Isolation and characterization of intranuclear ribonucleoprotein complexes associated with double-stranded RNA adenosine deaminase from brain cells: implications for RNA-editing and hypermutation of viral RNA in the CNS

Zhigang Chen, Akiko Hirano and Timothy C Wong

Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195, USA

Double-stranded RNA adenosine deaminase (DsRAD), which converts adenosine in duplex RNA to inosine, has been implicated in editing of cellular mRNA and hypermutation of viral RNA in the central nervous system (CNS). We used subcellular fractionation to show that DsRAD in bovine brain tissues is associated with high-molecular-weight ribonucleoprotein (RNP) complexes in the nuclei. DsRAD-associated RNP complexes have apparent molecular mass of up to 500 kDa and buoyant density of 1.35 to 1.42 g cc^{-1} in CsCl solution. In human glioma cells, DsRAD is also found exclusively in intranuclear RNP complexes that co-sediment with the largest RNA species. These DsRAD-associated RNP complexes are dissociated by RNase A or high salt. The RNA component is not essential for DsRAD activity, and the protein component can be separated by dsRNA-affinity column, gel filtration column, and glycerol gradient into enzymatically active protein species with apparent molecular mass ranging from 120 kDa to 70 kDa in polyacrylamide gel. The bovine brain DsRAD has no apparent requirement for low-molecular-weight cofactors or metal ions. These results provide insight into the native state of DsRAD in brain cells and have interesting implications for its putative roles in RNA-editing and hypermutation of viral RNA in the CNS.

Keywords: double-stranded RNA adenosine deaminase; RNA-editing; RNP

Introduction

Measles virus (MV), which typically causes an acute infection, can occasionally cause a chronic infection in the central nervous system (CNS), leading to a fatal degenerative neurological disease subacute sclerosing panencephalitis (SSPE) (ter Meulen and Carter, 1984; Kristensson and Norrby, 1986). The minus-sense RNA genomes of MV strains that cause CNS infection often show numerous adenosine (A)-to-guanosine (G) transitions. These unusual mutations, known as biased hypermutation, were first described in a case of measles inclusion body encephalitis (Cattaneo et al, 1988) and subsequently observed in many MV strains that cause SSPE (Ayata et al, 1989; Cattaneo et al, 1989; Wong et al, 1989, 1991; Billeter and Cattaneo, 1991; Baczko et al, 1993).

Biased hypermutation can drastically change the properties and functions of the MV proteins. A well studied example is the Biken strain SSPE virus (Ayata et al, 1989), which was derived from a progenitor closely related to the Yamagata strain acute MV (Wong et al, 1991). Most of the differences that distinguish the matrix (M) genes of the Biken and Yamagata strains are A-to-G transitions (in the minus-sense genomic RNA). These mutations account for nearly all the mis-sense mutations in the Biken M gene and drastically alter the Biken M protein (Wong et al, 1991). The Biken M protein is not recognized by antibodies against the M protein of Yamagata strain MV (Wong et al, 1991), and it cannot bind to the MV nucleocapsids during virus assembly (Hirano et al, 1992). Thus, hypermutation

may lead to a non-productive mode of replication in MV strains that chronically infects the CNS (Hirano et al, 1993).

Although the precise mechanism of biased hypermutation in MV remains to be established, circumstantial evidence points to the involvement of a cellular RNA-modifying enzyme. Specifically, a MV (Yamagata strain) that has been passaged in human neuroblastoma IMR-32 cells shows numerous A-to-G transitions that are not found in the same MV strain passaged in African green monkey kidney Vero cells (Wong et al, 1989). It is postulated that the genomic RNA and mRNA of MV form aberrant duplexes which become substrates for a cellular double-stranded RNA-unwinding/modifying

enzyme which was first reported in *Xenopus laevis* (Bass and Weintraub, 1987; Bass et al, 1989). This enzyme, now called double-stranded RNA adenosine deaminase (DsRAD), has subsequently been found in a wide range of mammalian somatic cells (Rebagliati and Melton, 1987; Bass and Weintraub, 1988; Wagner and Nishikura, 1988; Wagner et al, 1989, 1990; for review: Bass 1993; Kim and Nishikura, 1993). DsRAD catalyzes the conversion of A residues to inosine (I), by hydrolytic deamination of the purine at the C-6 position (Polson et al, 1991). Interestingly, cells and tissues of neural origin typically show high DsRAD activities (Wagner et al, 1990). The IMR-32 human neuroblastoma cells used for passaging the highly hypermutated
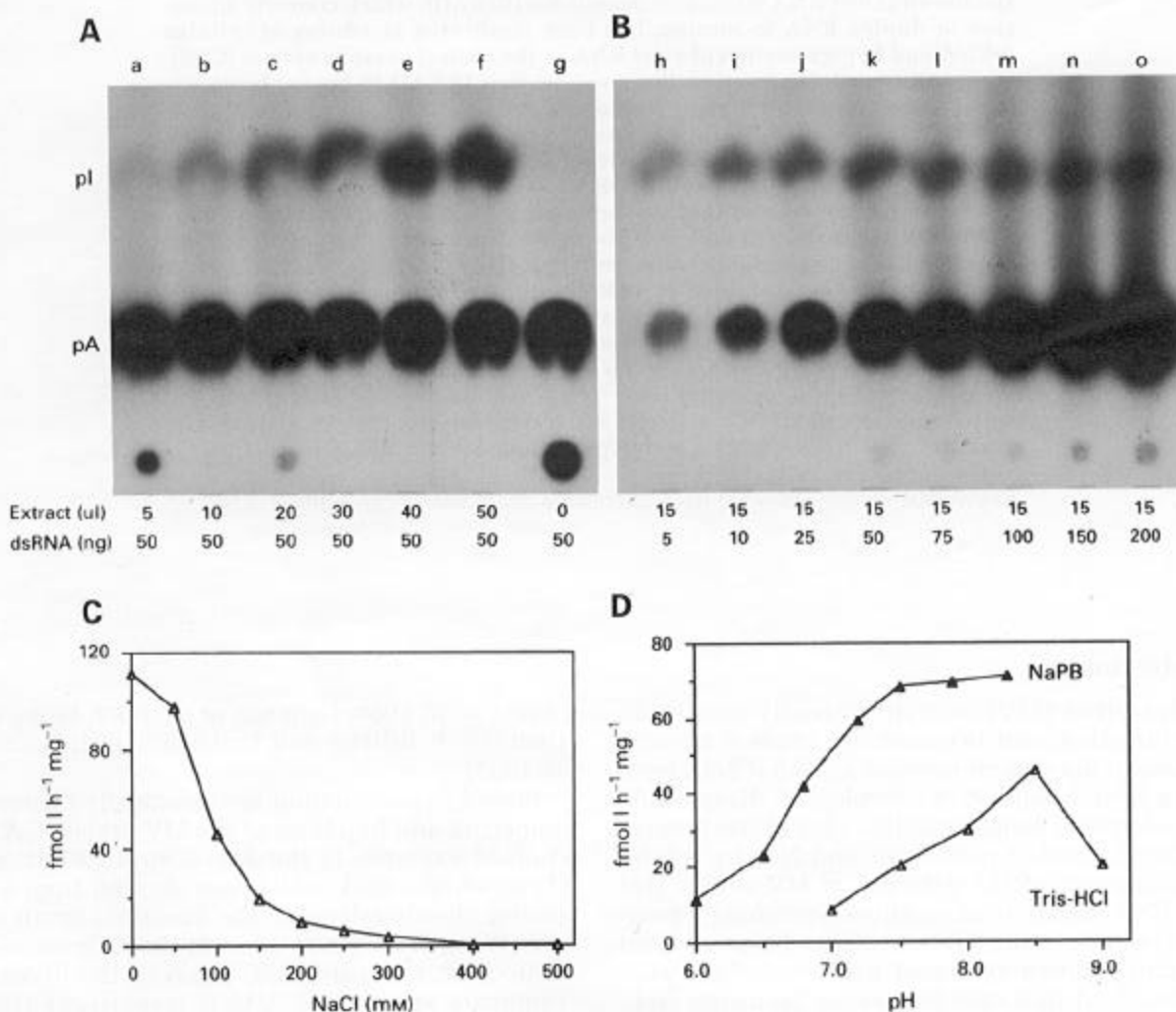

Figure 1 Effects of excess substrates, salt, and pH on bovine brain DsRAD activity. (A) [^{32}p]ATP-labeled dsRNA (50 ng) was incubated with increasing amounts of bovine brain nuclear extract at 37°C for 2 h. The RNA was recovered and digested with nuclease P1 and analyzed by TLC. (B) Constant amounts (15 μl) of bovine brain nuclear extract was incubated with increasing amounts of [^{32}p]ATP-labeled dsRNA at 37°C for 2 h. The RNA was digested with nuclease P1 and analyzed by TLC. (C) Bovine brain nuclear extract (14 μg of proteins) was incubated with 50 ng of [^{32}p]ATP-labeled dsRNA at 37°C for 2 h in a reaction buffer containing varying concentrations of NaCl. The A-to-I conversion was analyzed by TLC as in (A) and quantified by a PhosphorImager. (D) Same as in (C) except the reactions were carried out at different pH.

Yamagata MV stock in fact contain higher DsRAD activity than Vero cells used for passaging the less hypermutated Yamagata MV stock (Rataul et al, 1992). These findings suggest that DsRAD may play a role in the generation of MV mutants that infect the CNS. Thus, studying DsRAD in brain cells may provide insight into the mechanism of hypermutation of RNA virus that infects the CNS.

Recently, DsRAD has been purified from *Xenopus* oocytes, bovine liver, and bovine thymus (Hough and Bass, 1994; Kim *et al*, 1994a; O'Connell and Keller, 1994), and the human and rat DsRAD genes have been cloned (Kim *et al*, 1994b; O'Connell *et al*, 1995). Those studies have provided significant insight into the molecular nature of the DsRAD enzyme. However, little is known about the native

state of DsRAD in brain cells, where the enzyme serves putative roles in RNA modification. In this study, we show that DsRAD does not exist as soluble monomers in bovine brain tissues or cultured glioma cells. Instead, DsRAD is associated with intranuclear ribonucleoprotein (RNP) complexes that consist of non-enzymatic RNA and enzymatic protein components. We have characterized these components and the possible mode of action of DsRAD is discussed in light of the findings.

Results

Enzymatic properties of DsRAD in bovine brain
Preliminary studies indicated that the optimal conditions for DsRAD from brain tissues are somewhat
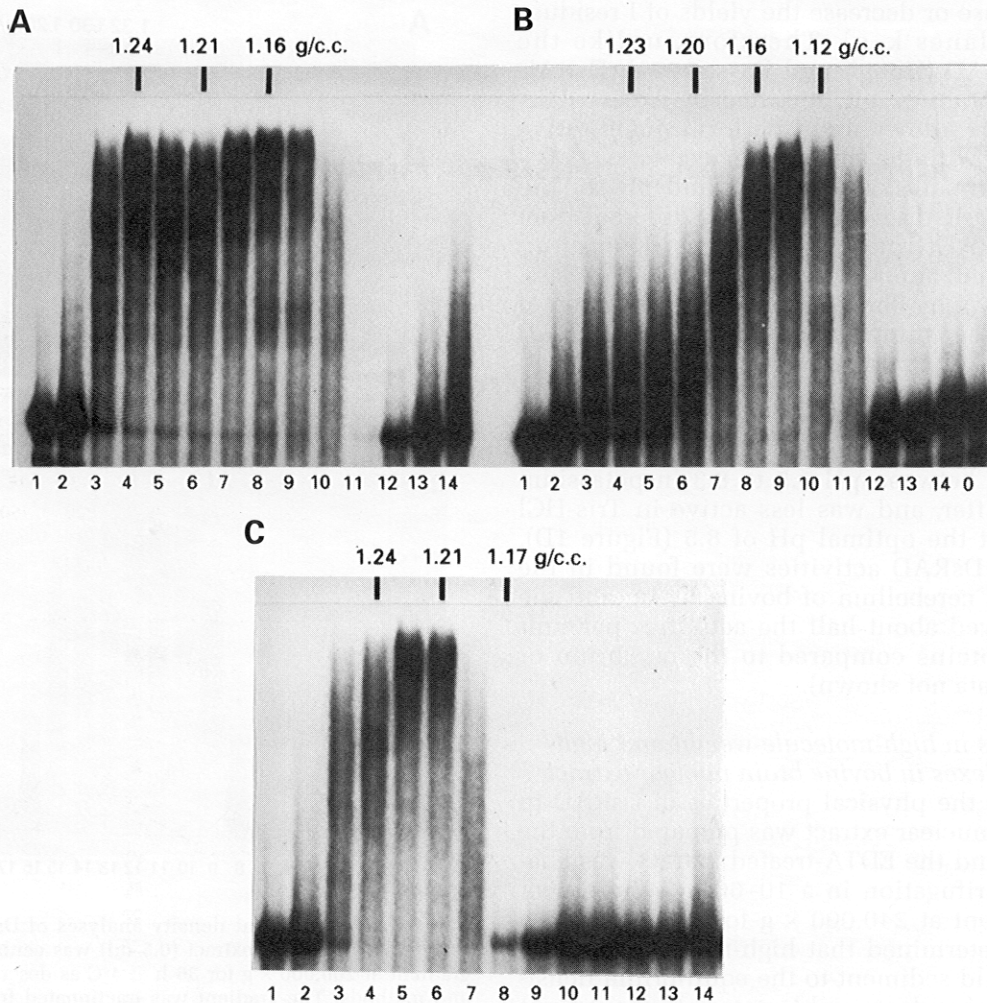

Figure 2 Sedimentation and equilibrium sucrose gradient analyses of bovine brain DsRAD. (A) Crude bovine brain nuclear extract (200 μl) was centrifuged through a 10–60% (w/w) linear sucrose gradient at 240,000 × g for 20 h at 4°C. The gradient was fractionated from bottom (fraction 1) and an aliquot from each fraction was incubated with [^{32}P]-ATP-labeled duplex RNA. DsRAD-catalyzed denaturation of the duplex RNA was analyzed by non-denaturing PAGE. Density of the gradient fractions was determined by measuring the refractive index. (B) Crude bovine brain nuclear extract (200 μl) was adjusted to 0.3 M NaCl and centrifuged in a gradient identical to that in (A) but containing 0.3 M NaCl. Lane O is a control reaction without the nuclear extract. (C) The bovine brain nuclear extract was centrifuged in the same gradient as in (A) but at 240,000 × g for 65 h.

different from those reported for DsRAD from other sources. We have therefore established the optimal conditions for our studies. DsRAD reactions were carried out with increasing amounts of nuclear extract from bovine brain or increasing amounts of $[\alpha\text{-}^{32}P]$ATP-labeled duplex RNA substrate representing the 5' portion of the M gene of MV (Rataul et al, 1992). After the reaction, the RNA was exhaustively digested with nuclease P1 and analyzed by thin-layer chromatography (TLC). DsRAD from bovine brain converted A residues in the duplex RNA to I residues in a dose-dependent manner (Figure 1A, lanes a–f). No A-to-I conversion occurred in the absence of the nuclear extract (Figure 1A, lane g). With a constant amount of nuclear extract, the yields of I residues were proportional to the RNA substrate up to about 25 ng (Figure 1B, lanes h–j). Increasing the RNA substrate from 50 ng to 200 ng did not increase or decrease the yields of I residues (Figure 1B, lanes k–o). Therefore, unlike the *Xenopus* DsRAD (Hough and Bass, 1994), DsRAD from bovine brain is not inhibited by excess RNA substrate. This allows us to perform quantitative DsRAD assays with the substrate in excess.

The optimal salt concentration and pH for the bovine brain DsRAD are also slightly different from those for DsRAD from other sources (Kim et al, 1994a; O'Connell and Keller, 1994; Hough and Bass, 1994). As shown by PhosphorImager quantitation of the A *versus* I residues resolved by TLC, DsRAD activity in bovine brain was reduced by more than 50% at 0.1 M NaCl, and was inhibited by more than 90% at NaCl concentrations higher than 0.2 M (Figure 1C). The bovine brain DsRAD had an optimal pH range between pH 7.5 to 8.3 in potassium phosphate buffer, and was less active in Tris-HCl buffer even at the optimal pH of 8.5 (Figure 1D). Equally high DsRAD activities were found in the cerebrum and cerebellum of bovine brain, and the medulla showed about half the activities per unit weight of proteins compared to the cerebrum or cerebellum (data not shown).

DsRAD resides in high-molecule-weight and high-density complexes in bovine brain nuclear extract
To investigate the physical properties of DsRAD in bovine brain, nuclear extract was prepared from the whole brain and the EDTA-treated extract was analyzed by centrifugation in a 10–60% (w/w) linear sucrose gradient at 240,000 × g for 20 h. We have empirically determined that high-molecular-weight materials would sediment to the equilibrium density but low-molecular-weight materials would not reach equilibrium under these conditions. DsRAD was assayed by monitoring the retardation of the partially denatured ^{32}P-labeled dsRNA substrate in native polyacrylamide gel (Rataul et al, 1992). The results showed that DsRAD activity was distributed broadly in the gradient (Figure 2A). When the extract was treated with 0.3 M NaCl and fractionat-

ed in a parallel sucrose gradient containing 0.3 M NaCl, DsRAD activity in the high-density region was reduced, and activity remained near the top of the gradient (Figure 2B). When the extract was centrifuged in a 10–60% (w/w) sucrose gradient at 240,000 × g until equilibrium of 65 h, all the DsRAD activity was banded at a density around 1.23 g cc^{-1} (Figure 2C). These results indicate that DsRAD in bovine brain form higher order complexes that are dissociated by high salt.

We further determined the density of these complexes by equilibrium density centrifugation in CsCl gradients in the absence or presence of 20% glycerol. In the absence of glycerol, DsRAD activity was banded at a density of 1.30 to 1.32 g cc^{-1} in CsCl, consistent with the typical densities of pro-
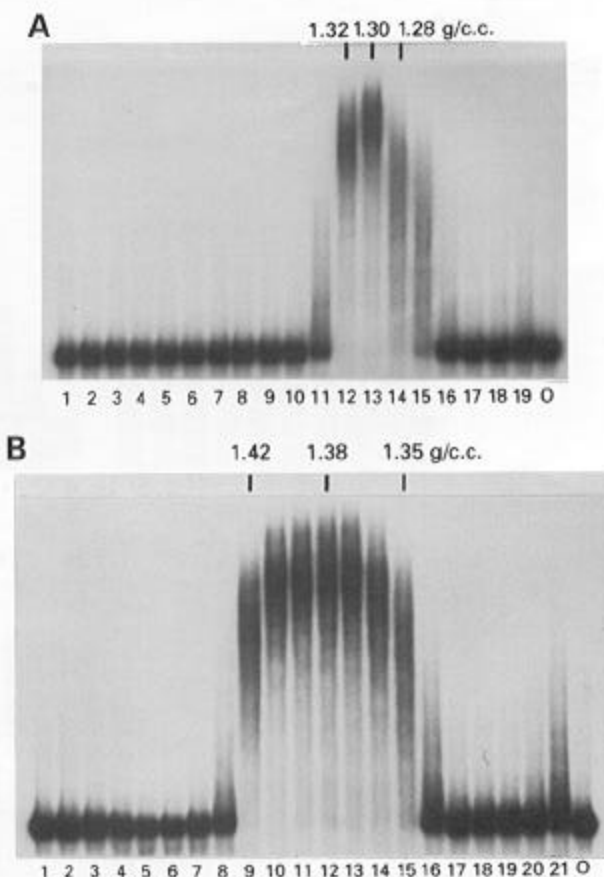

Figure 3 CsCl buoyant density analyses of DsRAD. (A) Crude bovine brain nuclear extract (0.5 ml) was centrifuged in a CsCl gradient at 200,000 × g for 36 h at 4°C as described in Materials and methods. The gradient was fractionated from bottom (fraction 1) and CsCl were removed by extensive dialysis. An aliquot from each fraction was assayed for DsRAD activity by studying denaturation of ^{32}P-labeled duplex RNA by PAGE as in Figure 2. Density was determined by measuring the refractive index and weighing a unit volume of each fraction. Lane O is control without the nuclear extract. (B) The experiment was repeated as in (A) except that the gradient contained 20% glycerol and the density was determined by directly weighing a unit volume of each gradient fraction.

teins (Figure 3A). However, when glycerol was included in the gradient to preserve intermolecular complexes, very little DsRAD activity was found to be lighter than 1.35 g, and most of the activity was in materials ranging in density from 1.35 to 1.52 g cc^{-1}, suggestive of RNP complexes (Figure 3B; Christofori and Keller, 1988; Greeve et al, 1991). Since the nuclear extract was prepared in the presence of glycerol, the stabilizing effect of glycerol

A −RNase A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 O

B +RNase A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 O

C

a b c d e f g h i j k l m n o p

Figure 4 Effects of RNase A on sedimentation and activity of the bovine brain DsRAD. Crude bovine brain nuclear extract (400 μl) was incubated for 1 h at room temperature without (A) or with 7 mg prewashed RNase A Enzygel (100 units g^{-1}) (B). After removal of RNase A Enzygel by centrifugation, the treated nuclear extracts were centrifuged in two identical 10–60% (w/w) linear sucrose gradient at 180 000 × g for 22 h at 4°C. The gradients were fractionated from bottom (fraction 1) for DsRAD assay. Lane O is control without the nuclear extract. (C) Crude bovine brain nuclear extract (200 ml) was incubated with 3.5 mg prewashed RNase A Enzygel for 0, 5, 15, 30, 50, 80, 120 min in the absence (lanes b–h, respectively) or presence of 0.3 M NaCl (lanes i–o, respectively). An aliquot from each sample was assayed for DsRAD activity in the presence of 22 unit RNasin. Lane a is a control reaction without RNase A treatment. Lane p is a control without the nuclear extract.

may explain why DsRAD-associated complexes were not completely dissociated during $(NH_4)_2SO_4$ precipitation of the extract. The association of DsRAD with RNP complexes was not an artifact of $(NH_4)_2SO_4$ precipitation, since nuclear extract prepared without high salt treatment gave identical results (see below).

DsRAD in bovine brain is associated with RNA

To test for the presence of RNA in DsRAD-associated complexes, the brain nuclear extract was treated with RNase A coupled to polyacrylamide beads. Following this treatment, the RNase A-polyacrylamide beads were removed by centrifugation, and the extract was analyzed in parallel with an untreated extract by sedimentation centrifugation in a 10–60% (w/w) linear sucrose gradient at 180,000 × g for 22 h. As described above, the native DsRAD was broadly distributed in the sedimentation gradient (Figure 4A). RNase A treatment eliminated the fast-sedimenting DsRAD activities and the remaining activity was shifted towards the top of the gradient (Figure 4B). This demonstrates that much of DsRAD in bovine brain in fact resides in RNPs that are sensitive to RNase A.

The RNA component is not essential for DsRAD activity

Micrococcal nuclease (MN) commonly used for studying the nucleic acid components in RNPs (Krainer and Maniatis, 1985; Mowry and Steitz, 1987; Kass et al, 1990) strongly inhibited the bovine brain DsRAD activity (data not shown). However, the inhibition was dependent on the concentration of MN but not on the time of incubation. Furthermore, even after the nuclease activity was completely blocked by EGTA, MN continued to show inhibitory effects on DsRAD (data not shown). Therefore the inhibition by MN is not due to destruction of an essential RNA component, but may be due to non-specifically binding of MN to the substrate dsRNA, a phenomenon known as substrate masking (Wang and Gegenheimer, 1990).

Since RNase A decreased the fast-sedimenting DsRAD activity but had no effect on the slow sedimenting DsRAD activity (Figure 4B), RNase A may destroy the RNA component and release DsRAD from the RNP complexes. Alternatively, the RNP-associated DsRAD may require RNA for activity. To distinguish between these possibilities, we incubated bovine brain nuclear extract with RNase A coupled to polyacrylamide beads, which were removed by centrifugation after various times of incubation. As shown in Figure 4C, incubating the extract with RNase A for up to 2 h did not reduce DsRAD activity (Figure 4C, compare lanes b–h to lanes a and p), although prolonged incubation led to release of RNase A from the polyacrylamide beads and degradation of the substrate RNA during enzyme assay (Figure 4C, lane h). The latter observation shows
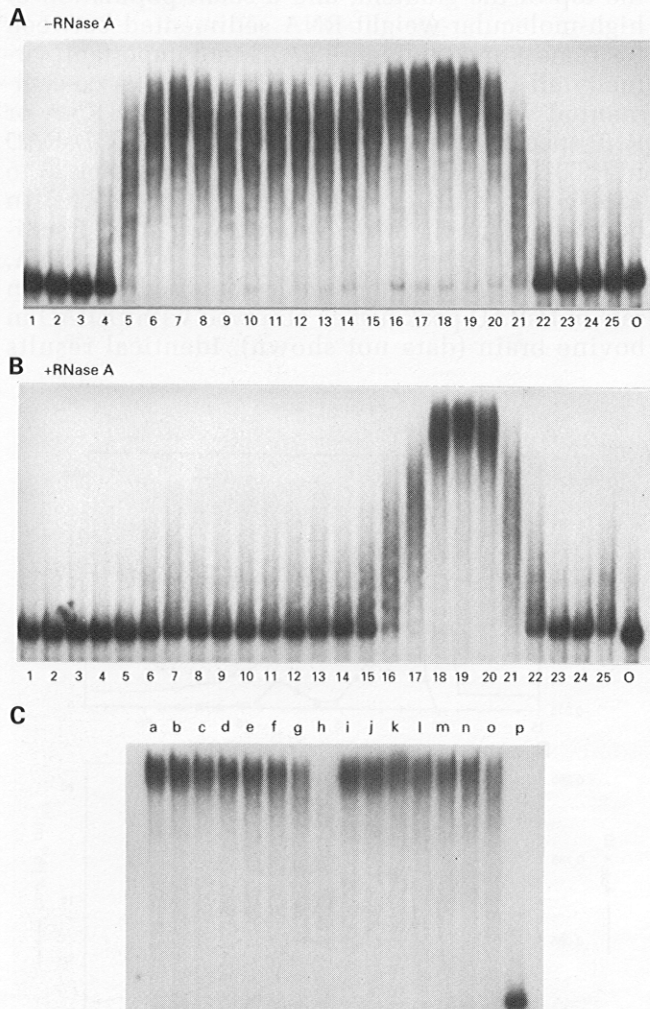
300

that the RNase A used in this experiment can degrade dsRNA; yet had no effects on DsRAD activity. To rule out the possibility that an essential RNA component was protected by the proteins in the RNP, we incubated the bovine brain nuclear with RNase A in the presence of 0.3 M NaCl, which partially dissociated DsRAD-associated RNP complexes (Figure 2B), and assayed for DsRAD activity after desalting the samples. Again, RNase A failed to destroy the bovine brain DsRAD activity even in the presence of 0.3 M NaCl (Figure 4C, compare lanes i–o to lanes a and p). Treating the bovine brain extract with DNase I or a combination of both RNase A and DNase I also did not decrease DsRAD activity (data not shown). These results show that the RNA component in the RNP is not essential for DsRAD activity.

DsRAD in cultured neural cells resides exclusively in RNP complexes

The heterogeneous sizes of DsRAD-associated materials suggest that some DsRAD may not be associated with RNA. Alternatively, the size heterogeneity

may be due to degradation of the RNA in the RNP complexes, which is difficult to avoid in the bovine brain. Therefore, we examined DsRAD in cultured neural cells. Human glioma (HTB-14) cells were labeled with [³H]uridine and the whole-cell extract was concentrated and centrifuged in a 10–60% (w/w) sucrose linear gradient at 180,000 × g for 22 h. The gradient fraction aliquots were assayed for DsRAD activity and RNA contents. The bulk of the ³H-labeled RNA in HTS-14 cells sedimented near the top of the gradient, and a small population of high-molecular-weight RNA sedimented between fractions 4 and 10 (Figure 5A). More important, virtually all DsRAD activity in HTB-14 cells co-sedimented with the high-molecular-weight RNA or RNP species in fractions 4 to 6 (Figure 5B). DsRAD in HTB-14 cells had a density in sucrose of 1.23 to 1.24 g cc⁻¹, similar to the density of DsRAD in bovine brain (Figures 5A and 2C). The fast-sedimenting DsRAD is physically associated with RNA, since RNase A treatment reduced the sedimentation rate of DsRAD peak just as observed with DsRAD in bovine brain (data not shown). Identical results
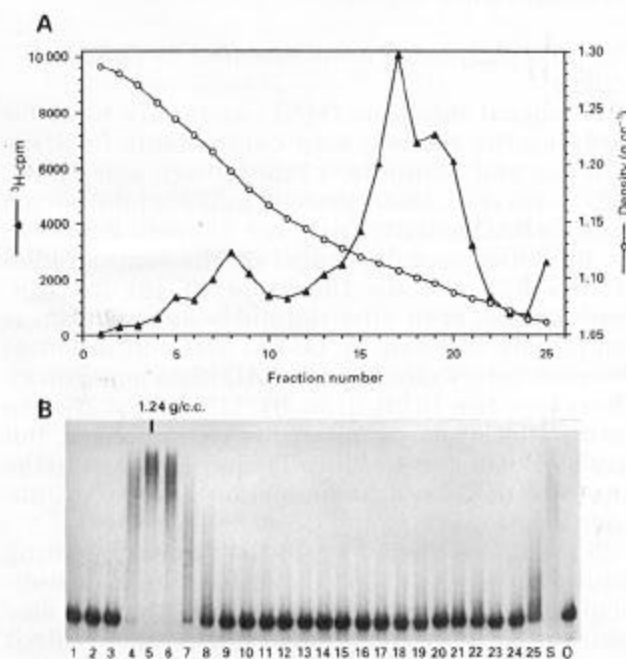

Figure 5 Sucrose gradient analysis of DsRAD in HTB-14 cells. Cultures containing 3 × 10⁷ HTB-14 cells were labeled with 0.3 mCi [³H]uridine for 15 h. Whole-cell extract was concentrated by Centriprep-10 and centrifuged through a 10–60% (w/w) linear sucrose gradient at 180,000 × g for 22 h at 4°C. The gradient was fractionated from the bottom (fraction 1) and the ³H-labeled RNA extracted by phenol-chloroform from an aliquot (100 μl) of each fraction was precipitated and counted in a scintillation counter (A). Another aliquot from each fraction was assayed for DsRAD activity with ³²P-labeled dsRNA and analyzed by PAGE (B). Density of the gradient fractions was determined from the refractive index. Lane S contains unfractionated HTB-14 extract. Lane O is control without cell extract.
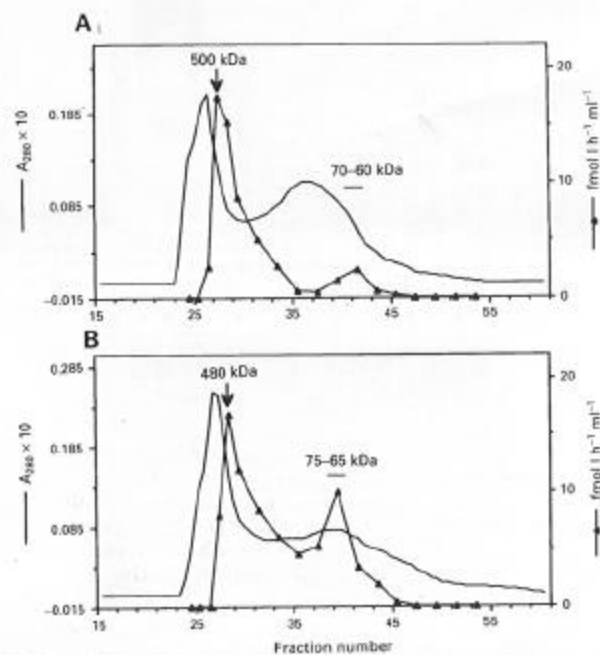

Figure 6 Gel filtration chromatography of DsRAD-associated complexes. (A) Crude bovine brain nuclear extract (200 μl) was fractionated in a Superose-12 (10 × 300 mm) column equilibrated with 25 mM Tris-HCl, pH 7.9, 50 mM KCl, 0.25 mM MgCl₂, 20% glycerol, and 0.2% Brij 35 at 0.4 ml min⁻¹. Aliquots of the collected fractions were assayed for DsRAD activity by PhosphorImager quantitation of the A and I residues resolved by TLC (closed triangles). Protein concentration was monitored by spectrophotometric absorbance at 280 nm. Molecular weight was calculated by comparing the retention time to protein standards (Materials and methods). (B) Crude brain extract was fractionated in a Superose-12 column as in (A), except the buffer contains 300 mM KCl.
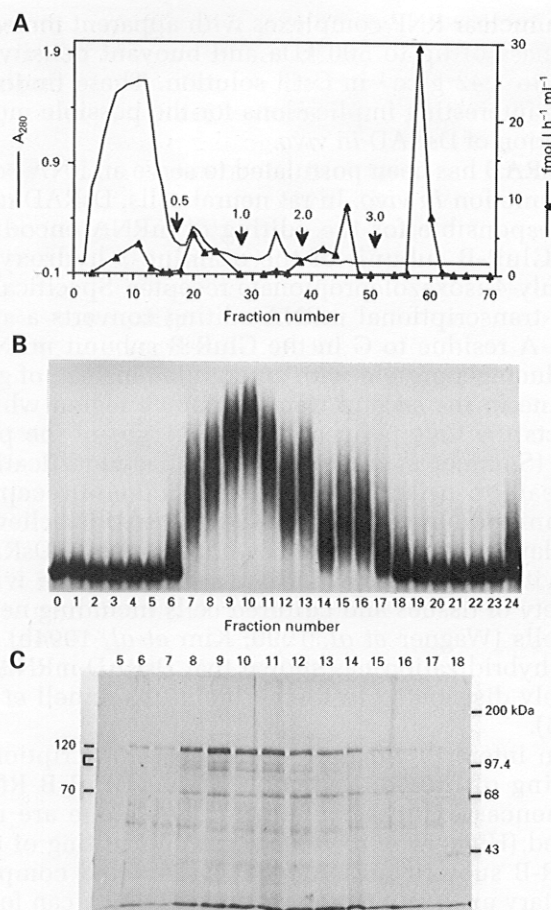
A

B

C

Figure 7 Purification of the active component of DsRAD. (A) Protein and DsRAD activity profiles of poly(I).poly)C)-agarose column fractions. Crude bovine brain nuclear extract (46 ml) was adjusted to 0.35 M KCl and passed through a ss-DNA cellulose column (2.6 × 7.0 cm). The flow through was applied to a poly(I).poly(C)-agarose column (2.6 × 7.0 cm) equilibrated with buffer A with 0.35 M KCl. The flow-through fraction and the fractions eluted with buffer A containing 0.5, 1.0, 2.0 M KCl, or 3.0 M NaCl and 0.2% Brij-35 from the poly(I).poly(C) column were desalted by Microcon-10 twice and assayed for DsRAD activity by measuring the A-to-I conversion by TLC (closed triangles). Protein concentration was monitored by spectrophotometric absorbance at 280 nm. The arrows indicate starting positions of the different elution buffers. (B) 200 μl of DsRAD purified twice by chromatography on poly(I).poly(C) agarose was centrifuged through 10–30% glycerol linear gradient at 180,000 × g at 4°C for 36 h. The sedimentation gradient was fractionated from bottom and assayed for DsRAD activity with ^{32}P-labeled dsRNA substrate analyzed by PAGE. Lane Ø is a control reaction without DsRAD. (C) Protein associated with DsRAD activity. Glycerol sedimentation fractions (200 μl) were precipitated with TCA (15%) and the pellets were washed twice with acetone and dissolved in loading buffer. Proteins were electrophoresed on 8% polyacrylamide gel with SDS and visualized by staining with silver staining.

were obtained with another human glioma cell line U373 (data not shown).

These results show that DsRAD resides almost exclusively in high-molecular-weight RNP in human glioma cells. The heterogeneous size of the DsRAD-associated complexes in bovine brain is most likely due to RNA degradation. The association of DsRAD exclusively with high-molecular-weight RNA strongly suggests that the association is not an artifact of non-specific RNA binding. To further rule out the possibility that the DsRAD-associated RNP complexes were formed during desalting of the nuclear extracts prepared under high salt conditions, we also obtained DsRAD from sucrose gradient purified nuclei of bovine brain and HTB-14 cells without exposing the nuclei to high salt (see Materials and methods). DsRAD prepared under these conditions had sedimentation profiles identical to those of DsRAD prepared by high-salt extraction (data not shown). Therefore, DsRAD likely exists naturally in high-molecular-weight RNP complexes in neural cells.

Active component in DsRAD-associated RNP complexes

To gain knowledge of the enzymatic component(s) of the DsRAD-associated RNP complexes, we fractionated the brain nuclear extract in a Superose-12 gel filtration column (Pharmacia) under low or high salt conditions. In low salt, the bulk of DsRAD-associated materials had apparent molecular mass of up to 500 kDa, beyond the resolving range of Superose-12. A small fraction of DsRAD was found around 60–70 kDa region (Figure 6A). In the presence of 0.3 M KCl, DsRAD-associated complexes were partially dissociated to give increased activity in the 70 kDa region and a broad distribution of activity between 200 and 480 kDa (Figure 6B). However, the elution profiles of DsRAD in gel filtration columns are surprisingly broad and variable as compared to the protein standards. This may be due to binding of DsRAD to the chromatographic matrix through hydrophilic interactions which retard its elution from the column. Similar results were obtained using purified DsRAD-associated RNP as the starting materials.

When the brain nuclear extract was fractionated by poly(I).poly(C)-affinity column chromatography as described for the bovine liver DsRAD (Kim et al, 1994a), the highest DsRAD activity was eluted in 3 M KCl just like the bovine liver enzyme, and lower activities were detected in the flow through, 0.5 and 2 M KCl fractions as well (Figure 7A). The materials eluted in 3 M (KCl) were repurified by poly(I).poly(C)-affinity column chromatography and further fractionated in a 10–30% glycerol gradient. DsRAD activities in the glycerol gradient fractions were assayed (Figure 7B) and proteins in the fractions were precipitated with trichloroacetic acid (TCA) and analyzed by polyacrylamide gel elec-

trophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (Figure 7C). A major protein (about 120 kDa) and a minor protein (about 70 kDa) were copurified with DsRAD activities (Figures 7B and C, lanes 8–13). The 120 kDa protein species was also observed in the 0.5 and 2 M KCl fractions from the poly(I).poly(C) column (data not shown). Several faint bands below the 120 kDa protein were also seen in the fractions with highest DsRAD activity (Figure 7C, lanes 9–11). The 120 and 70 kDa protein species and the fainter bands below the 120 kDa protein were invariably co-purified upon further fractionation by gel filtration and Heparin column chromatography (data not shown). We were unable to prevent the appearance of multiple protein species by including the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, and pepstatin A, in the protein purification scheme.

These results indicate that DsRAD in neural tissues exists in high-molecular-weight RNP complexes. The RNA component is non-essential for the enzyme activity, at least under *in vitro* assay conditions. One or more of the protein species ranging from 120 to 70 kDa constitute the enzymatic component.

Discussion

The present study shows that DsRAD activity in bovine brain is associated with protein species with apparent molecular mass ranging from 120 to 70 kDa. These values are in general agreement with the reported sizes of DsRAD purified from calf thymus (116 kDa; O'Connell and Keller, 1994), bovine liver (93, 88, and 83 kDa; Kim *et al*, 1994a), and Xenopus laevis (120 kDa; Hough and Bass, 1994). The human DsRAD gene has recently been cloned by two groups. It encodes a single protein of 1226 amino acid residues with a calculated molecular mass of 139 kDa (Kim *et al*, 1994b; O'Connell *et al*, 1995). A single DsRAD RNA species of about 7 kb is detected in all human tissues analyzed (Kim *et al*, 1994b; O'Connell *et al*, 1995). Assuming the bovine brain DsRAD gene encodes a similar protein, the 120-kDa protein corresponds well with the expected size of bovine DsRAD, and the smaller protein species may be degradative products similar to those purified from bovine liver (Kim *et al*, 1994a). Like DsRAD purified from other tissues, DsRAD from bovine brain retains activity even after extensive dialysis against o-phenanthroline and thus appears to have no cofactor requirements. In fact, Zn^{2+}, Cu^{2+}, Co^{2+}, and Fe^{3+} ions strongly inhibit the bovine brain DsRAD activity (data not shown). DsRAD monomers from different sources are enzymatically active and the bovine liver DsRAD shows first-order reaction kinetics (Kim *et al*, 1994a; Hough and Bass, 1994). However, the present study indicates that DsRAD does not exist as soluble monomers in neural cells. Instead, it resides almost exclusively in

intranuclear RNP complexes with apparent molecular mass of up to 500 kDa and buoyant density of 1.35 to 1.42 g cc^{-1} in CsCl solution. These findings have interesting implications for the possible mode of action of DsRAD *in vivo*.

DsRAD has been postulated to serve an RNA-editing function *in vivo*. In rat neural cells, DsRAD may be responsible for the editing of mRNA encoding the GluR-B subunit of the α-amino-3-hydroxy-5-methly-4-isoxazolepropionate receptor. Specifically, post-transcriptional mRNA-editing converts a specific A residue to G in the GluR-B subunit mRNA, producing a protein with an arginine instead of glutamine in the second transmembrane region which affects the Ca^{++} permeability properties of the protein (Sommer *et al*, 1991). A similar modification appears to occur in a human glutamate receptor subunit mRNA (Cha *et al*, 1994). DsRAD is believed to play a role in this mRNA-editing event. DsRAD activities and DsRAD-mRNA are found in a wide variety of tissues and cultured cells including neural cells (Wagner *et al*, 1990; Kim *et al*, 1994b). *In situ* hybridization has shown that DsRAD-mRNA is widely distributed in the rat brain (O'Connell *et al*, 1995).

An interesting feature of the post-transcriptional editing of the GluR-B gene is that GluR-B RNA sequences expressed from a cDNA clone are not edited (Higuchi *et al*, 1993). Specific editing of the GluR-B subunit mRNA requires inverted complementary exon and intron sequences which can form a potential duplex. Mutations that disrupt the base-pairing curtail editing, and complementary mutations that allow base-pairing restore editing (Higuchi *et al*, 1993). These features, and the finding of additional A-to-G changes in the intron, strongly suggest that the FluR-B mRNA is edited shortly before or during mRNA splicing, since the intron sequences essential for proper editing are absent in the mature mRNA. In light of the extraordinarily high efficiency of the editing event in neural cells (>99% of the endongenous GluR-B mRNA and 30–50% of GluR-B mRNA expressed from a cloned gene by transfection; Higuchi *et al*, 1993) and the low abundance of DsRAD (0.005% of total proteins; Kim *et al*, 1994a), it seems unlikely that soluble monomeric DsRAD can adequately accomplish the mRNA-editing function. Furthermore, the size of monomeric DsRAD cannot explain the need for a relatively long RNA duplex (over 100 base-pairs) to perform the deamination function efficiently *in vitro* (Nishikura *et al*, 1991). These observations suggest that DsRAD may normally function in a higher order complex. An intriguing possibility is that DsRAD may interact with spliceosomal complexes and performs the editing function during mRNA splicing. Coupling the RNA-editing mechanism to the mRNA splicing machinery may allow the enzyme to achieve the high efficiency and specificity required for the RNA-editing function.

The apparent lack of sequence specificity of DsRAD enzyme *in vitro* may be due to uncoupling of the components responsible for the deamination reaction and target specificity. Consistent with this hypothesis, immunofluorescence studies show that DsRAD is localized in granulated structures in the nuclei but is excluded from the nucleolus, where rRNA rather than mRNA is synthesized (O'Connell *et al*, 1995). Interestingly, the human DsRAD predicted from the nucleotide sequences contains a partial RNP core consensus sequence found in many single-stranded RNA binding proteins (Kim *et al*, 1994b). It is tempting to speculate that this sequence may interact with the RNA component of the DsRAD-associated RNP complexes.

DsRAD is thought to be involved in biased hypermutation of MV strains that infect the CNS (Bass *et al*, 1989; Cattaneo *et al*, 1989). Hypermutation also occurs in other RNA viruses. Recently, an intriguing example of hypermutation was found in a defective recombinant avian retrovirus containing retroviral splice sites and sequences that can form a potential stem and loop structure. Remarkably, a non-retroviral construct carrying similar potential stem-and-loop sequences but lacking known splice sites appeared not to be susceptible to hypermutation (A Hajjar and M Linial, personal communication). These results support the hypothesis that RNA splicing may play a role in hypermutation of retroviral RNA. Unlike retroviruses, MV replicates in the cytoplasm and does not utilize the cellular mRNA-splicing machinery. Furthermore, the RNA genome of MV is encapsidated by the viral nucleocapsid protein and should not form duplexes (Norrby and Oxman, 1990). Thus, the precise mechanism of hypermutation in MV remains to be elucidated. In any event, hypermutation of MV appears to be a far rarer event than editing of the GluR-B subunit mRNA. This is best illustrated in the Yamagata strain MV passaged in neuroblastoma cells, which has acquired numerous A-to-G transitions strictly within a 400-nucleotide region in its M gene (Wong *et al*, 1989). The remarkably localized nature of these mutations indicate that the mutations did not accumulate slowly over many rounds of virus replication, but were all introduced in a single rare event. The normally cytoplasmic localization of MV and the lack of splicing in the MV mRNAs may explain the rarity of hypermutated MV mutants, which may be selected during persistent infection of the CNS.

Materials and methods

Preparation of nuclear and whole-cell extracts
Nuclear extract was prepared from bovine brains (Pel-Freez Biologicals) by the method described by Gorski *et al* (1986) with modifications as follows. A bovine brain (about 700 g) stored at −80°C was smashed into small pieces and quickly thawed in 1600 ml phosphate buffer saline (PBS). The thawed pieces were collected by centrifugation at 4,000 × g at 4°C for 10 min and homogenized in one volume of packed cell (PCV) of homogenization buffer [25 mM Tris-HCl, pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol and 2 M sucrose] by 6 or 7 strokes in a Dounce (Bellco, New Jersey) homogenizer with a B pestle. The homogenate was diluted with one PCV of the homogenization buffer and centrifuged at 85,000 × g at 4°C for 30 min. The nuclear pellet was collected and suspended in 530 ml nuclear extraction buffer [50 mM Tris-HCl, pH 7.9, 10 mM KCl, 5 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF. One-tenth volume of saturated $(NH_4)_2SO_4$ was added while mixing and the mixture was incubated at 0°C for 1 h. The nuclear extract was cleared of debris by centrifugation at 85,000 x g at 4°C for 1 h, and the proteins were precipitated by adding 0.35 g ml^{-1} solid $(NH_4)_2SO_4$ and stirring for 30 min at 0°C. After centrifugation at 22,000 × g at 4°C for 15 min, the pellet was resuspended in 45 ml of 20 mM Tris-HCl, pH 7.9, 10 mM KCl, 5 mM EDTA, 10% glycerol and dialyzed overnight at 4°C against two changes of 1,800 ml of the same buffer. The final extract was cleared by centrifugation at 85,000 × g at 4°C for 30 min and aliquots were kept at −80°C.

Whole cell extract was prepared from HTB-14, a malignant glioma cell line, by suspending the cells in the nuclear extraction buffer and bursting the cells by addition of one-tenth volume of saturated $(NH_4)_2SO_4$. The cell extract was processed by the same procedure as outline above.

Nuclear extracts were also prepared from HTB-14 cells and bovine brain under low salt condition as described by Dignam *et al* (1983) and Kay *et al* (1972) with modification as follows. HTB-14 cells were harvested from cell culture media by centrifugation for 5 min at 700 × g and washed twice in five volumes of PBS at 4°C and once with 10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1 mM EDTA, and 0.5 mM DTT. The cells were suspended in five PCV of the same buffer and allowed to stand for 10 min and lysed by 10 strokes in a Dounce homogenizer with a B type pestle. The homogenate was centrifuged at 700 × g at 4°C for 15 min. The crude nuclear pellet was collected and resuspended in 2.4 M sucrose with 25 mM Tris-HCl, pH 7.9, 10 mM KCl, and 1 mM EDTA. The mixture was centrifuged at 100,000 × g at 4°C for 60 min to purify the nuclei. Nuclei were prepared from bovine brain as outlined above but further centrifuged through a second 2.4 M sucrose cushion. The final nuclear pellet was drained and resuspended in 10 mM Tris-HCl, pH 7.9, 0.1 mM MgCl$_2$, 5 mM 2-mercaptoethanol, 0.29 M sucrose, and 0.25 mM PMSF. The purified nuclei were centrifuged at 1000 × g at 4°C for 5 min and suspended in 2 ml of the same buffer containing 1 μg ml^{-1} freshly dissolved DNase I. The mixture was digested for 15 min at 22°C and the digested

304

materials were diluted with an equal volume of ice cold water and spun for 15 min at 2000 × g at 4°C. The supernatant was saved. The pellet was redigested with the same buffer containing DNase I. The supernatants from both digested samples were pooled and cleared by centrifugation at 100,000 × g at 4°C for 30 min and concentrated by Centriprep-10 (Amicon) to 0.5 ml for analysis by 10–60% sucrose gradient.

DsRAD assay

DsRAD assay was performed as previously described (Rataul et al, 1992) with the following modifications. Reactions were carried out at 37°C for 2–3 h in 60 ml 50 mM potassium phosphate buffer, pH 7.5, 2 mM DTT, 0.5 mM GTP, 0.25 mM $MgCl_2$ 0.25 mM $CaCl_2$, 20% glycerol, 15 μg yeast tRNA, 0.2% Brij-35, and 5 or 50 ng of $[\alpha\text{-}^{32}p]ATP$-labeled RNA duplex consisting of plus- and minus-sense RNA representing the 5' region of the matrix gene of Edmonston strain measles virus (Rataul et al, 1992). Samples containing a high concentration of salt were desalted by concentrating to less than 5 μl in a Microcon-10 concentrator (Amicon) and replacing with low salt buffer (LSB: 20 mM Tris-HCl, pH 7.9, 5 mM KCl, 1 mM $MgCl_2$, and 15% glycerol). The reactions were stopped by addition of 100 μl of 0.8 mg ml^{-1} proteinase K in 0.1 M Tris-HCl, pH 7.5, 5 mM EDTA, and 0.5% SDS. After incubation at 37°C for 30 min, RNA was extracted with phenol and chloroform, and precipitated with ethanol. The RNA products were analyzed by electrophoresis in non-denaturing 4% polyacrylamide gel (PAGE).

To determine the A-to-I ratios in the products, the reaction mixture was extracted directly with phenol and chloroform and precipitated with ethanol. RNA was resuspended in 55 μl of 50 mM NH_4OAc, pH 5.3, 0.2 mM $ZnCl_2$, heated at 95°C for 3 min and chilled to 0°C quickly. Nuclease P_1 (3 U) and 0.1% Brij-35 were added to a total volume of 60 μl. The mixture was incubated at 37°C for 5 h to overnight. The digested samples were extracted by phenol and chloroform, dried in a Speed Vac concentrator, and resuspended in 5 μl water. The resulting mononucleotides were chromatographed in one dimension on a cellulose TLC plate with saturated $(NH_4)_2SO_4$-01 M sodium acetate, pH 6.0-isopropanol (79:19:2, by volume) as solvent (Bass and Weintraub, 1988). Quantitative image analysis was performed with a Molecular Dynamics Model 400E PhosphorImager using Image Quant Software (Molecular Dynamics, Palo Alto, California).

Sucrose gradient analysis

Bovine brain nuclear extracts (0.2–0.5 ml) were centrifuged through a 10–60% (w/w) linear sucrose gradient containing 20 mM Tris-HCl, pH 7.9, 10 mM KCl, 1 mM $MgCl_2$ in Beckman SW-55 rotor at 240,000 × g at 4°C for 20 h or SW-41 rotor at 180,000 × g at 4°C for 22 h. For equilibrium density centrifugation, the gradients were centrifuged in a Beckman SW-55 rotor (Beckman Instruments, Fullerton, California) at 240,000 × g at 4°C for 65 h. The gradients were fractionated from the bottom and the fractions were assayed for DsRAD activity.

Analysis of radiolabeled cellular RNA

A culture containing 3×10^7 HTB-14 cells was labeled with 0.3 mCi [³H]uridine for 15 h. The whole-cell extract was concentrated to 0.6 ml by Centriprep-10 and centrifuged through a 10–60% (w/w) linear sucrose gradient in a Beckman SW-41 rotor as described above. RNA was extracted from 100 μl aliquots from each fraction by phenol and chloroform and precipitated with ethanol. The radioactivity in each fraction was quantified by a scintillation counter.

Buoyant density determination of DsRAD in CsCl gradient

CsCl gradient centrifugation was performed using conditions for analysis of small nuclear RNP and the apolipoprotein B mRNA-editing enzyme (Christofori and Keller, 1988; Greeve et al, 1991). Briefly, 0.5 ml of the nuclear extract was adjusted to a total volume of 2.5 ml containing 40 mM Tris-HCl, pH 7.9, 15 mM $MgCl_2$, 5 mM DTT, and 1.45 g cc^{-1} CsCl with or without 20% glycerol. This was overlaid by 2.5 ml of 1.33 g cc^{-1} CsCl in the same buffer, and centrifuged until equilibrium in a Beckman SW-55 rotor at 200,000 × g at 4°C for 36 h. Because the inclusion of 20% glycerol affected the refractive index, density of the collected fractions was determined directly by weighing. The fractions were desalted by extensive dialysis against several changes of a buffer containing 40 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 1 mM DTT, and 20% glycerol at 4°C over 24 h. Aliquots of the fractions were assayed for DsRAD activity.

RNase A analysis of DsRAD

The crude nuclear extract (200–400 μl) was incubated at room temperature with 17.5 mg ml^{-1} of polyacrylamide beads coupled with RNase A (100 units g^{-1}, RNase A Enzygel, Boehringer Mannheim, Indianapolis) that had been prewashed extensively with a buffer containing 40 mM Tris-HCl, pH 7.9, 0.2 mM $MgCl_2$, 15% glycerol, 0.2% Brij-35. At various time points, the beads were removed by pelleting, and the supernatant was assayed for DsRAD activity in the presence of 22 units of RNasin RNase inhibitor (Promega Biotec, Promega Corp, Madison, Wisconsin). For sedimentation analysis, the nuclear extract was digested with RNase A Enzygel for 1 h. After removing the beads, the digested extract was centrifuged through 10–60% (w/w) sucrose linear gradient in 20 mM Tris-HCl, pH 7.9, 10 mM KCl, 1 mM $MgCl_2$ in a Beckman SW-41 rotor at 180,000 × g at 4°C for 22 h, and the collected fractions were

assayed for DsRAD activity.

Purification and characterization of DsRAD from bovine brain

The purification of DsRAD from bovine brain was performed as described by Kim *et al* (1994a). The nuclear extract prepared from 300 g of bovine brain as outlined above was precipitated by 0.3 g ml^{-1} solid $(NH_4)_2SO_4$ at 0°C for 30 min. The pellet collected by centrifugation at 85,000 × g at 4°C for 30 min was dissolved in 40 ml of buffer A (20 mM Hepes-HCl, pH 7.5, 5 mM EDTA, 150 mM KCl, 15% glycerol, 1 mM DTT, 0.25 mM PMSF) and dialysed against 1800 ml of the same buffer at 4°C overnight. After clearing at 85,000 × g at 4°C for 30 min, the extract was adjusted to 0.35 M KCl and passed through a ssDNA cellulose column (2.6 × 7.0 cm) equilibrated with buffer A containing 0.35 M KCl at 0.9 ml min^{-1}. The flow through containing the enzyme activity was loaded onto a poly(I).poly(C) agarose column (2.6 × 7.0 cm) that had been equilibrated by buffer A with 0.35 M KCl. The poly(I).poly(C) column was washed sequentially with 100 ml buffer A containing 0.5, 1, 2 M KCl and 3 M KCl plus 0.2% Brij-35 at a flow rate of 0.9 ml min^{-1}. The 3 M KCl elution was pooled, diluted to 0.35 M KCl with buffer A containing 0.2% Brij-35 and loaded to the same column for a second cycle of poly(I).poly(C) agarose column chromatography. The column was eluted as the first cycle except that all the elution buffers contained 0.2% Brij-35. The fractions of the second poly(I).poly(C) column were concentrated by Centriprep-10. The concentrated sample was divided into three portions. Two hundred microliter of one portion of this sample was applied to a 11.5 ml 10–30% glycerol gradient in a buffer containing 50 mM Hepes-HCl, pH 7.5, 5 mM EDTA, 0.35 M NaCl, 1 mM DTT, 0.7 mg ml^{-1} pepstain A, 0.4 mg ml^{-1} leupeptin, and 0.25 mM PMSF. The gradients were spun at 180 000 × g at 4°C for 36 h

and fractionated from bottom. A second portion of this sample was dialysed against buffer B (40 mM Tris-HCl, pH 7.9, 0.2 mM $MgCl_2$, 15% glycerol, 0.2% Brij-35) to remove salt and then applied to a 5 ml Heparin HiTrap column (Pharmacia Biotech, Picataway, New Jersey) equilibrated with buffer B. Bound proteins were eluted with a 0–0.7 M NaCl gradient. A third portion was analysed by gel filtration column chromatography.

Size exclusion analysis of crude and partially purified DsRAD

Crude or partially purified DsRAD was analyzed by gel filtration in Superose-12 HR 10/30 column (Pharmacia) under low or high salt conditions. For low salt chromatography, the column was equilibrated with a buffer containing 25 mM Tris-HCl, pH 7.9, 50 mM KCl, 0.25 mM $MgCl_2$, 20% glycerol and 0.2% Brij-35 at a flow-rate of 0.4 ml min^{-1}. High salt chromatography was performed with the same buffer containing 300 mM KCl, or with a buffer containing 50 mM potassium phosphate, pH 7.5, 250 mM NaCl, 5 mM EDTA, 20% glycerol, 0.2% Brij-35, and 1 mM DTT. The eluted fractions (0.3 ml) were desalted by Microcon-10 concentrator (Amicon, Beverly, Massachusetts), replaced with LSB, reconcentrated and resuspended in LSB for DsRAD analysis. The relationship between retention time and molecular mass was calibrated with jack bean urease hexamer (545 kDa) and trimer (272 kDa), bovine albumin dimer (132 kDa) and monomer (66 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa), and a-lactalbumin (14.2 kDa).

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306

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