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Purification of Native and Recombinant Double-Stranded RNA-Specific Adenosine Deaminases

Mary A. O'Connell,*,[1] André Gerber,† and Liam P. Keegan*

*MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom; and †Department of Cell Biology, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

ADAR1 and ADAR2 are members of a family of enzymes that catalyze the conversion of adenosine to inosine in double-stranded RNA. Unlike the other types of RNA editing that involve multiprotein editing complexes, the site-specific deamination of an adenosine to inosine is catalyzed by single enzymes. ADAR1 and ADAR2 have been purified and the genes cloned from various sources. Each gene encodes multiple splice variants. As it is crucial to have an adequate supply of pure protein to investigate this type of RNA editing, we describe in this article methods for both the purification and the overexpression of either full-length or partial ADAR1 and ADAR2 isoforms.

The different types of RNA editing are catalyzed by diverse multicomponent protein complexes. To date the only exceptions to this are the adenosine deaminases that act on RNA (ADAR1 and ADAR2) (1). Both catalyze site-selected hydrolytic deamination of adenosine to inosine in particular precursor mRNAs (pre-mRNAs) [see reviews (2–4)]. In the target pre-mRNAs that have been identified so far, the site specificity of editing is dependent on intramolecular base pairing between exon sequences containing the editing site and a sequence within the adjacent 3′ intron to form a double-stranded (ds) RNA structure. Mutations disrupting the base pairing interfere with editing, whereas mutations restoring complementarity also restore editing (5, 6). Inosine is read as guanosine by the translational machinery (7); therefore, the site-selected deamination of adenosine to inosine changes the amino acid incorporated at functionally critical sites in the encoded proteins (8–10).

Site-specific editing by ADAR1 and ADAR2 was first observed in the pre-mRNAs encoding subunits of the glutamate gated ion channel receptors (GluR) (5, 11). In one case ADAR1 converts the adenosine to inosine at hotspot 1 in intron 11 of GluR-B pre-mRNA (12–14). However, ADAR2 converts an adenosine several nucleotides away at the Q/R (Glu/Arg) site in exon 11, resulting in the incorporation of arginine at this position. Both enzymes are capable of the conversion of the same adenosine at the R/G (Arg/Gln) site in exon 13 of GluR-B pre-mRNA. These enzymes therefore have overlapping substrate specificities. The ability to edit the adenosines at either the Q/R or hotspot 1 site is the basis of the specific assay for either ADAR2 or ADAR1.

ADAR1 and ADAR2 are members of a family of adenosine deaminases the genes of which encode highly similar proteins. Each member contains dsRNA-binding domains and a deaminase domain. There exists different spliced variants of human (h) ADAR1 and hADAR2 (Fig. 1). Three variants of hADAR1 (a, b, c) (15) have been found in cDNA libraries, but it has not been established whether these

[1] To whom correspondence should be addressed. Fax: 44–131–343–2620. E-mail: Mary.O'Connell at hgu.mrc.ac.uk.

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spliced variants are actually expressed as proteins (15). The most abundant cDNA form is ADAR1a and the purification of bovine (b)ADAR1a is described below.

Of the four spliced variants of hADAR2 found (14, 16, 17) (see Fig. 1), it appears that the transcript hADAR2c, lacking an inserted *Alu* sequence and containing the short C terminus, is the most abundant form in HeLa cells (14) (Fig. 1). The two spliced variants hADAR2c/d, which have the short C terminus, cannot deaminate the adenosine at the Q/R site. The specific assay used to purify hADAR2 from HeLa cells was the ability to convert this adenosine; therefore, the presence of these spliced variants was

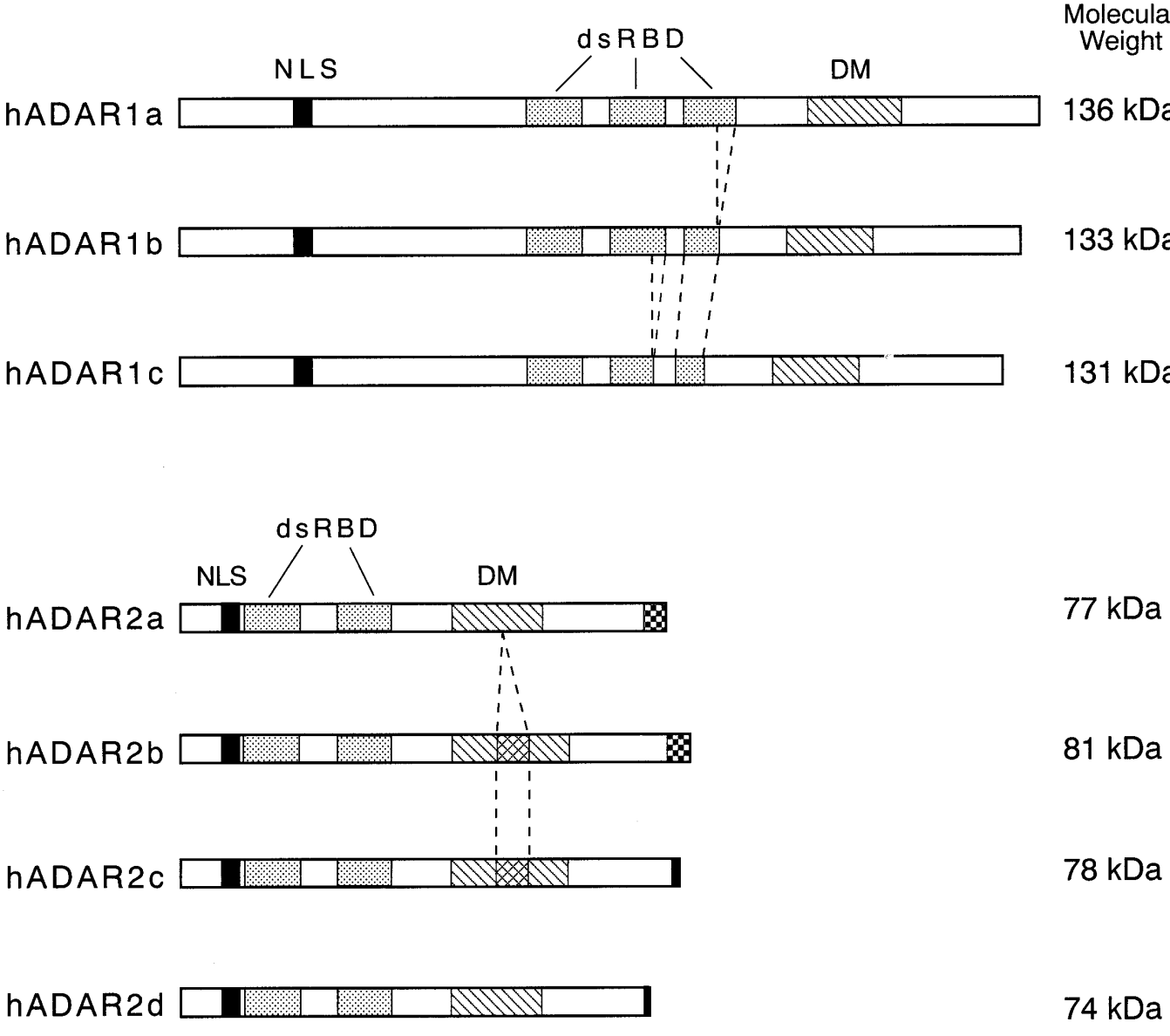

FIG. 1. Schematic diagram of the different spliced variants of hARAD1 and hADAR2. hADAR1 contains a nuclear localization sequence (NSL) at its N terminus, three dsRNA-binding domains (dsRBD), and a deaminase domain (DM). The three different isoforms of hADAR1 differ in their dsRBD; the third dsRBD of hADAR1b is lacking 26 amino acids present in hADAR1a, and hADAR1c is lacking 19 amino acids from the second dsRBD. hADAR2 has a configuration similar to that of hADAR1. hADAR2b/c have an inserted *Alu* sequence within their deaminase domain and hADAR2c/d have a shorter C terminus.

not assayed for in the purification (13). Only one form of hADAR2 was purified from HeLa cells, but as this was not subjected to amino acid sequencing, it is not yet certain whether this form is hADAR2a or hADAR2b. Hence in the purification described below the protein is referred to as hADAR2.

hADAR1a and hADAR2 are not very abundant proteins (13, 18), and so far the other spliced variants have been detected only at the level of RNA and not protein (14–17). Therefore it is necessary to overexpress the spliced variants of ADAR1 and ADAR2 in an expression system to investigate the functional differences between the different protein products. This article describes the overexpression of full-length hADAR2a/b in the yeast *Pichia pastoris* (16). The full-length protein overexpressed in *Escherichia coli* was nonfunctional but the protein was valuable for the generation of antibodies (13) and is also described briefly.

DESCRIPTION OF THE METHOD: GENERAL GUIDELINES FOR PURIFICATION OF ADAR1a OR ADAR2

Even though ADAR1a and ADAR2 have been purified to homogeneity from a variety of sources (13, 18–21), it is still a formidable task as these enzymes are present at low levels, are sensitive to losses in activity, and require very extensive purification from large amounts of starting material. Before commencing the purification, it is necessary to ensure that ADAR1a or ADAR2 is expressed to reasonable levels in the starting material; the enzyme should be assayed from the chosen source and compared with another source, for example, HeLa cell nuclear extract.

The simplest assay procedure should be chosen to assay for the activity of these enzymes as this will significantly reduce the work involved in the purification. If only one enzyme is present, then it is simplest to assay for the conversion of adenosine to inosine by non-sequence-specific deamination in a long artificial dsRNA (18). In brief, complementary RNA strands are transcribed *in vitro* with the incorporation of [α-^{32}P]ATP and annealed together to form dsRNA, which is then gel-purified and incubated with extract containing deaminase activity. The dsRNA is afterward digested with P1 nuclease, and the resulting mononucleotides are chromatographed on thin-layer chromatography (TLC) plates. Inosine has a different migration rate than adenosine.

If both ADAR1 and ADAR2 are present, it is necessary to perform a specific assay for whichever enzyme is being purified. If antibodies are available that can distinguish between the enzymes, it is then possible to monitor the purification by Western blot analysis. A word of caution: if Western blot analysis is used to follow the purification procedure, it is necessary to use an activity assay in conjunction with it. Specific assays that are routinely used are primer extension analyses that can distinguish between ADAR1 and ADAR2 by the ability of the primer to bind near either the Q/R or hotspot 1 site of GluR-B (13, 16).

All steps in the purification should be carried out at 4°C. The flow rate of the columns is generally one column volume per hour, unless the manufacturer recommends a higher flow rate. The fraction size is adjusted so that approximately 100 fractions are collected per column and usually every third fraction is assayed. Aliquots of fractions are used in the activity assay, whereas the fractions themselves are frozen immediately in liquid nitrogen and not thawed until they are required for the next column. The aliquots to be assayed are dialyzed against buffer A [50 mM Tris–HCl (pH 7.9), 5mM EDTA, 10% (v/v) glycerol] containing 200 mM KCl so that the final concentration in the assay is 100 mM KCl. Microdialysis dishes are very convenient for dialyzing small aliquot volumes and are available from Millipore. These dishes are similar to microtiter dishes and float on the surface of the buffer. They contain 96 slots, and it is possible to dialyze 75 μl/slot. Two hours of dialysis is normally sufficient.

Tissue extracts contain higher levels of RNases than do nuclear extracts. To partially overcome this problem addition of a general RNase inhibitor, for example, RNAguard (Boehringer-Mannheim), to the activity assays in the initial stages of purification is recommended. Degradation of the RNA substrate is also reduced if the assay is performed at a lower temperature (30°C versus the optimum 37°C). It is not possible to accurately calculate the total units from the starting pool due to the presence of RNases, and often the total units increase after the first column. When the pure protein is being assayed, it is also beneficial to include bovine serum albumin

(BSA 0.2 mg/ml) in the assay to increase the stability of the enzymes.

The principal buffer used in the purification is buffer A. The concentration of KCl varies and that is indicated below. Protease inhibitors are added to all buffers: 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.7 μg/ml pepstatin, and 0.4 μg/ml leupeptin. It is also essential to add a 1 mM concentration of the reducing agent dithiothreitol (DTT). All buffers contain 10% glycerol. Pure hADAR2 is not as stable as bADAR1a so the concentration of glycerol is increased to 20% for chromatography over the final column. Small aliquots of the pure ADAR2 are made to decrease the number of freeze–thaw cycles that inactivate the enzyme. At later stages in the purification when the total concentration of protein is low, it is necessary to add 0.02% Nonidet-P40 (NP-40) to all buffers to increase protein stability.

PURIFICATION OF bADAR1a FROM CALF THYMUS

Even though ADAR1a has been purified by four groups from a variety of sources (18–21), a review of the different purification procedures immediately reveals the similarities between the purification schemes. Ion-exchange chromatography was followed by affinity chromatography. The purification from calf thymus extract is reviewed in this article, a very brief synopsis of the other purification schemes is given, and the cited references should be referred to for further details.

ADAR1 has been purified from *Xenopus laevis* eggs but it should be noted that 4.7 liters of *Xenopus* eggs is required to obtain 6 μg of the pure 120-kDa protein (19). Bovine liver nuclear extracts have also been used; 1 kg of liver yields 5 g of nuclear extract, which after purification gives 80 μg of protein (20). Unfortunately, this source does not give a full-length protein but a mixture of three active proteolytic forms of 93, 88, and 83 kDa. Purification from chicken lung extracts yields a protein of 140 kDa, which is probably full-length; 200 g of tissue yields approximately 10 μg of protein (21).

Purification from 2 kg of calf thymus yields 3–6 μg of ADAR1, which has a molecular mass of 116 kDa (Fig. 2) (18). Even though this yield is not very high, one advantage of using calf thymus is that it is a cheap and readily available source of raw material. The protein is purified from whole-cell extract, not nuclear extract, as the nucleus in the thymus is very large and takes up most of the cell volume. The calf thymus obtained from freshly slaughtered animals is dropped in liquid nitrogen and stored frozen at −70°C.

Experimental Procedure

A summary of the purification is given in Table 1. The enzyme is purified from 2 kg of calf thymus by chromatography over six columns. One kilogram of calf thymus is thawed in 2 liters of buffer A/100 mM

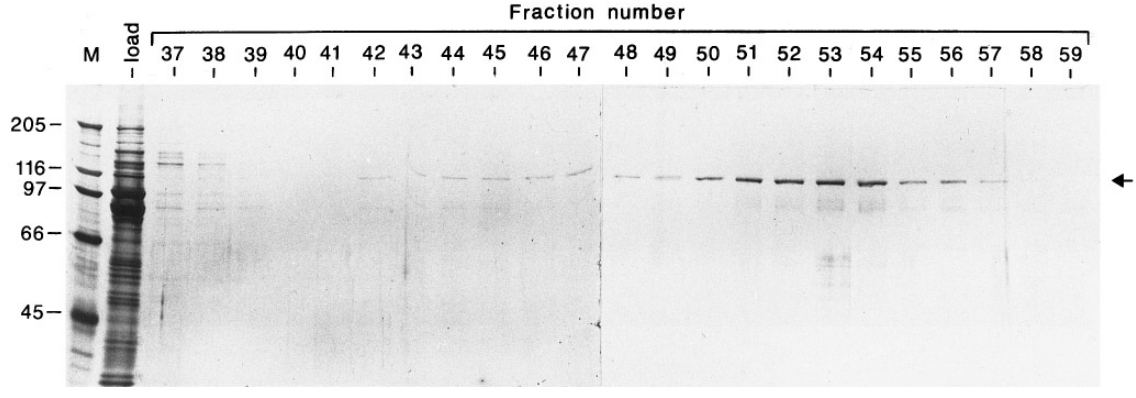

FIG. 2. Affinity chromatography of bADAR1a on poly(G)·poly(C). Column fractions (150 μl) were precipitated with 15% TCA, electrophoresed on an SDS/8% polyacrylamide gel, and visualized by silver staining. The arrow on the right points to bADAR1a, which is 116 kDa; the molecular masses (in kDa) of the molecular weight markers are on the left. Reproduced with permission from O'Connell, M.A., and Keller, W., 1994, *Proc. Natl. Acad. Sci. USA* **91,** 10596–10600. Copyright (1994) Nationl Academy of Sciences, USA.

KCl, homogenized in a Waring blender for 1.5 min, and then centrifuged for 1 h at 15,000*g*. The supernatant is poured through eight layers of cheesecloth and loaded onto a 4-liter column of DEAE–Sepharose Fast Flow (Pharmacia) that has been previously equilibrated in this buffer. Once the supernatant has been loaded, the column matrix is stirred with a metal rod as lipid from the tissue forms a layer on top of the column matrix and prevents the buffer from entering the matrix uniformly. The column is then washed with 1 column volume of buffer A/100 mM KCl; the bADAR1a activity is eluted with a 10-liter gradient from 100 to 500 mM KCl, with the peak of activity at approximately 300 mM KCl. The conductivity of the pool is checked, adjusted to 300 mM KCl, and the pool is loaded directly on a preequilibrated 1-liter Blue Sepharose column (Pharmacia). This column is washed with 2 column volumes of buffer A/300 mM KCl and developed with a 4-liter gradient from 300 to 1000 mM KCl, with the peak of bADAR1a activity eluting between 480 and 770 mM KCl. A second kilogram of calf thymus is treated in a similar fashion, and both active pools from the Blue Sepharose are combined. $CaCl_2$ (10 mM) is added to the bADAR1a pool to neutralize the 5 mM EDTA that is present before it is loaded on a 40-ml Ultrogel hydroxyapatite column (IBF Biotechnics). This column is preequilibrated in buffer B [20 mM Tris–HCl (pH 7.9), 50 mM KCl, 10% (v/v) glycerol, 1 mM DTT, protease inhibitors], washed with 2 column volumes, and developed with a 400-ml gradient from 0 to 500 mM potassium phosphate (pH 7.9). The peak of activity elutes between 90 and 185 mM potassium phosphate.

The advantage of running the three columns in this order is that dialysis is not required, and considering the large volumes involved, this is a great advantage. After chromatography over hydroxyapatite, however, the bADAR1a pool is dialyzed as the phosphate buffer is not suitable for heparin–Sepharose chromatography. The active pool is precipitated with ammonium sulfate (55% saturation), as the protein concentration is rather low, and subsequently dialyzed against buffer A/50 mM KCl. One problem with ammonium sulfate precipitation is that it inhibits bADAR1a activity and it is difficult to eliminate the salt despite extensive dialysis. Therefore, as a result of the residual ammonium sulfate, total units are often higher after heparin–Sepharose chromatography than before.

After dialysis, the bADAR1a pool is applied to a preequilibrated 10-ml heparin–Sepharose column (Pharmacia). The column is washed with 2 column volumes and developed with an 80-ml gradient from 50 to 500 mM KCl. The activity elutes between 150 and 220 mM KCl. The conductivity of the active bADAR1a pool is checked and adjusted to 50 mM KCl by addition of buffer A minus KCl. NP-40 (0.02%) is added to the active pool and to all buffers. The active pool is then loaded onto a preequilibrated 1-ml Mono Q column (Pharmacia), washed with 13 ml buffer A/50 mM KCl, and developed with a 30-ml gradient from 50 to 500 mM KCl, and the peak of activity elutes between 150 and 230 mM KCl. Unfortunately, Mono Q chromatography of bADAR1a is not highly reproducible, and bADAR1a is sometimes found in the flow-through fraction. This is not due to overloading of the column as the same result is obtained if less material is loaded. Neither is it the result of proteolysis as the same 116-kDa protein is purified either from the flowthrough fraction or from the bound material.

The final column is a 7-ml dsRNA affinity column of poly(G)·poly(C) (see below for details of affinity

TABLE 1

Purification Scheme for ADAR1a from Calf Thymus Whole-Cell Extracts

Fraction	Gradient	Peak of activity	Protein (mg)	Purification factor
Calf thymus extract			72,930	1
DEAE–Sepharose	100–500 mM KCl	~300 mM KCl	4,470	14
Blue Sepharose	300–1000 mM KCl	480–770 mM KCl	114.6	226
Hydroxyapatite	0–500 mM KH_2PO_4	90–185 mM KH_2PO_4	36.5	335
Heparin–Sepharose	50–500 mM KCl	150–220 mM KCl	12.6	1,163
Mono Q	50–500 mM KCl	150–230 mM KCl	2.9	3,314
ds poly(G)·poly(C)	300–1000 mM KCl	~600 mM KCl	0.003	341,731

column preparation). The conductivity of the active pool is adjusted to 300 mM KCl and applied to the preequilibrated affinity column. The column is washed with 14 ml buffer A/50 mM KCl and bADAR1a activity is eluted with a gradient of 300 to 1000 mM KCl; the peak of activity elutes at approximately 600 mM KCl. As shown in Fig. 2, a single protein with a molecular mass of 116 kDa is present in the active fractions. The protein concentration is very low, and for visualizing the protein on silver-stained gels, 100 μl of peak fraction is precipitated with trichloroacetic acid (TCA) to a final concentration of 15% and electrophoresed on an SDS/8% polyacrylamide gel.

dsRNA Affinity Chromatography

Two groups independently employed the same poly(G)·poly(C) affinity matrix for the purification of ADAR1a (18, 19). This matrix purified ADAR1a between 40- and 100-fold (Table 1). Poly(G) can form a four-stranded helical hydrogen-bonded complex (22), and this is probably the crucial feature that makes this particular affinity column so effective. Poly(G) alone competes against dsRNA substrates in bADAR1a activity assays, suggesting that ADAR1a binds to the unusual poly(G) structure. Poly(G) and poly(C) can either be annealed together and then coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) or be coupled first and then annealed together. It was found that the length of the poly(G) is important; it should be short (approximately 20 nucleotides). If long poly(G) is used, the column does not bind the enzyme efficiently. The length of poly(C) is not critical. To increase the efficiency of the dsRNA affinity column, one can pass the active ADAR1a pool first over a dsDNA–cellulose and then over a ssDNA–cellulose column (19). ADAR1a activity will be in the flowthrough of both columns but ssDNA-binding proteins will be removed that can otherwise also bind to the dsRNA affinity column and decrease its binding efficiency.

PURIFICATION OF hADAR2

hADAR2 has been purified from HeLa cell nuclear extract by a scheme similar to that described for bADAR1a (13). Ion-exchange chromatography is followed by two affinity chromatographic steps, first poly(I)·poly(C) and then poly(G)·poly(C). Unlike calf thymus, where bADAR1a is expressed to higher levels than bADAR2, in HeLa cell nuclear extract the levels of the enzymes are similar. Therefore, the activity assay used in the purification has to be specific for hADAR2.

The purification described below is for a large-scale preparation. The starting material is HeLa cell nuclear extract containing a total of 14.7 g of protein, which is the equivalent of approximately 2 kg of packed HeLa cells. This purification yields approximately 100 μg of pure protein, which illustrates the fact that this protein is not very abundant. Much smaller quantities of protein are sufficient for carrying out routine assays and unless large quantities of protein are required the purification scheme outlined should be scaled down.

Experimental Procedures

The buffer used in the purification is the standard buffer A (see above for details). The first two matrices used, Macro-Prep High Q (Bio-Rad) and Affi-Gel

TABLE 2

Purification Scheme for ADAR2 from HeLa Cell Nuclear Extract

Fraction	Gradient	Peak of activity	Protein (mg)	Purification factor
HeLa NXT			14,670	
Macroprep High Q	50–500 mM KCl	130–280 mM KCl	5,610	1
Affi-Gel Blue	300–1000 mM KCl	480–800 mM KCl	657	4
Hydroxyapatite	0–500 mM KH_2PO_4	90–160 mM KH_2PO_4	147	13
Mono S	50–500 mM KCl	190–330 mM KCl	51	28
Mono Q	50–500 mM KCl	50–280 mM KCl	33	34
ds poly(I)·poly(C)	500–2500 mM KCl	600–1800 mM KCl	1	1,034
ds poly(G)·poly(C)	250–1000 mM KCl	370–500 mM KCl	0.1	7,501

Blue (Bio-Rad), have binding properties very similar to those of DEAE-Sepharose and Blue Sepharose but are manufactured by a different company. A summary of the purification scheme is given in Table 2. HeLa nuclear extract is prepared by the standard method (23) and dialyzed in buffer A/50 mM KCl. The nuclear extract is applied to a preequilibrated 3-liter MacroPrep High Q column, washed with 1 column volume, and developed with a 10-liter gradient from 50 to 500 mM KCl. The peak of activity elutes between 130 and 280 mM KCl. The conductivity of the active hADAR2 pool is adjusted to 300 mM KCl and applied to a preequilibrated 600-ml Affi-Gel Blue column. The column is washed with 2 column volumes and then developed with a 3-liter gradient from 300 to 1000 mM KCl. The peak of activity elutes between 480 and 800 mM KCl. As the next column is Ultrogel hydroxyapatite (IBF Biotechnics), 10 mM $CaCl_2$ is added to the pooled fractions before loading. The buffer used with this matrix is buffer B (see above for details), and the active pool is applied to a preequilibrated 350-ml Ultrogel hydroxyapatite column, washed with 2 column volumes, and developed with a 2.8-liter gradient from 0 to 500 mM potassium phosphate. The peak of hADAR2 activity is between 90 and 160 mM potassium phosphate. Peak fractions are dialyzed against buffer C/50 mM KCl, which is the same as buffer A except that 50 mM Tris–HCl is replaced by 50 mM Hepes–KOH, pH 7.9. The hADAR2 fractions are loaded onto an 8-ml preequilibrated Mono S column (Pharmacia), washed with 3 column volumes, and developed with a 240-ml gradient from 50 to 500 mM KCl. The peak of activity elutes between 190 and 330 mM KCl and is then dialyzed against buffer A/50 mM KCl. To increase hADAR2 stability, 0.02% NP-40 is added to all buffers at this stage. The dialyzed hADAR2 pool is applied to an 8-ml preequilibrated Mono Q column (Pharmacia), washed with 2 column volumes, and developed with an 80-ml gradient from 50 to 500 mM KCl. Usually hADAR1a activity is found in the flowthrough and hADAR2 activity binds to Mono Q, but unfortunately this is not always the case, and hADAR2 activity can elute between 50 and 280 mM KCl. The conductivity of the Mono Q hADAR2 pool is then adjusted to 500 mM KCl and loaded on a 5-ml preequilibrated poly(I)·poly(C) column (Pharmacia), washed with 2 column volumes, and developed with a 40-ml gradient from 500 to 2500 mM KCl. hADAR2 activity elutes between 600 and 1800 mM KCl. The conductivity of the active pool is adjusted to 250 mM KCl by diluting with buffer A minus KCl. The glycerol concentration in the buffers is increased to 20% and the active pool is applied to a preequilibrated 6-ml poly(G)·poly(C) column (see above for details on preparation), washed with 2 column volumes, and developed with a 43-ml gradient from 250 to 1000 mM KCl. hADAR2 activity elutes between 370 and 500 mM KCl. Fractions should be collected as small as possible so that they are used once, as the pure hADAR2 is very sensitive to freeze–thaw cycles.

As can be seen in Fig. 3, hADAR2 is not homogeneous at this stage as two other unrelated HeLa proteins are also present in the active fractions. Unfortunately, one of these proteins is very abundant and has a molecular mass similar to that of hADAR2. To be able to visualize hADAR2, it is necessary to electrophorese the fractions on an SDS/7.5% polyacrylamide gel and to allow the 45-kDa molecu-

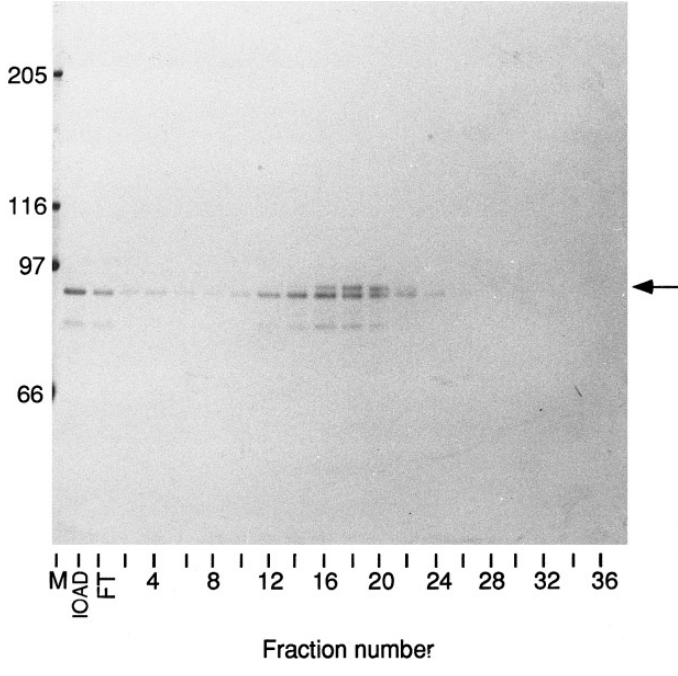

FIG. 3. Affinity chromatography of hADAR2 on poly(G)·poly(C). Aliquots of 15 μl of column fractions were electrophoresed on a large SDS/7.5% polyacrylamide gel and visualized by silver staining. The arrow on the right points to a protein of approximately 90 kDa, which is the upper part of a doublet. The molecular masses (in kDa) of the molecular weight markers are indicated on the left. It should be noted that the 45-kDa molecular weight marker was allowed to migrate out of the gel so that the 90-kDa protein could be seen. Reproduced with permission from O'Connell *et al.* (13).

lar mass marker to migrate out of the gel. It should then be possible to detect a 90-kDa protein whose abundance comigrates with enzymatic activity in different fractions.

OVEREXPRESSION OF PEPTIDE-TAGGED PROTEINS

As both ADAR1 and ADAR2 have been cloned, it is convenient to obtain pure protein by overexpressing the proteins in a recombinant system. To aid in their subsequent purification, it is advantageous to tag the recombinant proteins with one of the many epitope tags that are now commercially available. Commonly used are the FLAG and histidine hexamer epitope tags. The expression vectors have the peptide tags surrounded by convenient restriction sites to aid in the introduction of the cDNA. The tag can be attached to either the C or the N terminus of the protein, and it is often advantageous to have one at either end. A series of expression vectors are available for the three different open reading frames.

OVEREXPRESSION OF ADAR1 AND ADAR2: GENERATION OF ANTIBODIES IN *Escherichia coli*

Several members of the ADAR family containing a histidine epitope tag were overexpressed in *Escherichia coli*, and the full-length protein was detected by Western blot analysis with different antibodies in the whole-cell extracts. The recombinant proteins were partially purified by passage over a Ni^{2+}-nitrilotriacetic acid agarose (Ni^{2+}-NTA, Qiagen) affinity column but always failed to be catalytically active *in vitro*. Possibly some posttranslational modifications of the protein are missing or the folding is affected in *E. coli*. Nevertheless, the expression of fragments or domains of the proteins and subsequent purification under denaturing conditions generate suitable quantities of protein for antibody production (13, 24).

The extent of overexpression of proteins in *E. coli* is highly variable; therefore, it is advisable to subclone different regions of the protein to determine which region is expressed best. Often there are problems with expressing large proteins in *E. coli*, but small polypeptides of approximately 30 kDa are usually produced without difficulty. If possible more than one rabbit should be immunized with different antigens, as it is impossible to predict which domain will generate the best antibodies.

One expression system that is commercially available is pTrcHis Xpress from Invitrogen. The vector polylinker is 3′ of the histidine hexamer; therefore, the fusion protein contains the histidine hexamer at the N terminus. Also available from Invitrogen is a monoclonal antibody to the Xpress leader peptide that is useful for monitoring expression of the fusion protein.

Different domains of hADAR2a/b were subcloned into the appropriate sites of pTrcHis and used to transform the *E. coli* strain BL-21. A single transformed *E. coli* colony was picked and grown overnight at 37°C in 3 ml 2YT/ampicillin (25). Two hundred fifty milliliters 2YT/ampicillin was inoculated with 1:100 of the preculture (2.5 ml) and induced at A_{600} = 0.6 with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h at 37°C or for 7 h at 25°C. Cells were harvested by centrifugation and resuspended in 50 ml of lysis buffer [50 mM Tris–HCl (pH 7.9), 200 mM KCl, 10% glycerol, 1 mM PMSF, 10 μg/ml DNase I, 0.01% NP-40]. Lysozyme was added to a final concentration of 0.4 mg/ml. The suspension was mixed and incubated at room temperature for 15 min. Cells were sonicated on ice for 3 min (three pulses of 1 min at 40% power output with a 1-min break between each pulse) (Branson Sonifier 250). The lysate was centrifuged in a Sorvall GSA or SS-34 rotor at 4°C, 12,000 rpm for 30 min and the supernatant was directly mixed with 1 ml of a 50% slurry of Ni^{2+}-NTA agarose in buffer D [50 mM Tris–HCl (pH 7.9), 200 mM KCl, 10% glycerol, 0.01% NP-40, 1 mM 2-mercaptoethanol], adsorbed for 30 min on ice, and poured into a column. The column was washed and the proteins were eluted as described below. Aliquots from the fractions were analyzed by electrophoresis on an SDS–polyacrylamide gel stained with Coomassie Blue as well as by immunoblot analysis with anti-Xpress antibody 1:5000 (Invitrogen). If no contaminating proteins were present, then after dialysis against buffer A/50 mM KCl and concentration with Centricon 10 (Amicon), the protein was injected into rabbits. Although this protocol is suitable for purifying active protein, no enzymatic activity was obtained.

EXPRESSION IN EUKARYOTIC SYSTEMS

To obtain catalytically active recombinant ADAR proteins, several laboratories have chosen different mammalian expression systems (6, 12, 15, 26–28). The yield is often very low and this prompted us to seek more effective means of expressing active proteins.

We have been very successful in overproducing and purifying active hADAR1a (S. Krause, unpublished results), hADAR2a/b (16), as well as the related yeast deaminase Tad1p (HRA400; A. Gerber, unpublished results) using the yeast *Pichia pastoris*. *Pichia* has many of the advantages of higher eukaryotic expression systems, for example, protein processing, protein folding, and posttranslational modifications. It is faster and less expensive than the other eukaryotic expression systems, such as baculovirus systems and mammalian tissue culture. In addition, extracts from *Pichia* cells do not contain any measurable endogenous dsRNA-specific adenosine deaminase activity.

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. Two homologous alcohol oxidases encoded by the genes *AOX1* and *AOX2* perform the first step in this metabolic pathway by the oxidation of methanol to formaldehyde. The *AOX1* gene product is responsible for the majority of alcohol oxidase activity in the cell. The expression of the *AOX1* gene is regulated and induced by methanol and its promoter can be used to drive heterologous protein expression. The standard procedure involves substituting the *AOX1* gene on the chromosome with the gene to be overexpressed. Even though *AOX2* is highly homologous to *AOX1*, growth on methanol is much slower when only *AOX2* is functional. This phenotype allows the isolation of Mut^S (methanol utilization slow) strains that have the expression construct at the *AOX1* locus.

The following detailed protocol describes the hADAR2 expression but can be adapted to any RNA-specific adenosine deaminase as well (16). In general, the manufacturer's instructions were followed (Invitrogen, San Diego, CA). A detailed manual can be downloaded from the Internet at http://www.invitrogen.com/manuals.html.

Pichia pastoris Overexpression Constructs

The coding sequence of hADAR2a was amplified by polymerase chain reaction (PCR) with primers containing *Spe*I sites on their 5′ ends and subsequently cloned into this site in the polylinker of pBluescript KS (Stratagene). The constructs were sequenced and PCR errors are repaired by exchange with cDNA sequences. A hADAR2b construct was generated by introducing a *BstE*II/*Mlu*I fragment of the hADAR2b cDNA in the respective restriction sites of the hADAR2a construct. These preconstructs were further used for subcloning the coding sequences into *E. coli* or *P. pastoris* expression vectors.

The *Pichia* expression vector pHIL-D2 (Invitrogen) carries the *AOX1* promoter for high-level expression, the *HIS4* gene for selection, and the 3′ *AOX1* gene for targeted integration into the *Pichia* genome. In the single *Eco*RI restriction site of the pHIL-D2 shuttle vector we inserted an epitope-tagged cassette encoding an N-terminal FLAG epitope (Eastman Kodak), a *Spe*I restriction site, and a C-terminal histidine hexamer (Fig. 4). The FLAG epitope comprises the octapeptide DYKDDDDK, which is highly charged, promoting solubility of expressed proteins, and can be detected with a mouse anti-FLAG monoclonal antibody (Eastman Kodak). The amino acids DDDK specify an enterokinase cleavage site and thus allow removal of the tag. The histidine tag at the C terminus permits rapid purification of recombinant proteins with a Ni^{2+}-NTA agarose affinity column (Qiagen). The coding sequences of the different deaminases were subcloned into this epitope-tagged expression vector pSK-$FLIS_6$ via *Spe*I restriction sites. The resulting expression vectors pSK-FLIS6-hADARn were linearized with *Not*I and electroporated into the *P. pastoris* strain GS 115. The *Not*I linearized and transformed vector leads to gene replacement at the *AOX1* locus in GS115 by homologous recombination. In particular, this event arises from a double crossover event between the *AOX1* promoter and 3′ *AOX1* regions of the vector and the genome. The His^+ phenotype is achieved with introduction of the HIS4 gene, which is also encoded by the transformed plasmid.

Transformation and Selection

GS115 *his4* cells were grown in 100 ml yeast extract peptone dextrose medium (YPD: 1% yeast extract, 2% peptone, 2% glucose), at 230 rpm at 30°C until $A_{600} = 1–2$. The cells were centrifuged for 5 min at 3000 rpm, and the pellet was resuspended in 20 ml YPD. Two milliliters of sterile 1 M Hepes (pH 8.0) and 0.5 ml sterile 1 M DTT were added and

incubated at 30°C for 15 min with gentle shaking. The cells were washed three times with 100 ml cold sterile water and once with 5 ml of sterile 1 M sorbitol and centrifuged as above. Finally, the pellet was resuspended in 200 μl of 1 M sorbitol. One-half microgram of linearized, salt-free plasmid was mixed with 40 μl of the *P. pastoris* cell suspension and electroporated at 1.5 kV, 25 μF, 200Ω (Bio-Rad Gene Pulser) in a cold 0.1-cm electroporation cuvette. The time constant (t) was approximately 5. The transformation mix was spread on minimal dextrose plates without histidine (MD; 1.34% yeast nitrogen base, 4 $\times 10^{-5}$% biotin, 1% dextrose, 1.5% agar). After 2 days at 30°C single colonies were picked and replated on MD plates.

Screening for His^+/Mut^S phenotypes is performed by restreaking 50–200 independent colonies on MD and minimal methanol plates (MM; same as MD but containing 0.5% methanol instead of dextrose) and incubating at 30°C for 2–4 days. Colonies with normal growth on MD but slow growth on MM plates were considered His^+/Mut^S. Twelve His^+/Mut^S transformants were picked as single colonies from MD plates and grown in 50-ml pilot cultures in buffered glycerol–complex medium [BMGY containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4 $\times 10^{-5}$% biotin, 1% glycerol] at 30°C, 250 rpm. After 2 days the cells were pelleted by centrifugation at 3000g for 5 min and induced by resuspension in the same volume of buffered methanol–complex medium (BMMY: BMGY containing 0.5% methanol instead of glycerol). The cells were grown for another 2 days at 30°C, at 260 rpm. Liquid nitrogen extracts of the cells (see below) were tested for overexpression by immunodetection on Western blots with anti-FLAG M2 monoclonal antibodies (Eastman Kodak). The extracts can be further tested for their ability to convert adenosines to inosines on extended dsRNA (18).

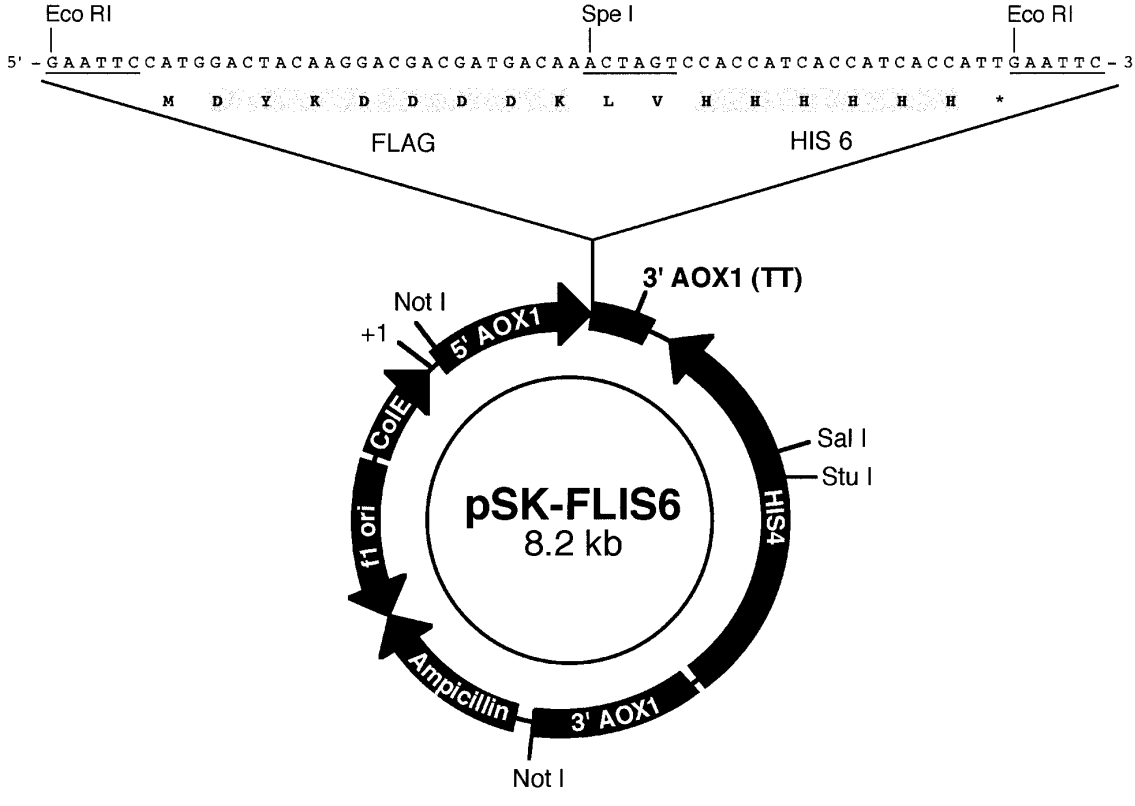

FIG. 4. Constructs used for overexpression of ADAR proteins in *Pichia pastoris*. The coding sequences of the deaminases were cloned into the expression vector pSK-FLIS6. This vector has the FLAG epitope and a $(His)_6$-tag cassette inserted in the polylinker of the *P. pastoris* shuttle vector pHIL-D2 (Invitrogen). Restriction sites are underlined.

Large-Scale Expression

Twelve milliliters of BMGY was inoculated with a single colony and incubated as described above. The entire culture was used to inoculate 300 ml of BMGY in a 2-liter Erlenmeyer flask. Culture growth is always performed at 30°C, 260 rpm. The cells were washed with sterile water, induced in 1 liter of BMMY, and further incubated. Due to the evaporation of methanol from the culture, an additional 5 ml of methanol was added after 24 h. Alternatively, the 300-ml BMGY preculture was used to inoculate 5 liters of BMGY for an additional day. For this purpose, 3-liter buffled Erlenmeyer flasks containing 600 ml (20% volume) of culture were used, as good aeration is very important. After being washed, the cells were induced in the same volume of BMMY for another 24 h. Longer induction leads to intracellular degradation of the recombinant protein. The cells were harvested by centrifugation at 3000*g* for 10 min, washed twice with 0.5 culture volume of sterile water, and 0.25 culture volume of buffer A/80 mM KCl. The wet weight of the pellets was on the order of 250 g/5 liters of culture. Cells were resuspended in 1 ml/g buffer A/80 mM KCl on ice and mechanically broken with a pestle in a mortar filled with liquid nitrogen. The cell suspension was dropped into the liquid nitrogen and portions of 25 ml (maximum) were broken to a fine powder. The frozen powder can be stored in aliquots at −70°C. For the purification from extracts, the powder was thawed on ice and centrifuged twice at 4°C, 12,000 rpm for 15 min in a SS34 rotor. The supernatant was subsequently loaded on the first column.

Purification of Recombinant Proteins

All manipulations were carried out at 4°C. Aliquots or extracts were frozen in liquid nitrogen. The extracts from 1-liter cultures were loaded on a 40-ml Macro-Prep High Q column that had been equilibrated with buffer A/80 mM KCl. The column was washed with 10 column volumes of buffer D (buffer A without EDTA)/80 mM KCl and developed with a 5-column-volume gradient from 80 to 500 mM KCl. Most *P. pastoris* proteins do not bind to this matrix and recombinant hADAR2a/b eluted between 200 and 350 mM KCl. The fractions were analyzed by immunoblot analysis with an appropriate antibody or tested in an assay specific for hADAR2a/b activity. The peak fractions were mixed with 2 ml of a 50% slurry of Ni^{2+}-NTA agarose that had been equilibrated in buffer A/200 mM KCl and 0.01% NP-40, absorbed for 30 min on ice with mixing, and poured into a column. The column was washed with 10 column volumes of the above buffer containing 15 mM imidazole hydrochloride (pH 8.0). hADAR2a/b were eluted in the same buffer containing 250 mM imidazole hydrochloride. Fractions were dialyzed against buffer A/200 mM KCl. Protein concentrations were determined according to Bradford (29) with BSA as a standard. Alternatively, proteins and BSA can be titrated and separated on a SDS/7.5% polyacrylamide gel and stained with Coomassie brilliant blue R-250 (Bio-Rad). Approximately 1 mg of purified hADAR2 protein per liter of yeast culture is obtained (Fig. 5).

Peak fractions of the Ni^{2+}-NTA column can be loaded directly on a poly(I)·poly(C) column. The column is washed with 2 column volumes of buffer A/250 mM KCl and developed with an 8-column-volume gradient from 250 to 2500 mM KCl as described. This additional column is included to obtain protein that is suitable for crystallization trials.

CONCLUDING REMARKS

To date only a small number of specific pre-mRNA targets have been identified for the ADAR family

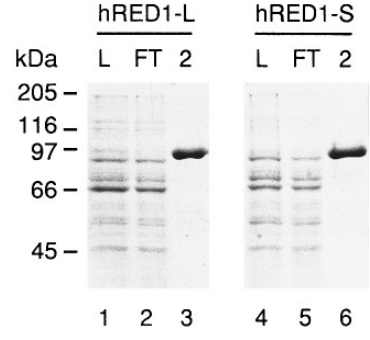

FIG. 5. SDS–PAGE analysis of the Ni/NTA purification of hADAR2a (hRED1-S) and hADAR2b (hRED1-L). Aliquots were electrophoresed on a SDS–7.5% polyacrylamide gel and proteins were stained with Coomassie brilliant blue R-250. Lanes 1 and 4: 15 μl pooled Macro-Prep High Q fractions of hADAR2a/b (load); lanes 2 and 5: 15 μl flowthrough of the Ni^{2+}-NTA column; lanes 3 and 6: 10 μl of the Ni^{2+}-NTA fraction 2 containing pure hADAR2b (lane 3) or hADAR2a (lane 6). Reproduced from Gerber, A., O'Connell, M.A., and Keller, W., 1997, *RNA* **3,** 453–463, by permission of Cambridge University Press.

of enzymes, and the full range of target sequences remains to be determined. To investigate what governs the specificity of the ADAR family of enzymes, it is necessary to have pure protein. While the advantages of overexpression of recombinant protein are obvious, purification of the native enzyme may shed some light on other factors that could modulate its activity. Even though the purification procedure described is for native bADAR1a and hADAR2, it should be applicable to other members of the ADAR family as they are expected to have similar properties. It is possible that other members of the deaminase family or deaminases from other organisms may not behave in the same manner on ion exchange matrices but they should bind to the dsRNA affinity columns, either poly(I)·poly(C) or poly(G)·poly(C).

The advantages of using the yeast *P. pastoris* for the overexpression of recombinant proteins have been outlined above, and it is fast becoming a very powerful tool. The method described has given 1 mg/liter hADARa/b that is pure and is now being used for crystallization trials.

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