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Cloning of cDNAs Encoding Mammalian Double-Stranded RNA-Specific Adenosine Deaminase

MARY A. O'CONNELL,[1] SABINE KRAUSE,[1] MIYOKO HIGUCHI,[2] J. JUSTIN HSUAN,[3] NICHOLAS F. TOTTY,[3] ANDREAS JENNY,[1] AND WALTER KELLER[1]*

Department of Cell Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland[1]; Center for Molecular Biology, University of Heidelberg, D-69120 Heidelberg, Federal Republic of Germany[2]; and Ludwig Institute for Cancer Research, University College London, Medical School Branch, London W1P 8BT, England[3]

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Double-stranded RNA (dsRNA)-specific adenosine deaminase converts adenosine to inosine in dsRNA. The protein has been purified from calf thymus, and here we describe the cloning of cDNAs encoding both the human and rat proteins as well as a partial bovine clone. The human and rat clones are very similar at the amino acid level except at their N termini and contain three dsRNA binding motifs, a putative nuclear targeting signal, and a possible deaminase motif. Antibodies raised against the protein encoded by the partial bovine clone specifically recognize the calf thymus dsRNA adenosine deaminase. Furthermore, the antibodies can immunodeplete a calf thymus extract of dsRNA adenosine deaminase activity, and the activity can be restored by addition of pure bovine deaminase. Staining of HeLa cells confirms the nuclear localization of the dsRNA-specific adenosine deaminase. In situ hybridization in rat brain slices indicates a widespread distribution of the enzyme in the brain.

Double-stranded RNA (dsRNA)-specific adenosine deaminase is a mammalian RNA-modifying enzyme that was discovered upon performing antisense experiments with *Xenopus laevis* (2, 36). The enzyme destabilizes duplex RNA by converting adenosine to inosine by hydrolytic deamination (34), which results in unstable U · I base pairs so that the dsRNA becomes unwound. While in vitro there is no sequence specificity for substrate RNAs, the activity is specific for dsRNAs, and a minimum of 100 bp of either intermolecular or intramolecular duplex seems to be required for efficient modification (30).

The biological function of the enzyme as well as the full range of its physiological substrate RNAs are not known. The deaminase has been implicated in editing the RNAs of certain subunits of glutamate-gated ion channel receptors in the brain, because of the dependence of this editing event on intramolecular RNA · RNA base pairing (19, 24, 37). The dsRNA adenosine deaminase appears also to be responsible for the generation of defective measles virus with biased hypermutations that result in a lethal central nervous system disease, measles inclusion body encephalitis (4).

This enzyme is of interest not only because of its assumed biological functions but also because its mechanism of action is intriguing. dsRNA differs from duplex DNA in forming an A-type helix. In A-form RNA, the minor groove is shallow and wide (10 to 11 Å [1 Å = 0.1 nm]) and the major groove is deep and narrow (3.5 Å) (38). RNA-binding proteins usually bind to the major groove at the end of a helix or at a bulge in RNA structures where the major groove is more accessible (38). The binding of human immunodeficiency virus Tat protein to its target RNA TAR is an example, as the binding occurs on the major groove in the region of a bulge (35, 47, 48). The dsRNA adenosine deaminase binds to continuous duplex RNA; therefore, it must be able to recognize and access the adenosines that it deaminates even though this amino group is at position 6 of the base and lies in the major groove. This position is inaccessible to diethyl pyrocarbonate modification and by inference to amino acid side chains as well (48).

Recently, the dsRNA adenosine deaminase has been purified from different sources; a 120-kDa protein was purified from *X. laevis* (20), three polypeptides which vary in size from 90 to 96 kDa were purified from bovine liver nuclear extracts (22), and we have purified a 116-kDa protein from calf thymus (31). Amino acid sequence derived from the pure 116-kDa protein was used to clone a short gene sequence by PCR. A calf aorta endothelium cDNA library was screened with this probe, and a partial cDNA clone was obtained. This clone was used as a probe to screen HeLa and rat libraries, and full-length HeLa and rat cDNA clones were obtained. Antibodies raised against a fusion protein made with the partial bovine cDNA clone recognized a 116-kDa protein in partially purified calf thymus preparations. These antibodies could also deplete an extract of dsRNA adenosine deaminase activity, and the activity could be restored by addition of purified bovine dsRNA adenosine deaminase.

MATERIALS AND METHODS

Amino acid sequencing. Seven kilograms of calf thymus was used to purify sufficient dsRNA adenosine deaminase to obtain peptide sequences. The protein was purified as previously described (31), with minor modifications. The first DEAE-Sepharose column was omitted, and instead the calf thymus extract was precipitated with ammonium sulfate to 25 to 50% saturation and batch absorbed to Blue Sepharose at 300 mM KCl in buffer A (50 mM Tris-HCl [pH 7.9], 5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg of pepstatin per ml, 0.4 μg of leupeptin per ml).

Approximately 20 μg of pure protein was precipitated with 10% trichloroacetic acid and washed twice with acetone, and the dry pellet was resuspended in electrophoresis buffer (27). The protein was applied to a sodium dodecyl sulfate (SDS)-polyacrylamide gel, and the 116-kDa protein was excised after staining with Coomassie blue. The protein was digested with trypsin in the gel slice, and the peptides were eluted and separated by high-pressure liquid chromatography on a Michrom Reliasil C18 reverse-phase column (150 by 1 mm). A gradient of acetonitrile concentration in 0.08% trifluoroacetic acid was developed at 50 μl/min on a Michrom UMA apparatus. Fractions were applied directly to an Applied Biosystems 477A automated liquid pulse sequencer which had been

* Corresponding author. Mailing address: Department of Cell Biology, Biozentrum, University of Basel, CH-4056, Basel, Switzerland. Phone: 41-61-267 2060. Fax: 41-61-267 2078. Electronic mail address: Keller2@ubaclu.unibas.ch.

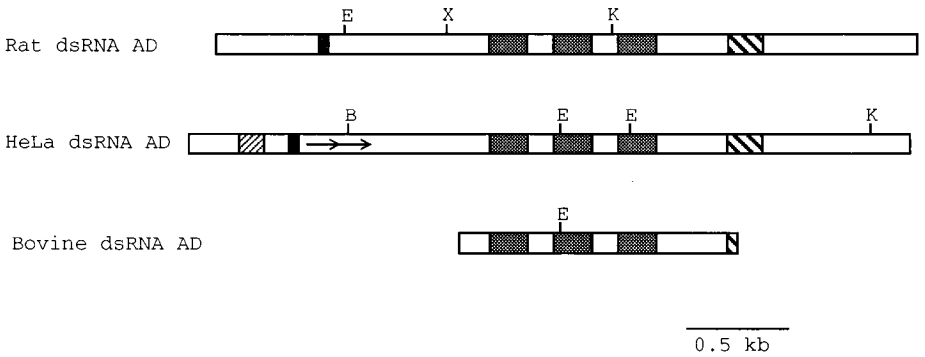

FIG. 1. Schematic diagram of the human, rat, and partial bovine dsRNA adenine deaminase (AD) cDNA clones. ▨, RG-enriched domain; ▬, nuclear targeting signal; →→, duplication of 48 amino acids; ▭, dsRNA binding domain; ▩, deaminase motif; E, *Eco*RI; X, *Xba*I; K, *Kpn*I; B, *Bam*HI.

modified as described previously (42). All sequences were obtained at less than a 2-pmol starting signal.

PCR, libraries, isolation of cDNA clones, and sequencing. The 14 amino acids of peptide 5 (AIMEMPSFYSHGLP) were used to design two partially degenerate PCR primers: 5′-CCGAATTCGCIATHATGGARATGCC-3′ and 5′-TTGTCGACGGNARNCCRTGRCT-3′ (I = inosine; H = A, C, or T; R = A or G; N = A, C, G, or T). All PCR primers had restriction sites at their 5′ termini for subcloning. The 50-μl PCR mixture contained 30 mM Tricine (pH 8.4), 2 mM $MgCl_2$, 0.01% Triton X-100, 0.2 mM deoxynucleoside triphosphates, 50 pmol of PCR primers, 250 ng of calf thymus genomic DNA, and 2.5 U of *Taq* polymerase. Thirty cycles were carried out, with annealing at 50°C for 30 s and extension at 72°C for 2 min. The PCR products were analyzed first on an agarose gel; bands of the correct size were excised, subcloned into the *Sal*I-*Eco*RI sites of the polylinker of Bluescript KS (Stratagene), and further analyzed by sequencing one strand with Sequenase (United States Biochemical Corp.). One of the PCR products had the predicted amino acid sequence of peptide 5, and this was then used to screen a bovine aorta endothelium cDNA library in λZap II vector (Stratagene). The nitrocellulose filters were hybridized overnight at 42°C in hybridization buffer as described previously (43), with minor modifications (5× Denhardt's solution, 5× SSC [0.75 M NaCl, 0.075 M sodium citrate], 50 μg of sonicated herring sperm DNA per ml, 50 mM sodium phosphate, 20% formamide, 1% SDS), and approximately 7×10^5 cpm of denatured probe per ml. The filters were washed at room temperature for approximately 1 h with five changes of buffer, three times in 2× SSC–0.1% SDS, once in 1× SSC–0.1% SDS, and finally in 0.2× SSC–0.1% SDS and then exposed overnight with two intensifying screens. One positive clone (CAE13) was obtained and was used to screen two HeLa cDNA λgt11 libraries (Clontech and Gary McMaster, Ciba-Geigy) and a rat hippocampus λgt10 cDNA library (Peter Seeburg, Center for Molecular Biology, Heidelberg, Germany). Four clones from the HeLa library and three clones from the rat library were further analyzed and sequenced. Structures of the human, rat, and partial bovine cDNA clones are shown in Fig. 1.

Northern (RNA) analysis. A premade human multiple tissue Northern blot of poly(A)$^+$ RNA (Clontech) was probed with a 1.2-kb fragment from *Eco*RI to *Bam*HI of the human 33.1B cDNA clone. The blot was washed twice in 0.1× SSC–0.1% SDS at 55°C for 30 min and exposed to autoradiographic film for 4.5 h with an intensifying screen. The blot was also reprobed with amyloid precursor-like protein 2 (APLP2) and alternatively spliced Kunitz protease inhibitor domain in APLP2 (APLP2-KP1) (46) to ensure equal loading of poly(A)$^+$ RNA in all lanes (data not shown).

Histidine tag fusion protein. The 1.5-kb partial bovine clone CAE13 was subcloned in both orientations (pQH-T3 and pQH-T7) into the *Bam*HI-*Sal*I sites of the polylinker of the histidine tag expression vector pQE9 (Qiagen) with complementary PCR primers that had restriction sites at their termini. Another construct, pQH-TE1, derived from pQH-T3, that contained only the first 520 nucleotides ending at the *Eco*RI site was subcloned into *Bam*HI-*Hin*dIII sites of the polylinker of pQE9. *Escherichia coli* SG13009 (DIAGEN) was used to overexpress pQH-T3 and pQH-T7; *E. coli* BMH71-80 (H. Bujard, University of Heidelberg) was used to overexpress pQH-TE1. Bacteria containing plasmids pQH-T3 and pQH-T7 were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density at 600 nm of 0.9 U and allowed to grow overnight at 37°C. pQH-TE1 was induced with 1 mM IPTG at an optical density at 600 nm of 0.7 to 0.8 U and was harvested after a further 4 h of growth at 37°C. After harvesting by centrifugation, the overexpressed proteins were purified under denaturing conditions suggested by the manufacturer (Qiagen) and chromatographed on a nitrolotriacetic acid-Ni$^+$ affinity column. The fusion protein was eluted in 10 ml of buffer C (1 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl [pH 6.3], 250 mM imidazole). Aliquots of the fractions from the nitrilotriacetic acid-Ni$^+$ affinity column were analyzed by electrophoresis on an SDS-polyacrylamide gel. Purified protein from plasmid pQH-T3 was electroeluted from a preparative SDS-polyacrylamide gel and injected into rabbits.

Antiserum preparation. Two New Zealand White rabbits were initially bled to obtain preimmune serum; then one was injected intradermally with approximately 5 μg of purified protein expressed from plasmid pQH-T3, and the other was injected with 100 μg of recombinant protein from plasmid pQH-TE1. The antigens were emulsified with Specol adjuvant (Central Veterinary Institute, Lelystad, The Netherlands). Both rabbits were subsequently boosted with recombinant protein from plasmid pQH-TE1 every 4 weeks, and blood was collected from the dorsal ear vein 2 weeks after every boost.

For affinity purification of dsRNA adenosine deaminase-specific antibodies, 500 μg of pure recombinant protein from plasmid pQH-TE1 was separated on a preparative SDS–8% polyacrylamide gel and blotted to nitrocellulose. After staining with Ponceau S, the 23-kDa band was excised and used for affinity purification as described by Burke et al. (8).

Immunoblot analysis. Proteins were fractionated on an SDS–8% polyacrylamide gel (27) and transferred to nitrocellulose by the semidry procedure (26). Blots were blocked in TNT buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. TNT buffer was also used for incubation with antisera and washing. The antisera were diluted 1:15,000, incubated for 1 h at room temperature, and then washed for 1 h with five changes of buffer. Proteins were detected by peroxidase-conjugated swine antibodies directed against rabbit immunoglobulins (DAKO, Glostrup, Denmark) and chemiluminescence staining (ECL kit; Amersham).

Immunodepletion of dsRNA-specific adenosine deaminase from calf thymus extracts. To deplete calf thymus extracts of dsRNA adenosine deaminase activity, 20 and 100 μl of both dsRNA adenosine deaminase antibody and preimmune serum were coupled to 130 μl of protein A–Sepharose CL-4B (Pharmacia) for 3 h at room temperature with constant mixing. The resins were washed three times with buffer 1 (100 mM Na_2HPO_4-HCl [pH 8], 0.2% bovine serum albumin [BSA], 0.1% NaN_3) and three times with buffer A containing 50 mM KCl (31) and incubated with partially purified dsRNA adenosine deaminase for 6 h at 4°C with gentle mixing. The resin was then centrifuged, and the supernatant was removed, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined by the Bradford method (6), with BSA as a reference standard.

dsRNA adenosine deaminase assay. The dsRNA substrate was prepared by in vitro transcription as previously described (31). dsRNA containing either 45 or 200 fmol of labeled adenosine was used per assay. The assay was performed at 37°C with either pure enzyme or partially purified fractions as previously described (31).

Immunofluorescence staining of HeLa cells. HeLa cells were grown on glass coverslips in minimum essential medium supplemented with 1% glutamine, 10% fetal calf serum, and antibiotics (Gibco BRL, Life Technologies, Paisley, Scotland). Cells were used after reaching a confluency of 50 to 70%. Fixation and immunolabeling were performed with affinity-purified antibody as described previously (9). The specimens were visualized on a fluorescence microscope (Axiophot; Carl Zeiss, Oberkochen, Germany) equipped with epifluorescence filters. Photographs were taken on Kodak Ektachrome 400 film. Primary and secondary antibodies were as indicated in the legend to Fig. 7.

In situ hybridization in brain slices. In situ hybridization was performed as described previously (50). Briefly, an oligonucleotide (5′-GGGTTCTTCAGCTGGCACTCAGTTAGCTTCTTGTAG-3′) complementary to rat dsRNA adenosine deaminase encoding nucleotides 1326 to 1361 (the A in the translation initiation codon ATG is nucleotide 1) was 3′ end labeled with terminal deoxynucleotidyltransferase and [α-^{35}S]dATP. Horizontal cryosections were hybridized overnight at 42°C in 50% formamide–4× SSC (0.6 M NaCl, 0.06 M sodium citrate)–10% (wt/vol) dextran sulfate with 1 pg of the probe per μl. Sections were washed in 1× SSC at 60°C for 20 min. Exposure to Kodak XAR-5 film was for 20 days. Control sections hybridized with radiolabeled probe in the presence of a 100-fold excess of unlabeled oligonucleotide produced no signal.

Nucleotide sequence. The nucleotide sequence of the rat clone has been submitted to the GenBank database; the accession number is U18942.

RESULTS

Cloning and sequence analysis of cDNAs encoding dsRNA-specific adenosine deaminase. Microsequence analysis of pure dsRNA adenosine deaminase yielded six peptides (Fig. 2). Peptide 5 contained 14 amino acids and was used to generate a specific probe by PCR (see Materials and Methods). After screening a bovine endothelium random and oligo(dT)-primed λZAP II library with this specific PCR probe, we isolated one 1.5-kb clone (CAE13). This clone was subsequently used as a probe for screening other cDNA libraries. HeLa cDNA libraries were screened to isolate human clones. A composite of the longest clones gave an open reading frame of 1,226 amino acid residues with a calculated molecular mass of 139 kDa containing the six peptides which were generated from microsequencing the pure bovine dsRNA adenosine deaminase (Fig. 2). The initiating methionine is preceded by an in-frame stop codon 32 amino acids upstream. The nucleotide sequence surrounding the start codon (CTCGCGGGCGCA) is in good agreement with the consensus sequence (25). A Southern blot analysis of human genomic DNA revealed that it was a single-copy gene (data not shown).

The rat homolog of dsRNA adenosine deaminase was also isolated. A rat hippocampus λgt10 library was screened, and positive clones were sequenced. A composite of the longest clones gave an open reading frame of 1,175 amino acid residues, which corresponds to a calculated molecular mass of 130 kDa. There are 17 nucleotides 5′ of the first methionine that carry no in-frame stop codon. Further screening is in progress to obtain more 5′ sequence.

Alignment of the amino acid sequence of the rat and human clones revealed a high degree of identity (79%); the identity between the human and partial bovine clone was even higher (91%), but this alignment is in a highly conserved region and a complete bovine clone would probably be less homologous (Fig. 2). The rat and human clones diverged at the N terminus, where there is also a 48-amino-acid duplication in the human clone that is not present in the rat clone.

There are conserved sequence motifs encoded in the deaminase clone (Fig. 1); a potential nuclear localization signal is present in both clones near the N terminus (Fig. 2). The human clone contains a cluster of RG repeats which are not present in the rat clone (Fig. 3C). There is a high degree of conservation between the human, rat, and partial clones in the three putative dsRNA binding domain motifs, with more divergency in the hinge region between the domains (Fig. 3A). The dsRNA binding motifs show a high degree of homology to the consensus sequence (39). The putative deaminase motif, which is present near the C terminus of each clone, is also highly conserved (Fig. 3B).

Northern blot analysis. Northern blot analysis of human tissue poly(A)$^+$ RNA with a probe derived from the middle region of the human dsRNA adenosine deaminase clone revealed only a single band of approximately 7.5 kb (Fig. 4). Even though this probe contained the sequence for the first dsRNA binding domain, it did not cross-hybridize with other mRNAs encoding dsRNA binding domains. The mRNA for the dsRNA adenosine deaminase was present in all tissues examined, in agreement with earlier reports that demonstrated a widespread expression of the deaminase (45). Northern blot analysis of poly(A)$^+$ RNA from calf thymus with the partial bovine cDNA clone as a probe also showed a band of >7.5 kb (data not shown). As a composite of the longest cDNA has an open reading frame of 3.9 kb, a substantial amount of either 5′ or 3′ untranslated sequence is lacking in the clones.

Verification of the dsRNA adenosine deaminase clone. To determine if the cDNA clones encode the dsRNA adenosine deaminase, a 1.5-kb fragment encoding the partial bovine clone was fused to a histidine tag (pQH-T3) and overexpressed in *E. coli*. Because of proteolysis, it was not possible to obtain high amounts of the entire fusion protein. Therefore, we constructed another clone (pQH-TE1) that consisted of the histidine tag fused to the first 520 nucleotides of the 5′ terminus of the bovine clone. This smaller fusion protein was more stable in *E. coli* and yielded high amounts of protein. Except for the initial immunization, this was the fusion protein used to boost the rabbits.

Antibodies raised against these fusion proteins were used in the immunoassay shown in Fig. 5. Peak fractions from a double-stranded poly(G) · poly(C) affinity column (31) were analyzed. A protein of the expected molecular mass of 116 kDa reacted with the anti-dsRNA adenosine deaminase serum but not with the preimmune serum (31). An activity profile of this column (Fig. 5) shows the coelution of activity with the band of 116 kDa. Silver staining of an SDS-polyacrylamide gel of these column fractions also revealed a polypeptide with a molecular mass of 116 kDa that was present only in the same lanes and in the same ratio as the protein in the immunoblot (data not shown). The peak fractions also contained degradation products that reacted with the antiserum; these degradation products were also present on the SDS-polyacrylamide gel that was silver stained. The antibody titer was low when this immunoassay was performed; hence, the antibodies react weakly with the load fraction, and therefore a longer exposure of the load fraction is shown in Fig. 5B.

The anti-dsRNA adenosine deaminase serum and preimmune serum were used to deplete a partially purified calf thymus fraction of deaminase activity (Fig. 6). The antibody clearly depleted the partially purified calf thymus extract of dsRNA adenosine deaminase activity (lanes 8 and 9); with higher amounts of antibody, less conversion of adenosine to inosine was observed (≈70% reduction; lane 9), whereas the preimmune serum led only to a lower reduction in activity (≈20% reduction; lanes 4 and 5). dsRNA adenosine deaminase activity was restored when pure bovine deaminase was added back to the antibody-depleted extract (lanes 10 and 11).

Immunofluorescence and rat brain in situ hybridization. HeLa cells were stained with affinity-purified anti-dsRNA adenosine deaminase antibodies (Fig. 7). A predominant nuclear staining with a granulated pattern that excluded the nucleolus was observed. Double-labeling experiments with anti-dsRNA adenosine deaminase and anti-Y12 (small nuclear ribonucleoprotein) indicated staining of similar subnuclear compartments (15, 32) (data not shown). This result is consistent with the finding that the deaminase activity is nuclear (45).

It has been proposed that dsRNA-specific adenosine deaminase is one of the components involved in the editing reactions that occur in the glutamate receptor subunits in the brain (19, 24, 37). To visualize the distribution of the dsRNA adenosine deaminase mRNA in the central nervous system, we performed in situ hybridization on horizontal sections of rat brain with an antisense oligonucleotide as a probe. As shown in Fig. 8, dsRNA adenosine deaminase transcripts were detected in all major areas of the developing brain. These included the cortex, hippocampus, striatum, thalamic regions, colliculi, and cerebellum. Expression levels of dsRNA adenosine deaminase were moderate around birth (Fig. 8A) and appeared to increase postnatally (Fig. 8B). The expression pattern seen in the adult brain corresponds closely to neuronal structures (Fig. 8C). However, the detection limits imposed by the procedure used may not have revealed expression in glial cells.

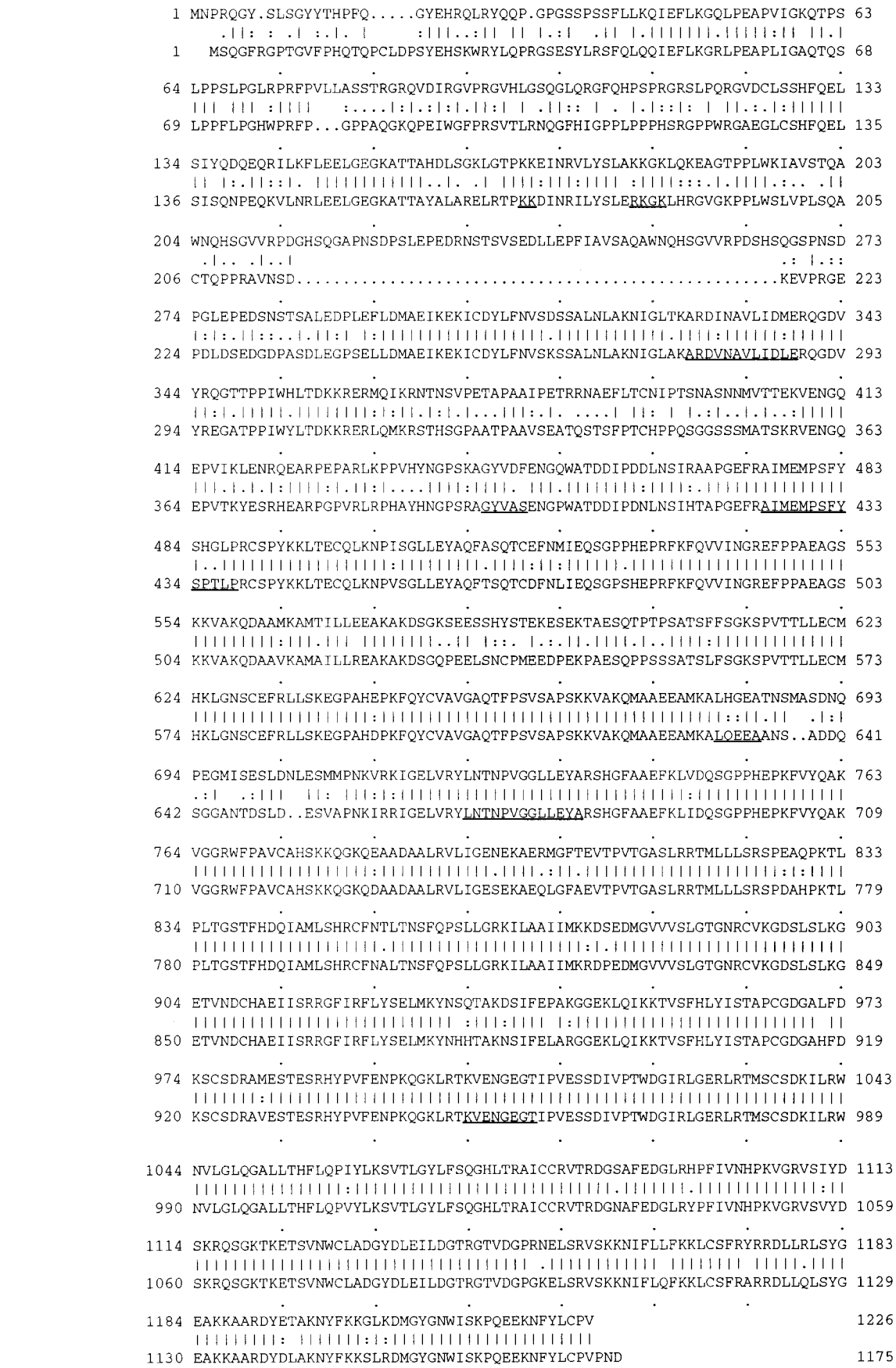

FIG. 2. Amino acid alignment of full-length human (top) and rat (bottom) clones. The nuclear targeting signal and the peptides corresponding to those that were sequenced are underlined.

A

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 Ct-dsRB       KNPISGLLEYA QFASQTCEFNMIEQSGPPHEPRFKFQVVINGREFPPAEAG SKKVAKQDAAMKAMTILL
 Hu-dsRB  502 KNPISGLLEYA QFASQTCEFNMIEQSGPPHEPRFKFQVVINGREFPPAEAG SKKVAKQDAAMKAMTILL
Rat-dsRB  452 KNPVSGLLEYA QFTSQTCDFNLIEQSGPSHEPRFKFQVVINGREFPPAEAG SKKVAKQDAAVKAMAILL

consensus        PV..LNEY..QK....P.Y.L...SGPAH.K.FTF.V.V..G.....G.G.SKK.AK..AAE.AL..L
                  I               F M               I                       M

 Ct-dsRB       KNPVTTLLECVH KLGSSCEFRLLSREGPAHDPKFQYCVAMGTHTFPTASAP SKKAAKQMAAEEAMKALQ
 Hu-dsRB  613 KSPVTTLLECMH KLGNSCEFRLLSKEGPAHEPKFQYCVAVGAQTFPSVSAP SKKVAKQMAAEEAMKALH
Rat-dsRB  563 KSPVTTLLECMH KLGNSCEFRLLSKEGPAHDPKFQYCVAVGAQTFPSVSAP SKKVAKQMAAEEAMKALQ


consensus        PV..LNEY..QK....P.Y.L...SGPAH.K.FTF.V.V..G.....G.G.SKK.AK..AAE.AL..L
                                  F               Y                       M

 Ct-dsRB       TNPVGGLLEYARSHGFAAEFK LVDQSGPPHEPKFVYQAKVG GRWFPAVCAHSKKQGKQEAADAALRVLI
 Hu-dsRB  725 TNPVGGLLEYARSHGFAAEFK LVDQSGPPHEPKFVYQAKVG GRWFPAVCAHSKKQGKQEAADAALRVLI
Rat-dsRB  671 TNPVGGLLEYARSHGFAAEFK LIDQSGPPHEPKFVYQAKVG GRWFPAVCAHSKKQGKQDAADAALRVLI

consensus        PV..LNEY..QK....P.Y.L...SGPAH.K.FTF.V.V..G.....G.G.SKK.AK..AAE.AL..L
                                                 Y                       D
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B

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Hu-dsRAD       909  CHAEILSRRGFIRFLYSELMK.....................TAPCGDGALFDKSCSD  978

HUMDEOXDEA      83  CHAELNAIMNKNSTDVKGC.......................LFPCNECAKLIIQAGI  122

MYPT4DDA       103  IHAELNAIFAARNGSSIEGA.......................LSPCPDCAKAIAQSGI  144

REPR            60  KHVENFIEKFTTERYFCPN.......................WSPCGECSRAITEFLS  105

HEPR            60  NHVENFIKKFTSERDFHPSI.......................WSPCWECSQAIREFLS  105

ADA-Hu         213  VHAGEVGSAEVVKEA  227
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C

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Hu-dsRAD   80 ASSTRGRQVDIRGVPRGVHLGSQGLQRGFQHPSPRGRSLPQRGVDCLSSHFQELSIYQDQEQ 141
              :....|:|.:|:|.||:| | .||:: | . |.|::|: | ||.:.|:|||||||| |:.||
Rat-dsRAD  82 GPPAQGKQPEIWGFPRSVTLRNQGFHIGPPLPPPHSRGPPWRGAEGLCSHFQELSISQNPEQ 143
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FIG. 3. (A) Alignment of the dsRNA binding domains (dsRB) present in the human (Hu), rat, and bovine (Ct) clones. The consensus sequence is shown below (39). Boldface letters indicate amino acids that are identical with the consensus sequence; letters underneath the consensus sequence are amino acids that are homologous to the conserved sequence and are found in the dsRNA adenosine deaminase. Gaps were introduced into the sequence so that the spacing would be conserved with the consensus sequence. (B) Alignment of protein sequences that contain either adenosine or cytosine deaminase. Boldface letters indicate the conserved amino acids. The top sequence is human dsRNA adenosine deaminase (Hu-dsRAD), and the sequences beneath are written in order of decreasing homology. Only the human clone is shown since the rat clone is nearly identical. HUMDEOXDEA, human deoxycytidylate deaminase, residues 83 to 122; MYPT4DDA, bacteriophage T4 dCMP deaminase, residues 103 to 144; REPR, rat 27-kDa cytidine deaminase component of the ApoB editing enzyme, residues 60 to 105; HEPR, human 27-kDa cytidine deaminase component of the ApoB editing enzyme, residues 60 to 105; ADA-Hu, human adenosine deaminase, residues 214 to 228. (C) Comparison of the human and rat clones in the composition of Gly and Arg residues at their N termini. Gly and Arg amino acids present in the human clone are in boldface letters.

DISCUSSION

In this study, we report the isolation of cDNA clones from human, rat, and bovine libraries that encode dsRNA adenosine deaminase. There is a high degree of sequence identity between the human and rat clones (79%) and the human and the partial bovine clones.

Binding to dsRNA. One of the most intriguing questions about this enzyme is how it can convert adenosine to inosine by hydrolytic deamination. As mentioned above, the major groove of duplex RNA is inaccessible to amino acid side chains. Since the RNA adenosine deaminase binds to duplex RNA, it must be able to recognize and gain access to the adenosine that it deaminates. One possibility of achieving this would be by melting the duplex structure by the binding of the three dsRNA binding domains. Another possible mechanism would be to flip out the adenine in a manner similar to that of *Hha*I DNA

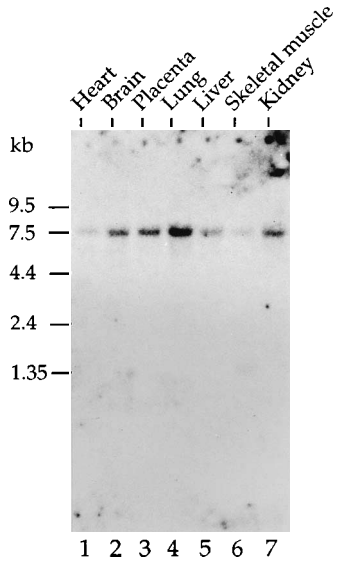

FIG. 4. Northern blot analysis of mRNA encoding dsRNA adenosine deaminase. A human multiple tissue Northern blot of poly(A)$^+$ RNA was probed with a 1.2-kb fragment from *Eco*RI to *Bam*HI of the human cDNA clone. The molecular weight markers are indicated on the left.

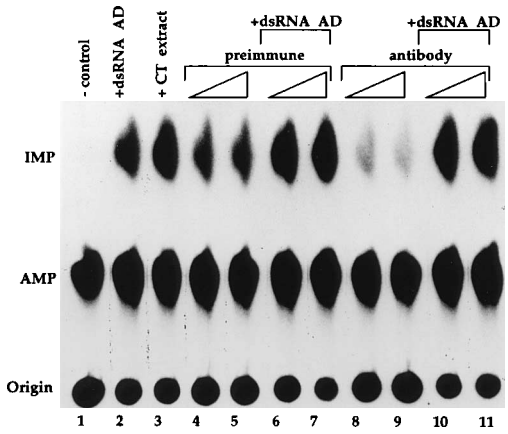

FIG. 6. Immunodepletion of calf thymus extracts with anti-dsRNA adenosine deaminase serum. Calf thymus extracts were incubated with either anti-dsRNA adenosine deaminase serum or preimmune serum that was attached to protein A-Sepharose beads and assayed for deaminase activity. After incubation of extracts with dsRNA, the dsRNA was digested with P1 nuclease and the products were chromatographed on thin-layer chromatography plates. The spot at the bottom is the origin, the one in the middle corresponds to AMP, and the one at the top corresponds to IMP. Lane 1, negative control (dsRNA incubated in the absence of extract). Lane 2, pure bovine dsRNA adenosine deaminase (AD). Lane 3, partially purified calf thymus (CT) extract that was used for the depletion. Lanes 4 to 7, extract incubated with 20 μl (lanes 4 and 6) and 100 μl (lanes 5 and 7) of preimmune serum. Pure bovine dsRNA adenosine deaminase was added to lanes 6 and 7. Lanes 8 to 11, extracts incubated with 20 μl (lanes 8 and 10) and 100 μl (lanes 9 and 11) of anti-dsRNA adenosine deaminase serum. Lanes 10 and 11 also contained pure bovine dsRNA adenosine deaminase. The protein concentration was adjusted so that all of the depleted extracts contained the same amount of protein.

methyltransferase (23, 44). The crystal structure of *Hha*I methyltransferase suggests that it is thermodynamically feasible and more favorable to severely distort nucleic acids than to reorganize the structure of a protein.

We have previously found that the deaminase prefers to bind poly(G), and it was this property that enabled us to purify the enzyme (31). Poly(G) is known to have higher-order structure (21); it is therefore probable that even though the deaminase can deaminate any adenosine in vitro in duplex RNA, it has a preference for certain conformations in the dsRNA that is provided by sequence or additional factors (33).

Motifs for dsRNA binding domains. Repeats of a 70-residue dsRNA binding domain have now been found in several proteins (1, 7, 14, 16, 17, 39). The number of dsRNA binding domains present in different proteins varies between one and three; also, the size of the domain differs if it consists of full-length or truncated domains and if additional sequences are required for binding (39). The C-terminal part of the dsRNA binding domain is the most conserved and contains a predicted α helix which may insert into the minor groove of the dsRNA since the major groove is too narrow (17).

The dsRNA adenosine deaminase sequence has three dsRNA binding domains (Fig. 3A). The C-terminal regions of the dsRNA binding motifs are more conserved than the N-terminal regions, and we found three predicted α helices in this region in each of the dsRNA binding domains with the Chou

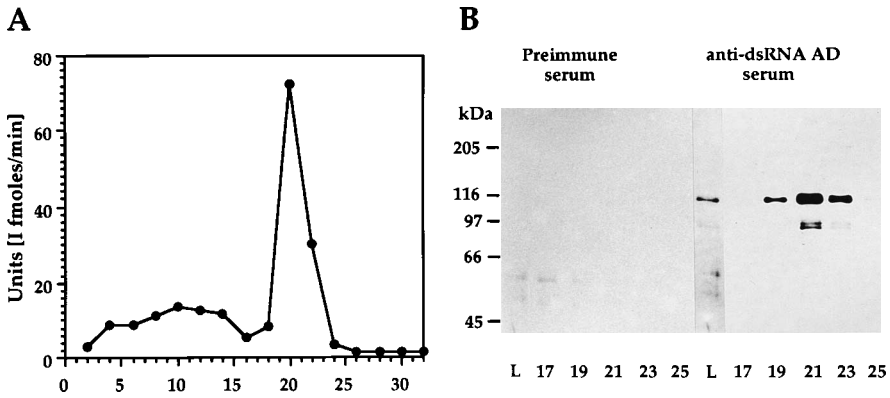

FIG. 5. Immunoblot of the peak fractions from the final chromatographic step in the purification of dsRNA adenosine deaminase. The dsRNA adenosine deaminase was purified as previously described (31); panel A is an activity profile of the poly(G) · poly(C) column. Fractions were assayed for dsRNA adenosine deaminase activity by determining the conversion of adenosine to inosine in dsRNA. (B) Load and peak fractions from the final poly(G) · poly(C) column that were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-dsRNA adenosine deaminase (AD) and preimmune serum. The bands were detected by chemiluminescence and were autoradiographed. Since the antibody titer was low and did not detect the load fraction very well, a longer exposure of this lane (L) is shown. Molecular weight markers are shown on the left.

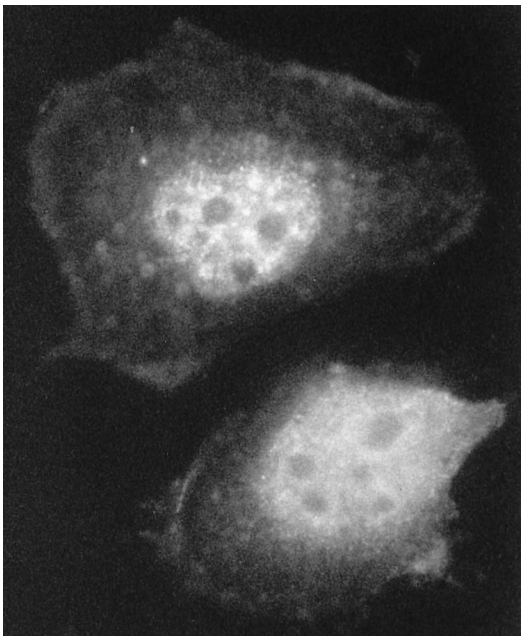

FIG. 7. Immunofluorescence staining of HeLa cells. HeLa cells were fluorescence labeled with affinity-purified anti-dsRNA adenosine deaminase serum (1:10). The secondary antibody was labeled with rhodamine. Magnification, ×800.

and Fasman algorithm (11). The spacers between the three dsRNA binding domains are greater than 40 amino acids, allowing sufficient space in the hinge regions between the three domains for binding of all domains to RNA simultaneously. It is difficult to predict from the sequence if all three domains are equivalent, as approximately the same number of amino acids are conserved in each domain, but domains 1 and 2 have better predicted α helices than domain 3.

Multiples of RNA binding domains are found in other proteins, and it is therefore not unusual that there are three dsRNA binding domains in dsRNA adenosine deaminase; this may give greater flexibility to a protein to bind a larger number of RNAs (7). It is also plausible that the three domains put more constraint on the enzyme so that the double-stranded region must have a certain length for all domains to bind and the deaminase to function. There is some experimental evidence to support this possibility, as it has been shown in vitro that the dsRNA adenosine deaminase prefers dsRNA that is at least 100 nucleotides in length (30). The binding of the three dsRNA binding domains to dsRNA may provide energy to unwind the duplex RNA so that the active site can access the adenosine that it deaminates. The positions of the three dsRNA binding domains may be important in positioning the deaminase active site. Changing the spacing between the deaminase domain and the dsRNA binding domains would indicate if the binding of the dsRNA domains is coupled to deamination.

Recent work of Polson and Bass (33) has shown that with short dsRNA substrates, the dsRNA adenosine deaminase has a 5′ preference for certain adenosines and also that adenosines near the 3′ end are not favored for deamination. This finding suggests that the deaminase has a binding polarity which is probably mediated by the dsRNA binding domains.

Nuclear targeting. There is a putative nuclear targeting motif in both the human and rat clones at the same position near the N terminus. This motif is bipartite, composed of two basic amino acids, a spacer of 10 amino acids, and a basic cluster in which three of five amino acids are again basic (12). The only difference between the rat and human clones is that one of the Lys residues in the second basic cluster is replaced by an Arg, which is also a basic amino acid. In agreement with this, immunofluorescence staining of HeLa cells with anti-dsRNA

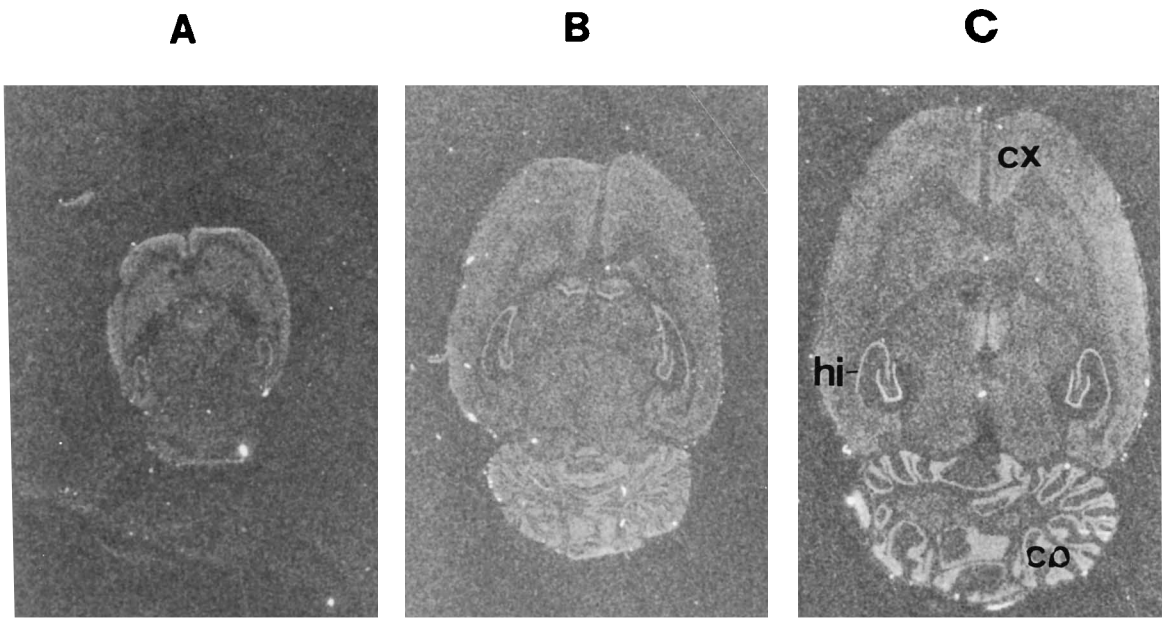

FIG. 8. Autoradiographs showing the expression of dsRNA adenosine deaminase mRNA in rat brain. Horizontal sections of rat brain at three developmental stages (birth [A], 12 days of age [B], and 40 days of age [C]) were probed with a dsRNA adenosine deaminase-specific 36-mer oligonucleotide. Note the ubiquitous expression of the deaminase throughout all major neuronal populations. cx, cortex; hi, hippocampus; cb, cerebellum.

adenosine deaminase affinity-purified antibody reveals a nuclear localization for the enzyme (Fig. 7). Also, previous results have shown that dsRNA adenosine deaminase activity is found in the nucleus (45).

Deaminase motif. Both adenosine and cytosine deaminases have Zn^{2+} ions in their active sites (5, 28, 29, 49). They also have some homology around the predicted bipartite active-site residues, especially the His and Cys residues which are thought to coordinate Zn^{2+} (Fig. 3B) (5, 10, 29, 52). A Glu residue is also conserved, and in the crystal structure of murine adenosine deaminase complexed to 6-hydroxy-1,6-dihydropurine ribonucleoside, this amino acid interacts with N-1 of the purine ring (49).

As dsRNA adenosine deaminase is inhibited by the Zn^{2+} chelator *o*-phenanthroline (22, 30a), one prediction would be that the residues surrounding the active site are similar to other deaminases. In the alignment shown in Fig. 3B, dsRNA adenosine deaminase also has the conserved Cys-909, His-910, and Glu-912 residues. Arg-921 is also conserved in many deaminases, but its role is unknown (52). Since dsRNA adenosine deaminase is not inhibited by deoxycoformycin (3), an inhibitor of adenosine deaminase, it is not surprising that the predicted active site of dsRNA adenosine deaminase does not resemble that of other adenosine deaminases (10). However, it is surprising that dsRNA adenosine deaminase resembles cytosine deaminases and also has some homology to the cytidine deaminase which mediates ApoB mRNA editing (5, 18, 29, 41, 52). dsRNA adenosine deaminase does not have the second Cys present in the C terminus of the motif that is conserved and believed to coordinate Zn^{2+}, but it has another Cys-976 nearby that could fulfill this function. Site-directed mutagenesis is required to determine if this predicted deaminase site is indeed the active site.

RG-enriched N terminus. Comparison between the rat and human clones indicates that their amino acid sequences are very similar except at the extreme N termini. The human clone encodes many Arg and Gly residues at the N terminus, but the Arg residues in particular are not conserved in the rat clone. It is possible that they are sites of posttranslational modification for the dimethylation of Arg residues, as a Gly residue is usually situated directly C terminal to the modified Arg residue and may be required for recognition by the methyltransferase (13, 40, 51). There are six Arg/Gly repeats within 40 amino acids in the human dsRNA adenosine deaminase. To determine if these are indeed sites for dimethylation of Arg, amino acid analysis will have to be performed.

Expression of dsRNA adenosine deaminase. The dsRNA-specific adenosine deaminase that we have cloned is a nuclear protein that is expressed in virtually all mammalian cells (Fig. 4). This is in agreement with earlier reports showing that the activity is ubiquitous (45). In situ hybridization shows a widespread distribution in the brain, with the expression levels increasing after birth (Fig. 8).

The open reading frame of the human dsRNA adenosine deaminase encodes a protein of 139 kDa, which is larger than the bovine protein that we have purified (116 kDa). Therefore, it is probable that the purified protein is a proteolytic product. The antibodies also recognize a protein of approximately 116 kDa in fractionated HeLa cell extract (data not shown). This proteolysis may occur at a very early stage of purification.

Antibodies raised against a fusion protein that was overexpressed in *E. coli* specifically recognized the dsRNA adenosine deaminase in calf thymus partially purified extracts (Fig. 5). The anti-dsRNA adenosine deaminase serum depleted a partially purified extract of deaminase activity, proving that we have indeed cloned the gene encoding the enzyme (Fig. 6). When purified bovine dsRNA adenosine deaminase was added to immunodepleted extract, it restored deaminase activity. The pure protein had previously been analyzed by SDS-polyacrylamide gel electrophoresis and visualized by silver staining to ensure that only the 116-kDa protein was present in the added fraction.

dsRNA adenosine deaminase requires no other factors in vitro to deaminate dsRNA (20, 22, 31), but as its substrate in vivo is probably mostly single-stranded RNA-containing duplex regions, its substrate specificity in vivo has yet to be determined. It is necessary to find the in vivo substrates for the deaminase, as only then we can determine the sequences and structures that are required by the enzyme for optimal activity and what its biological function is.

Site-specific editing of glutamate receptor B pre-mRNA has recently been shown to occur in HeLa cell nuclear extracts in vitro and leads to the conversion of selected adenosine residues to inosines (27a). Moreover, purification of the specific RNA-editing activity by column chromatography results in a fraction that contains both specific editing as well as nonspecific dsRNA-specific adenosine deaminase activity (31a). Thus, it is very likely that dsRNA-specific adenosine deaminase is responsible for the catalysis of adenosine-to-inosine conversion and is part of a specific RNA-editing complex.

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ADDENDUM IN PROOF

While the manuscript was under review, a paper reporting the cloning and expression of the human dsRNA-specific adenosine deaminase gene was published (U. Kim, Y. Wang, T. Stanford, Y. Zeng, and K. Nishikura, Proc. Natl. Acad. Sci. USA **91:**11457–11461, 1994).

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