of the ligation junction) showed that 3 was the minimum number that allowed specific amplification of the 5′-half cleavage product.

Rat brain RNA subjected to the inosine-specific cleavage protocol was amplified using the same procedures as for the synthetic RNA except a "CNA" DAP was used to detect cleavage at the R/G site, and the GluR-B-specific PCR primers were different; in particular, the primers used did not amplify the synthetic GluR-B RNA, eliminating concerns about contamination with the synthetic sequence. PCR primers: Q/R site detection, CCA GTC AAT CCA GCA CAG TTC NC (DAP) and TCA AGA AGC CTC AGA AGT CC (GluR-B-specific); R/G site detection, CCA GTC AAT CCA GCA CAG TTC NA (DAP) and ACA TTA GAC TCT GGC TCC ACT AAA GA (GluR-B-specific).

Sequencing of PCR Amplification Products. PCR products derived from two independent experiments using rat brain polyA$^+$ RNA were sequenced as follows. Gel slices and underlying Whatman 3 MM paper containing PCR products were excised from dried gels and soaked in 100 μL of water for 10 min at room temperature and then at 100 °C for 20 min. After it was spun at top speed in a microcentrifuge for 2 min, the supernatant was removed to a new tube. 4 μL of the eluate was added to a 25 μL PCR reaction, and the DNA was reamplified. The amplification conditions were as before except no radioactive nucleotide was included and dNTP concentration was 200 μM. The reamplified DNA was purified from 2% SeaPlaque GTG agarose by digesting with β-agarase (New England Biolabs) followed by precipitation. The PCR products were cloned using Stratagene's PCR-Script cloning kit. DNA was blunt-ended prior to ligation into the vector using Pfu DNA polymerase according to manufacturer's instructions. Four clones for each reamplified band were sequenced across the site of anchor ligation in one experiment, and three from each band in a second experiment. For the Q/R clones, the 247 nucleotides sequenced were identical to the rat gluR-B cDNA sequence (accession no. M36419). For the R/G clones, the 282 nucleotides sequenced were identical to the rat gluR-B cDNA sequence, except most clones showed a mismatch at the N of the CNX sequence of the DAP. The mismatch was likely due to the low stringency PCR conditions we used for these studies; our continuing studies indicate higher stringency conditions alleviate this problem.

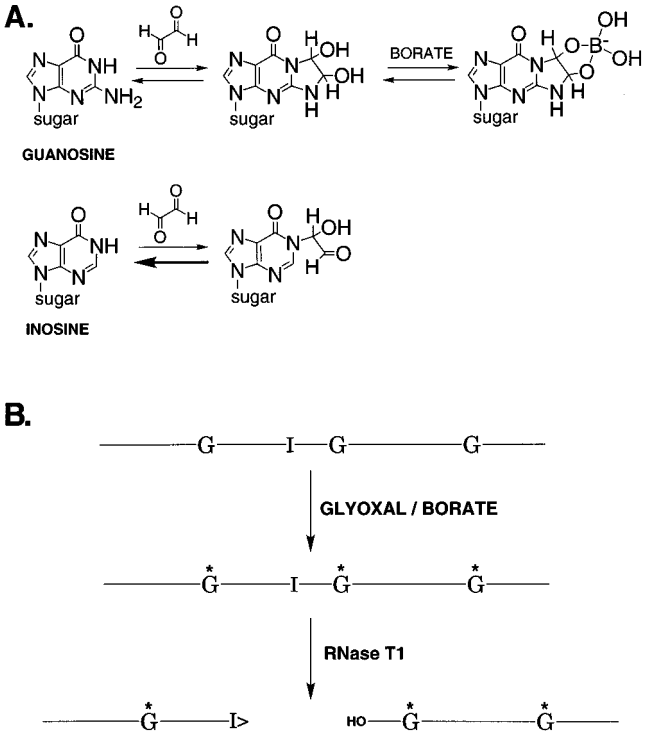

FIGURE 1: Strategy for inosine-specific cleavage of RNA. (A) The diagram shows the reaction of glyoxal ($C_2H_2O_2$) with guanosine and inosine and stabilization of the guanosine adduct with borate. As shown, inosine does not react stably with glyoxal. (B) Scheme for inosine-specific RNase T1 cleavage of glyoxalated RNA. Asterisks mark sites of glyoxalated guanosines which are resistant to RNase T1. The RNase T1 fragment that contains the inosine is shown with a 2′,3′ cyclic phosphate (>) because the RNase T1 reaction with inosine frequently does not go to completion.

RESULTS

The primary challenge in developing a method to specifically cleave RNA at inosine was the fact that most reagents that react with inosine also react with guanosine. However, as outlined in Figure 1, we accomplished inosine-specific cleavage by reacting RNA with glyoxal, stabilizing the glyoxal adducts with borate, and treating the RNA with ribonuclease T1. Glyoxal forms a stable adduct with guanosine but not with inosine, despite their structural similarity (Figure 1A; Broude & Budowsky, 1971). Further, glyoxal-modified guanosines are resistant to RNase T1 cleavage (Whitfeld & Witzel, 1963). Thus, although RNase T1 normally cleaves after both nucleotides, glyoxalated RNA is cut only after inosine (Figure 1B).

The procedure was optimized using a synthetic RNA that consisted of 386 nucleotides of the GluR-B mRNA, with a single inosine at the Q/R site. The synthetic RNA, which was labeled with ^{32}P immediately 5′ of the inosine, was spiked into 5 μg of carrier RNA, reacted with glyoxal and borate, and digested with RNase T1. Figure 2 shows a time course with increasing amounts of RNase T1. A single product was observed that migrated at the position expected for cleavage after the inosine. A 30 min incubation with 400 units of RNase T1 was determined to be optimal, since these conditions produced the most inosine-specific cleavage yet gave little nonspecific cleavage. Note that the same

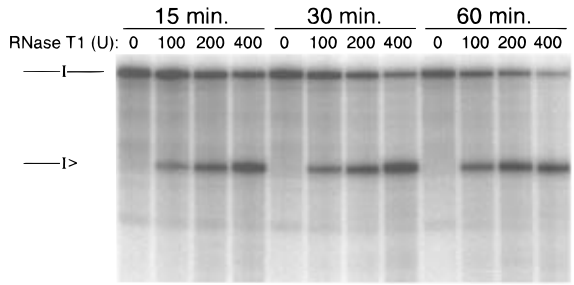

FIGURE 2: Inosine-specific cleavage of a synthetic GluR-B RNA. RNA containing a single inosine was synthesized by the method of Moore and Sharp (1992) and treated with glyoxal and borate as described in Materials and Methods. The glyoxalated RNA was incubated at 37 °C with increasing amounts (U) of RNase T1 and time, as indicated. The PhosphorImager image shows electrophoretically separated starting material and a single product of the size expected for cleavage after the inosine, which accumulated with increasing time and RNase T1. Positions of the full length and cleaved RNAs (determined in control experiments) are shown on the left. Only the phosphate 5′ of the inosine was radiolabeled, and thus the 3′ cleavage product is not visible.

concentration of RNase T1 with a 60 min incubation showed a lower amount of product, indicating nonspecific cleavage occurred during the longer incubation. In control experiments using the chosen optimal conditions, RNA that did not contain inosine was not cleaved (typically less than 5% digestion), and RNA not treated with glyoxal was completely degraded (data not shown).

In order to use our procedure on RNAs isolated from a cell, we needed a way to detect inosine-specific cleavage of non-radioactive RNA. Thus we devised a strategy to specifically amplify RNase T1 fragments that contained inosine at their 3′ ends (Figure 3). The method entailed ligation of an oligonucleotide (the anchor) to both the original and newly created 3′ ends to provide priming sites for reverse transcription and subsequent PCR (Tessier et al., 1986). Removal of phosphates at the newly created 3′ ends (the inosine end), which was required for anchor ligation, was accomplished with T4 polynucleotide kinase (step 1, Figure 3). RNase T1 normally produces a 3′ phosphate via a 2′,3′ cyclic phosphate intermediate, but cleavage after inosine results in a mixture of 3′ and cyclic phosphates (Whitfeld & Witzel, 1963; data not shown). T4 polynucleotide kinase has both 2′,3′ cyclic phosphodiesterase (C. L. Greer and O. C. Uhlenbeck, personal communication) and 3′ phosphatase (Cameron & Uhlenbeck, 1977) activities and thus can remove phosphates from both types of products. (Alkaline phosphatase was included in step 1 for a control described below.)

Following glyoxal removal (step 2) and anchor ligation (step 3), first-strand cDNA was synthesized using an anchor primer (step 4). In order to preferentially amplify cDNA derived from RNA molecules that had inosine at their 3′ ends, we used one of four discriminating anchor primers (DAPs) for the downstream primer required in the PCR of step 5. The DAPs ended with three extra nucleotides of the form "CNX", where the C was designed to pair with the G that corresponded to the I at the original ligation junction. To obtain the necessary specificity and discrimination (see Materials and Methods), we found it necessary to extend the DAPs three nucleotides beyond the ligation junction. Thus, in addition to the C, we included a randomized position (N) and a terminal position consisting of either A, G, C, or T (X). The original 3′ ends of mRNAs bearing poly-A tails

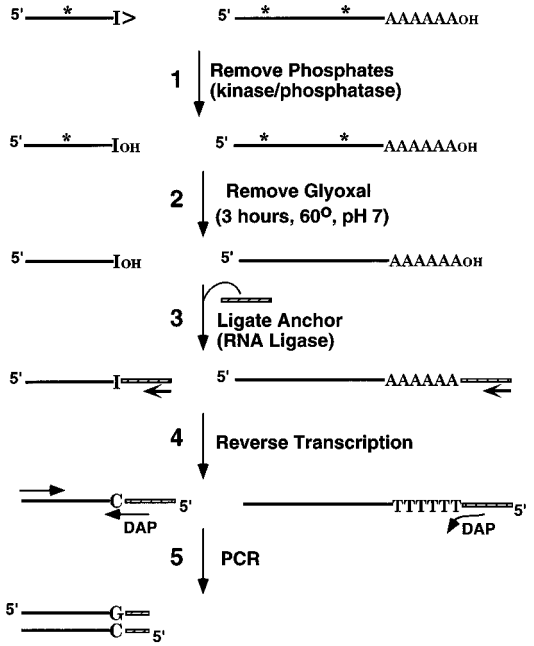

FIGURE 3: Strategy for amplifying RNase T1 cleavage products that have inosine at their 3′ ends. Step 1: removal of 3′ and 2′,3′ cyclic (>) phosphates with T4 polynucleotide kinase. The reason for including phosphatase is explained in the text. Step 2: removal of glyoxal adducts (*) by incubation at pH 7 for 3 h at 60 °C. Step 3: ligation of anchor to all 3′ hydroxyls using T4 RNA ligase (Tessier et al., 1986; Materials and Methods). Step 4: synthesis of first-strand cDNA using a primer that is complementary to the anchor. Step 5: amplification of the inosine-containing fragments using a sequence specific upstream primer and one of four possible DAPs as described in text. The curved arrow indicates that DAPs do not prime at that site (data not shown).

were amplified inefficiently, if at all, with these primers (data not shown). Although this strategy does not prevent amplification of RNA cleaved at guanosines that have escaped glyoxal protection, the observed background G cleavage was very low under the chosen optimal conditions (see Figures 2, 4 and 5).

The amplification strategy was tested using the synthetic GluR-B RNA that had been cleaved after the inosine at the Q/R site (see Figure 2). For the PCR, the DAP ("CNC", see Materials and Methods) was coupled with a GluR-B-specific primer that hybridized ~220 nucleotides upstream of the Q/R site. The "CNC" DAP was used because there is a guanosine two nucleotides upstream of the inosine at the Q/R site. A prominent band of the expected size (241 nucleotides) was observed and was largely dependent on cleavage of the RNA with RNase T1, treatment with T4 polynucleotide kinase, and ligation of the anchor (Figure 4). Between the Q/R site and the upstream priming site there are 14 guanosines that are two nucleotides downstream of another G. Thus, efficient cleavage at any of these G's would have been detected with the "CNC" DAP. However, very little PCR product smaller than 241 nucleotides was observed (Figure 4). The faint bands at 241 nucleotides in some of the negative control lanes are likely due to a small amount of contamination with the 5′-half molecule used to synthesize the RNA. Note that alkaline phosphatase, which can remove 3′ phosphates but not 2′,3′ cyclic phosphates, was relatively ineffective when used in step 1 in the absence of kinase (−KIN, Figure 4). This result is consistent with the presence of a cyclic phosphate at the RNase T1 cleavage site, and thus, the inclusion of the alkaline phosphatase

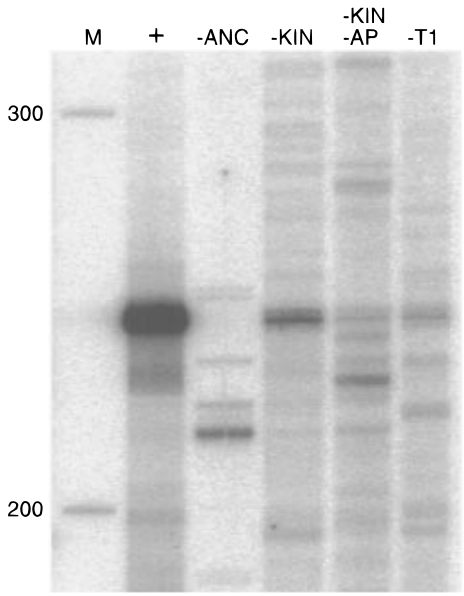

FIGURE 4: Amplification of synthetic gluR-B mRNA cleaved at inosine. The PhosphorImager image shows an electrophoretic separation of PCR products generated using the procedure outlined in Figure 3 (Materials and Methods). The starting material was synthetic GluR-B RNA specifically cleaved at inosine (400 units of RNase T1, 30 min; Materials and Methods). Production of the 241 bp PCR product depended on RNase T1, T4 polynucleotide kinase, and anchor ligation. (M) 100 base pair ladder; (+) all reagents included; (−ANC) anchor omitted from step 3 (see Figure 3 for steps); (−KIN) T4 polynucleotide kinase omitted from step 1 (alkaline phosphatase was included); (−KIN −AP) both kinase and alkaline phosphatase omitted from step 1; (−T1) RNA not treated with RNase T1.

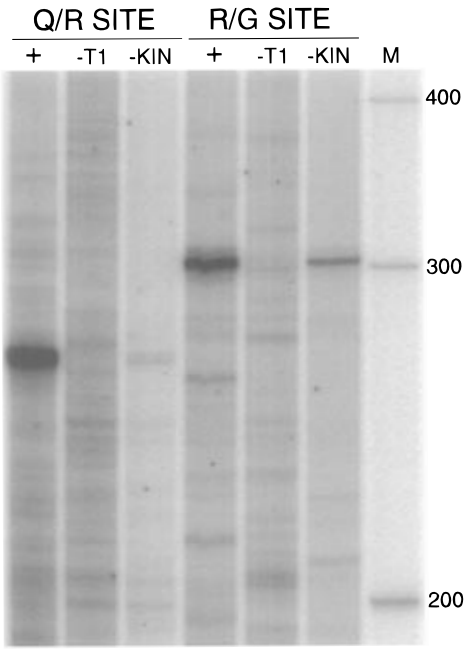

FIGURE 5: Detection of inosine at the Q/R and R/G editing sites of endogenous GluR-B mRNA from rat brain. The PhosphorImager image shows PCR products obtained from 5 μg of rat brain polyA$^+$ RNA that was subjected to the inosine-specific cleavage and amplification methods (Materials and Methods). As expected if inosine was present at the Q/R and R/G editing sites, prominent PCR products of the expected sizes (267 and 302 bp, respectively) were observed in the complete reaction (+) but not in the absence of RNase T1 (−T1) or T4 polynucleotide kinase (−KIN). (M) 100 base pair ladder.

control provided additional evidence that cleavage occurred 3′ of an inosine.

Next we asked if we could detect inosine at the Q/R and R/G editing sites in endogenous GluR-B mRNA isolated from rat brain (Figure 5). Rat brain polyA$^+$ RNA was subjected to the inosine-specific cleavage and amplification methods. PCR was carried out with two different GluR-B-specific primers coupled with DAPs ending with either "CNC" (for Q/R site detection) or "CNA" (for R/G site detection). The GluR-B-specific primers hybridized either ~250 nucleotides upstream of the Q/R site (first three lanes of Figure 5) or ~280 nucleotides upstream of the R/G site (second three lanes of Figure 5); neither priming site was present in the synthetic GluR-B RNA so as to eliminate possible artifacts due to contamination. Again, we observed prominent PCR products of the expected sizes (267 and 302 nucleotides) that depended completely on the addition of RNase T1. R/G site detection was less dependent on kinase than was Q/R site detection (compare −KIN lanes, Figure 5), suggesting a greater percentage of the Q/R site molecules contained cyclic phosphate intermediates at their cleavage sites; this difference may be due to the different sequences surrounding the two editing sites. To verify the identities of the observed PCR products, the bands were excised from the gel, reamplified, cloned, and sequenced. Since the anchor was ligated to the cleavage site, it was only necessary to sequence across the original RNA/anchor junction to identify the sequence and reveal the location of the inosine within the RNA. This feature of our protocol should prove extremely advantageous when applying these methods to inosine containing RNAs of unknown sequence (see below). In two independent experiments, the sequences of several clones from each band confirmed that the PCR products were, indeed, generated from GluR-B mRNA cleaved at the Q/R and R/G sites (data not shown).

DISCUSSION

Here we describe a simple method for specifically cleaving RNA at inosines using glyoxal and ribonuclease T1. We also describe a method by which RNAs cleaved at inosines can be selectively amplified using the PCR. Importantly, as a direct test of the utility of our cleavage and amplification methods, we showed that GluR-B mRNA, an RNA hypothesized to be edited by A to I conversion, can be cleaved at two known editing sites, and selectively amplified from polyA$^+$ RNA isolated from rat brain.

As mentioned, all of the RNAs proposed to be substrates for ADARs in vivo were identified indirectly by noticing discrepancies between genomic and cDNA sequences. Thus, in addition to verifying the efficacy of our protocol, detection of cleavage of the endogenous GluR-B message at the Q/R and R/G sites provides the first direct evidence that editing of this mRNA involves A to I conversion, and strongly supports the hypothesis that one or more ADAR family members are responsible for these RNA editing events. Although it could be argued that the edited nucleotide is another modified base, none of the known modified nucleic acid bases (Limbach et al., 1994), except inosine, easily satisfy all criteria required for detection with our method, namely, a requisite base pairing with cytidine, an inability to react stably with glyoxal, and a susceptibility to cleavage with T1 ribonuclease.

The few candidate ADAR substrates discovered to date suggest RNA editing by adenosine deamination may be an

extremely important way to achieve the diversity of gene expression required in metazoa (Sommer et al., 1991; Lomeli et al., 1994; Burns et al., 1997). Although many of the candidate substrates identified so far are expressed only in the mammalian nervous system, ADAR activity has been detected in every metazoan tissue tested (Wagner et al., 1990, Bass, 1993). Thus, there are likely many ADAR substrates yet to be discovered. The method described here not only provides a reproducible assay for the detection of inosine in known or candidate substrates, but with simple variations, should allow investigators to discover additional inosine containing RNAs in tissues and organisms of all types. For example, by replacing the specific upstream PCR primer (Figure 3, step 5) with a collection of arbitrary primers, populations of inosine-containing RNAs could be amplified using low-stringency PCR, as in the differential display method (Liang & Pardee, 1992). Alternatively, by using a combination of random primers and the discriminating anchor primers (DAPs) that selectively amplify newly created ends, it should be possible to construct cDNA libraries enriched for inosine-containing RNAs. Certainly, future studies will be directed toward these applications.

ACKNOWLEDGMENT

We are grateful to C. Greer and O. Uhlenbeck for helpful discussions about T4 polynucleotide kinase, M. Moore for helpful discussions about DNA ligase, S. Rogers for providing a GluR-B cDNA clone, A. Lingam for *C. elegans* RNA, and E. Meenen for assistance in designing oligonucleotides. We thank the members of our laboratory and colleagues at the University of Utah for helpful discussions concerning this work.

REFERENCES

Bass, B. L. (1993) in *The RNA World* (Gesteland, R., & Atkins, J., Eds.) pp 383−418, Cold Spring Harbor Laboratory Press, Plainview, NY.

Bass, B. L. (1997) *Trends Biochem. Sci. 22*, 157−162.

Bass, B. L., & Weintraub, H. (1987) *Cell 48*, 607−613.

Bass, B. L., & Weintraub, H. (1988) *Cell 55*, 1089−1098.

Broude, N. E., & Budowsky, E. I. (1971) *Biochim. Biophys. Acta 254*, 380−388.

Burns, C. M., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E., & Emeson, R. B. (1997) *Nature 387*, 303−308.

Cameron, V., & Uhlenbeck, O. C. (1977) *Biochemistry 16*, 5120−5126.

Hough, R. F., & Bass, B. L. (1997) *RNA 3*, 356−370.

Kim, U., Wang, Y., Sanford, T., Zeng, Y., & Nishikura, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11457−11461.

Lai, F., Chen, C.-X., Carter, K. C., & Nishikura, K. (1997) *Mol. Cell. Biol. 17*, 2413−2424.

Liang, P., & Pardee, A. B. (1992) *Science 257*, 967−971.

Limbach, P., Crain, P., & McCloskey, J. (1994) *Nucleic Acids Res. 22*, 2183−2196.

Lomeli, H., Mosbacher, J., Melcher, T., Hoger, T., Geiger, J. R., Kuner, T., Monyer, H., Higuchi, M., Bach, A., & Seeburg, P. H. (1994) *Science 266*, 1709−1713.

Melcher, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P. H., & Higuchi, M. (1996) *Nature 379*, 460−464.

Moore, M. J., & Sharp, P. A. (1992) *Science 256*, 992−997.

O'Connell, M. A., Gerber, A., & Keller, W. (1997) *J. Biol. Chem. 272*, 473−478.

O'Connell, M. A., Krause, S., Higuchi, M., Hsuan, J. J., Totty, N. F., Jenny, A., & Keller, W. (1995) *Mol. Cell. Biol. 15*, 1389−1397.

Patterson, J. B., & Samuel, C. E. (1995) *Mol. Cell. Biol. 15*, 5376−5388.

Polson, A. G., Bass, B. L., & Casey, J. L. (1996) *Nature 380*, 454−456.

Rebagliati, M. R., & Melton, D. A. (1987) *Cell 48*, 599−605.

Seeburg, P. H. (1996) *J. Neurochem. 66*, 1−5.

Simpson, L., & Emeson, R. (1996) *Annu. Rev. Neurosci. 19*, 27−52.

Sommer, B., Köhler, M., Sprengel, R., & Seeburg, P. H. (1991) *Cell 67*, 11−19.

Tessier, D. C., Brousseau, R., & Vernet, T. (1986) *Anal. Biochem. 158*, 171−178.

Wagner, R. W., Yoo, C., Wrabetz, L., Kamholz, J., Buchhalter, J., Hassan, N. F., Khalili, K., Kim, S. U., Perussia, B., McMorris, F. A., et al. (1990) *Mol. Cell. Biol. 10*, 5586−5590.

Whitfeld, P. R., & Witzel, H. (1963) *Biochim. Biophys. Acta 72*, 338−341.

Zalkin, H., & Dixon, J. E. (1992) *Prog. Nucleic Acid Res. Mol. Biol. 42*, 259−287.

BI9709607