Two forms of human double-stranded RNA-specific editase 1 (hRED1) generated by the insertion of an Alu cassette

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ABSTRACT

The double-stranded RNA-specific editase 1 (RED1/ADAR2) is implicated in the editing of precursor-mRNAs (pre-mRNA) encoding subunits of glutamate receptors (GluRs) in brain. Site-specific deamination of adenosine to inosine alters the codon at the Q/R site in GluR-B rendering the heteromeric receptor impermeable to Ca^{2+} ions. We cloned human RED1 (hRED1/hADAR2) cDNAs from a brain cDNA library. The human enzyme is 95% identical to the rat homologue. We characterized two alternatively spliced forms that differed by the presence of an Alu-J cassette in the deaminase domain. For the long form containing the Alu cassette, we isolated cDNA clones with an alternative C-terminus and 3'-UTR. An 8.8-kb transcript of hRED1 is most abundant in brain and heart, and lower levels are detected in other tissues. In vitro editing assays with purified recombinant hRED1 containing or lacking the Alu-J cassette revealed that both forms of the protein have the same substrate specificity, but differ in their catalytic activity.

Keywords: alternative splicing; double-stranded RNA-specific adenosine deaminase; GluR-B; *Pichia pastoris*; RNA editinga

INTRODUCTION

RNA editing refers to the alteration of the coding capacities of gene transcripts by insertion, deletion, or conversion of nucleotides (reviewed in Scott, 1995; Simpson & Emeson, 1996). RNA editing in mammals was first discovered in mRNA of intestinal apolipoprotein B (apoB), where a translational stop codon is generated by site-specific deamination, converting a cytidine to a uridine and resulting in a truncated protein with altered function (reviewed in Innerarity et al., 1996). This event is mediated by a protein complex (editosome) containing a cytidine deaminase as a catalytic component (APOBEC-1; Teng et al., 1993).

Another type of RNA editing found in mammals is the deamination of specific adenosines to inosines that occurs in pre-mRNAs of subunits of the glutamate-gated ion channel receptors (GluRs) in the brain (reviewed in Seeburg, 1996; Simpson & Emeson, 1996). These ionotropic receptors respond to ʟ-glutamate, the major neurotransmitter that mediates fast excitatory synaptic transmission in the central nervous system. Amino acid changes in receptor subunits introduced by RNA editing have a physiological impact on ion conductance, calcium permeability, and the kinetics of the ion channels (Seeburg, 1996). A physiologically important editing event occurs at the Q/R site of the the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit GluR-B (Sommer et al., 1991; Higuchi et al., 1993), where a glutamine codon CAG is converted to an arginine codon CIG (Melcher et al., 1995; Rueter et al., 1995; Yang et al., 1995). This arginine is localized in a channel-forming domain, and it renders the heteromeric receptor impermeable to Ca^{2+} ions (Seeburg, 1996).

Unlike the editing of apoB transcripts that requires specific sequence recognition, GluR pre-mRNA editing is dependent on a double-stranded RNA (dsRNA) structure formed by intramolecular base pairing between exonic and intronic sequences (Higuchi et al., 1993; Egebjerg et al., 1994; Lomeli et al., 1994; Brusa et al., 1995; Herb et al., 1996; Maas et al., 1996). Therefore, the ubiquitously expressed dsRNA-specific adenosine deaminase (DRADA/dsRAD/ADAR1), which was purified (Hough & Bass, 1994; Kim et al., 1994a; O'Connell & Keller, 1994) and cloned from different sources (Kim et al., 1994b; O'Connell et al., 1995; Patterson & Samuel, 1995), was considered a candidate enzyme responsible for the editing of GluR-B pre-mRNA. DRADA converts up to 50% of the adenosine to inosine in extended dsRNA in vitro. Indeed, it was

shown that recombinant DRADA edits the R/G site in exon 13 (Maas et al., 1996), as well as several intronic sites, such as the hotspot 1, in vitro (Dabiri et al., 1996; Maas et al., 1996). There are controversial reports in the literature concerning editing at the Q/R site in GluR-B. It was reported that DRADA can edit the Q/R site very inefficiently in vitro and that it may edit the site in vivo, but requires additional cofactors (Hurst et al., 1995; Dabiri et al., 1996) analogous to the editing of apolipoprotein B transcripts. Other groups found that DRADA could be separated partially from a highly specific Q/R editing activity by column chromatography (Yang et al., 1995; Maas et al., 1996).

Melcher et al. (1996b) reported the cloning of a second dsRNA-specific adenosine deaminase from a rat brain cDNA library that specifically edits the Q/R and the R/G site, but not the hotspot 1 in GluR-B pre-mRNA. This 80-kDa enzyme, termed dsRNA-specific editase 1 (RED1/ADAR2), has 31% amino acid sequence identity to DRADA and shares some common domain structures; DRADA contains three, and RED1 two, dsRNA-binding domains (dsRBD; St. Johnston et al., 1992) that are located amino terminal to a catalytic deaminase domain. The latter domain is characterized by conserved regions containing three putative Zn^{2+}-chelating and a proton-transferring amino acid (Kim et al., 1994b; Lai et al., 1995; O'Connell et al., 1995; Melcher et al., 1996b). Rat RED1 and DRADA can convert up to 50% of adenosines to inosines on long dsRNA, and both are capable of editing the R/G sites, suggesting that they have overlapping substrate specificities in vivo (Melcher et al., 1996b; O'Connell et al., 1997). Recently, a third, brain-specific member of the dsRNA-specific adenosine deaminase family was cloned from a rat brain cDNA library. In contrast to RED1 and DRADA, recombinantly expressed RED2/ADAR3 does not deaminate adenosines in extended dsRNA or GluR-B pre-mRNA (Melcher et al., 1996a)

Here we report the isolation of alternatively spliced cDNA clones of human RED1 (hRED1) from a brain cDNA library. We further characterized two alternatively spliced forms of hRED1 mediated by the insertion of an Alu cassette. We localized a complete Alu sequence in an apparent intron of the hRED1 gene. Both forms of hRED1 were overexpressed in the yeast *Pichia pastoris* and the recombinant proteins were purified. In vitro they showed the same substrate specificities as described for the recombinant rat RED1 (Melcher et al., 1996b) and the purified hRED1 (O'Connell et al., 1997), but had a twofold difference in their specific editing activity.

RESULTS

Cloning of human RED1 cDNAs

The two human expressed sequence tags (EST) L25485 and T70335 show high homology at the DNA level to the deaminase domain of RED1 (81.4% and 80.7%) and DRADA (Melcher et al., 1996b). L25485 was mapped previously to human chromosome 21 (HC21), region q22.2–q22.3, by hybridization to HC21 derived yeast artificial chromosomes (YACs; Cheng et al., 1994). Primers were designed to amplify these ESTs by PCR from a HeLa λgt11 library. Two products of 575 bp and 455 bp were obtained, corresponding to two forms of hRED1. The comparison of both fragments on an agarose gel indicated a slightly higher amount of the 575-bp DNA fragment compared to the 455-bp fragment. The larger PCR fragment was subcloned, sequenced, and used as a hybridization probe to screen a human brain λZAP II cDNA library. Out of 920,000 plaques screened, three positive clones of human homologues to rat RED1 were obtained. PCR and sequencing analysis revealed that the cDNA clones differed in their deaminase domain, as well as in their 3'-sequences (Fig. 1A). The full-length clone h-1 (2,728 bp) harbors the short PCR fragment (455 bp) and contains an open reading frame of 2,103 bp, encoding a protein of 701 amino acids termed hRED1-Short (hRED1-S) with a calculated molecular mass of 76.6 kDa and an isoelectric point of 9.1. The clone also contains 240 bp of 5' untranslated region (UTR) and a 3' UTR of 382 bp (UTR-1) with the AAUAAA polyadenylation signal consensus sequence preceding a poly-(A) tail. The cDNA sequence including both UTRs is 85% identical to rat RED1 and has analogous start and stop codon positions. The deduced amino acid sequence of hRED1 is presented in Figure 1B. hRED1 contains two dsRBDs (St. Johnston et al., 1992), a putative deaminase motif (DM), and a bipartite nuclear localization signal (NLS; Dingwall & Laskey, 1991). hRED1-S is 95% identical (97% similar) to rat RED1 and shows 33.1% identity (55.6% similar) to the previously cloned human DRADA (Kim et al., 1994b; O'Connell et al., 1995; Patterson & Samuel, 1995).

The partial clones h-7 and h-10 start at position 1123 of the h-1 cDNA. Interestingly, they encode 40 additional amino acids in the deaminase domain (Fig. 1A). The insertion of this additional amino acids in hRED1-S results in a larger hRED1 protein (hRED1-L) of 741 amino acids with a calculated molecular mass of 80.7 kDa and an isoelectric point of 9.1. Recently, a full-length cDNA sequence coding for hRED1-L followed by UTR-1 has been submitted to the GenBank database by other researchers (accession no. X99227). Compared with the rat RED1 protein (Melcher et al., 1996b), hRED1-S does not encode amino acids 466–475 of the rat RED1, whereas hRED1-L contains 38 different amino acids from residue 467 to 504. The 40-amino acid insertion (120 bp at the DNA level) is present in the human EST T70335 and is 88.1% identical to a segment of the human Alu-J subfamily consensus sequence (GenBank accession no. U14567).

In addition, these partial clones contain not only an insertion of an Alu-J cassette, but also show different

A

cDNA

Protein

B

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  1   MDIEDEENMS  SSSTDVKENR  NLDNVSPKDG  STPGPGEGSQ  LSNGGGGGPG

 51   RKRPLEEGSN  GHSKYRLKKR  RKTPGPVLPK  NALMQLNEIK  PGLQYTLLSQ

101   TGPVHAPLFV  MSVEVNGQVF  EGSGPTKKKA  KLHAAEKALR  SFVQFPNASE

151   AHLAMGRTLS  VNTDFTSDQA  DFPDTLFNGF  ETPDKAEPPF  YVGSNGDDSF

201   SSSGDLSLSA  SPVPASLAQP  PLPVLPPFPP  PSGKNPVMIL  NELRPGLKYD

251   FLSESGESHA  KSFVMSVVVD  GQFFEGSGRN  KKLAKARAAQ  SALAAIFNLH

301   LDQTPSRQPI  PSEGLQLHLP  QVLADAVSRL  VLGKFGDLTD  NFSSPHARRK

351   VLAGVVMTTG  TDVKDAKVIS  VSTGTKCING  EYMSDRGLAL  NDCHAEIISR

401   RSLLRFLYTQ  LELYLNNKDD  QKRSIFQKSE  RGGFRLKENV  QFHLYISTSP

451   CGDARIFSPH  EPILEGSRSY  TQAGVQWCNH  GSLQPRPPGL  LSDPSTSTFQ

501   GAGTTEPADR  HPNRKARGQL  RTKIESGEGT  IPVRSNASIQ  TWDGVLQGER

551   LLTMSCSDKI  ARWNVVGIQG  SLLSIFVEPI  YFSSIILGSL  YHGDHLSRAM

601   YQRISNIEDL  PPLYTLNKPL  LSGISNAEAR  QPGKAPNFSV  NWTVGDSAIE

651   VINATTGKDE  LGRASRLCKH  ALYCRWMRVH  GKVPSHLLRS  KITKPNVYHE

701   SKLAAKEYQA  AKARLFTAFI  KAGLGAWVEK  PTEQDQFSLT  P
                  VH
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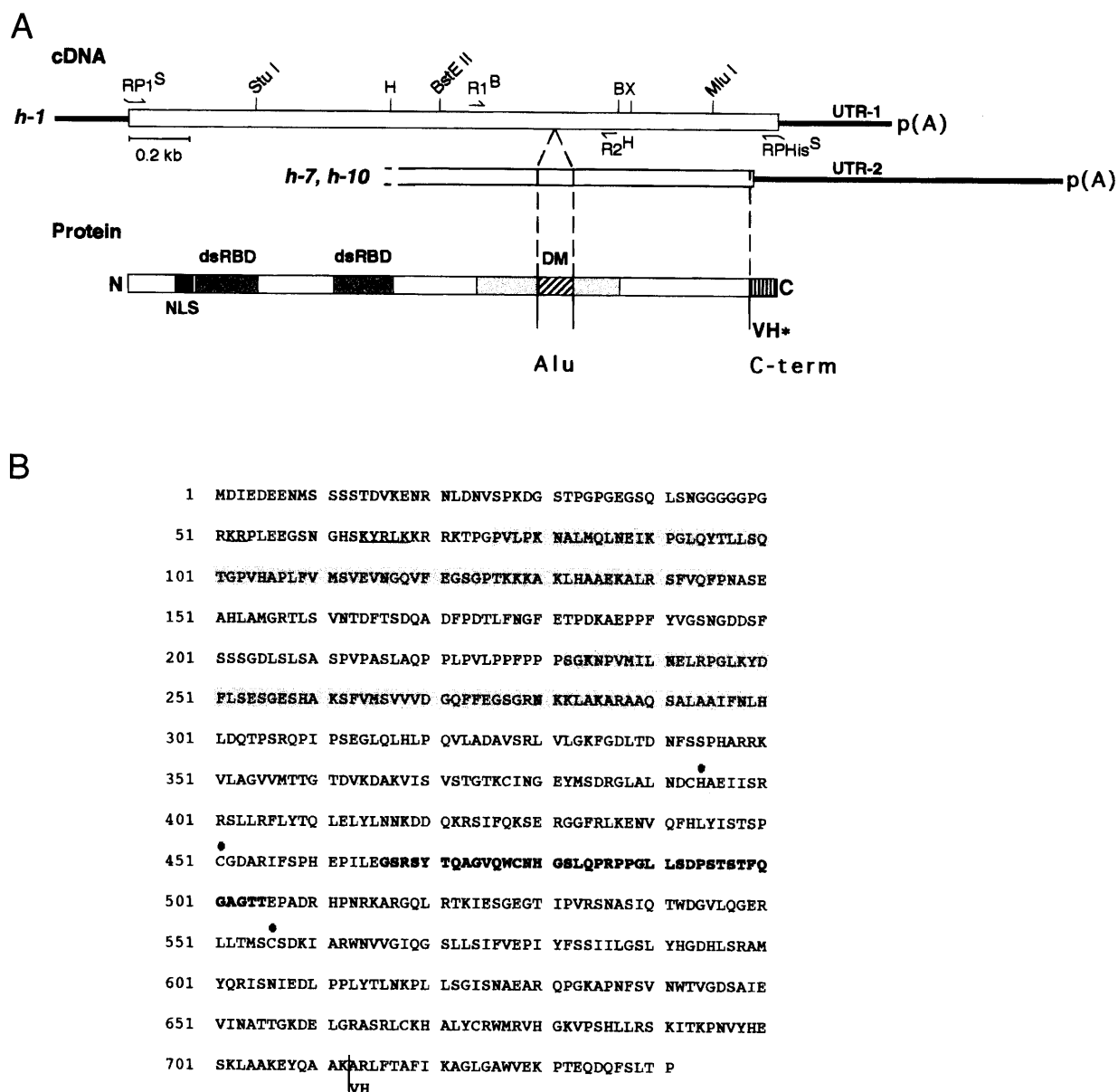
FIGURE 1. Human RED1 cDNAs and deduced amino acid sequence. **A:** hRED1 cDNAs and protein domains. The full-length cDNA clone h-1 coding for hRED1-S and the partial cDNA clones h-7 and h-10 are shown. The largest open reading frame is depicted as a box and the UTRs as thick lines. Some unique restriction sites and the primers used in PCR are indicated (B, *Bam*H I; H, *Hin*d III; X, *Xho* I; S, *Spe* I). The domain structure of the hRED1 protein shows the bipartite nuclear localization signal (NLS; black box), the two dsRNA binding domains (dsRBD; dark gray box), and the deaminase domain (DM; light gray box). The Alu-encoded 40 amino acids and the C-terminal truncations are marked with different cross-hatched boxes. Altered amino acids encoded by the partial clones are indicated below the domain structure. **B:** Deduced amino acid sequence of hRED1. Gray bars indicate the dsRBDs, filled circles show the putative Zn^{2+}-chelating residues in the DM. The NLS is underlined. Boldface letters mark the Alu-encoded 40 amino acids; altered amino acids in C-terminal truncated form are indicated below the sequence. Genbank accession numbers: hRED1-S, U82120; hRED1-L, U82121.

3'-sequences compared with the full-length clone of hRED1-S (h-1, Fig. 1A). They start to diverge after a putative exonic 5'-splice site (AG) at position 2256 of the full-length clone h-1 (amino acid 373 in hRED1-S), and continue with codons for two other amino acids, a valine and a histidine, followed by an in-frame stop codon and a different 3'-UTR (UTR-2). This UTR-2 shows no homology to the 3'-UTR of rat RED1, RED2, or DRADA, but is encoded in more than 20 human ESTs (as an example, N51725, AA026719, AA026607). Two ESTs, R07731 and T85478, match to the 5'-end of the UTR-2, but they continue with 5'-upstream sequences that are unrelated to hRED1. The significance of this is unknown, and we did not further characterize the altered C-termini of these clones, but they may originate from a second alternative splicing event.

Genomic analysis of the alternatively spliced Alu cassette in the hRED1 gene

To investigate the origin of the Alu-like sequence in hRED1 cDNAs, we amplified the corresponding region from genomic HeLa DNA by PCR and sequenced the products directly (for details, see Materials and Methods). The analysis showed that the 120-bp Alu cassette is a segment of a complete reverse intronic Alu-J sequence between the adjacent exons in the deaminase domain (Fig. 2A). The complete Alu sequence shows high homology to the Alu-J consensus sequence (88%) and contains the typical characteristics of an Alu sequence (Fig. 2B; Ullu & Tschudi, 1984). A part of the right arm of this Alu-J DNA sequence is inserted in antisense orientation in the cDNA of hRED1. The genomic sequence contains putative donor and acceptor splice sites in the Alu sequence flanking the 120-bp insert. The requirement for a polypyrimidine tract at the 3′ splice acceptor site can be fulfilled by the complement of the polyadenylate stretch of the Alu sequence (residues 276–288, referred to the Alu-J consensus). The same acceptor splice site delivered by an Alu-J sequence was found in decay-accelerating factor (DAF/CD44), resulting in two alternatively spliced forms of the protein (Caras et al., 1987; Post et al., 1990). The altered 5′ donor splice site AG/GCATGT, where the invariant GT dinucleo-

tide is changed to GC, may still permit splicing (Aebi et al., 1987). The same donor splice site with normal splicing activity is present in intron 8 of the mouse RNA polymerase II gene (Ahearn et al., 1987) and several human genes containing nonconsensus GC splice sites are described as well (Jackson, 1991; Nakai & Sakamoto, 1994). These aberrant donor and acceptor splice sites suggest that the 120-bp element in the intronic Alu-J sequence can be alternatively spliced and therefore constitutes an optional exon. Furthermore, most of the previously characterized examples of insertions of an antisense Alu cassette into a protein-coding region involve alternative splicing (Makalowski et al., 1994). We determined the distances of the Alu-J cassette to the neighboring exons by PCR. If the Alu-J cassette is considered an exon, the intron upstream of the Alu-J cassette is approximately 950-bp in length and the one downstream, approximately 330-bp in length.

hRED1 mRNA expression and abundance of the protein in different tissues

A 704-bp *Hin*d III/*Pst* I fragment (amino acids 281–515) of the hRED1-S coding sequence was used to probe a human multiple-tissue northern blot to determine expression levels in various tissues. The northern blot analysis revealed that an 8.8-kb mRNA is highly en-
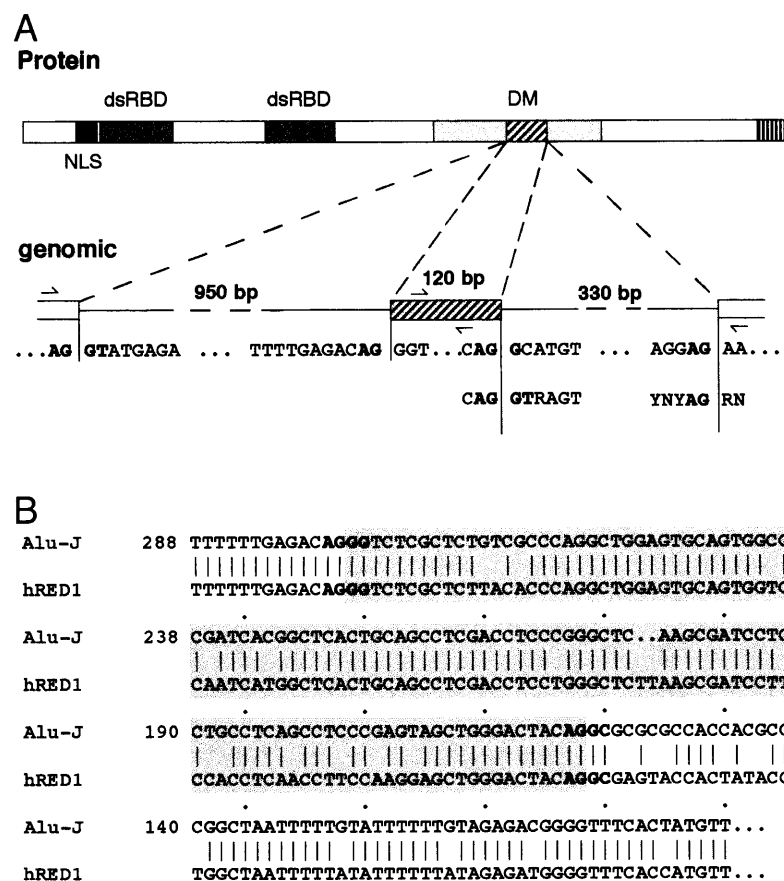

FIGURE 2. Exon/intron boundaries of the Alu cassette. **A:** The protein domain structure of hRED1 is depicted as in Figure 1A. In the "genomic" part of the map, exons are drawn as boxes and introns as lines (not drawn to scale). Primers for sequencing the exon/intron boundaries are indicated as arrows; boldface letters indicate conserved splice sites and a consensus is shown in the line below. **B:** DNA sequence alignment of the intronic Alu sequence in the gene of hRED1 and the Alu-J subfamily consensus sequence in reversed orientation (GenBank accession no. U14567). Boldface letters indicate the splice sites. The light gray box contains the alternatively spliced Alu cassette.

riched in brain and heart and is present in lower levels in placenta, pancreas, and skeletal muscle. A very faint signal was detected in lung, liver, and kidney (Fig. 3). In addition, we identified a minor transcript of 3.5 kb, which was present at a considerably lower level. The 8.8-kb transcript length is in good agreement with the size (approximately 7 kb) of the rat RED1 mRNA detected in a rat northern blot (Melcher et al., 1996b), but different RNA levels were found in several tissues. Compared to the rat RED1 northern blot, a stronger signal was seen in the lane with human heart and skeletal muscle RNA, whereas a weaker signal was found in lung. Reprobing the blot with a β-actin probe indicated that the amounts of RNA in the lanes differed by no more than a factor of two (data not shown). As a further control, the blot was hybridized with a *Hin*d III/*Pst* I fragment of the rat RED1 clone. The same distribution of the 8.8-kb band and some minor bands were detected as with the human probe (data not shown).

A human multiple-tissue western blot was probed with polyclonal anti-hRED1 serum recognizing both the Alu-J cassette-containing or lacking forms of the protein (O'Connell et al., 1997). A single 90-kDa band corresponding in size to purified hRED1 (O'Connell et al., 1997) was detected in the lanes containing brain, heart, kidney, lung, and liver extracts; a very faint signal was detected in skeletal muscle extract (data not shown).
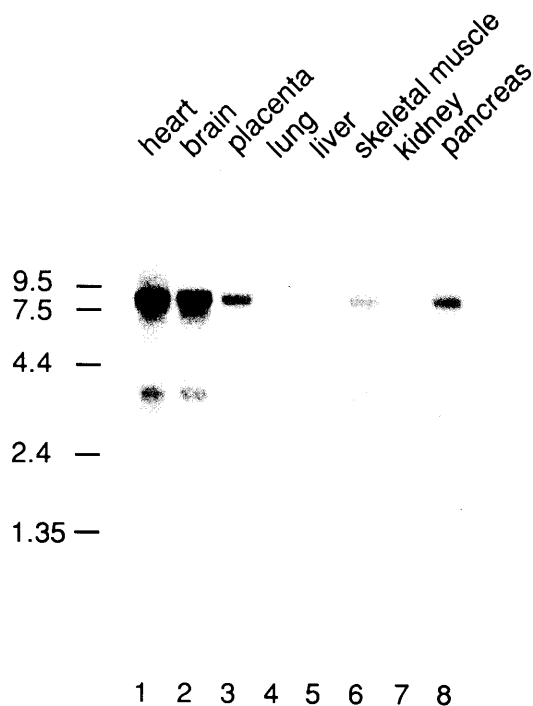
Overexpression in *P. pastoris* and purification of recombinant hRED1-S and hRED1-L

The coding sequences of hRED1-S and hRED1-L were subcloned into a *P. pastoris* expression vector (see Materials and Methods), which was engineered to encode a 5'-FLAG- and a 3'-histidine hexamer epitope. The coding sequence of hRED1-L is a chimeric hRED1-S/L construct, because we did not obtain a full-length hRED1-L cDNA. After transformation and screening for His[+]/Mut[S] phenotypes, 12 colonies were grown in pilot cultures and the extracts tested for their ability to convert adenosine to inosine on extended dsRNA (O'Connell & Keller, 1994). The amount of recombinant protein in the extract was estimated by immunodetection with an anti-hRED1 antibody (O'Connell et al., 1997). All extracts showed about the same ratio of recombinant protein to catalytic activity, but hRED1-S extracts contained a higher enzymatic activity compared to hRED1-L extracts (data not shown). To purify the recombinant proteins, extracts prepared from a 1-L culture were loaded on a Macro-Prep High Q column. The majority of proteins did not bind to the column and hRED1 was eluted with a salt gradient. Fractions containing hRED1-S or -L were pooled, loaded on a Ni^{2+}-nitrilotriacetic acid column, and eluted with imidazole (Fig. 4). Approximately 1 mg of purified protein per liter of yeast culture was obtained.

hRED1-S has a higher catalytic activity than hRED1-L

We tested the purified recombinant hRED1-S and hRED1-L proteins for their ability to deaminate adenosine on extended dsRNA and to edit the Q/R, hotspot 1, and R/G sites in GluR-B in vitro. No editing activity was detected with *P. pastoris* mock extracts.
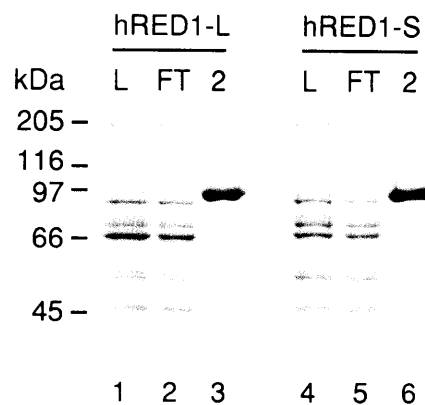

FIGURE 3. Northern blot analysis of hRED1 in human tissues. A northern blot containing 2 μg of human poly(A)[+] RNA from different tissues was hybridized with a hRED1-S DNA probe. Size markers in kilobases are shown on the left.

FIGURE 4. SDS-PAGE analysis of the Ni/NTA purification of hRED1-S and -L. Aliquots were electrophoresed on a 7.5% SDS-polyacrylamide gel (Laemmli, 1970) and proteins stained with Coomassie Brilliant Blue R-250. Lanes 1 and 4, 15 μL pooled Macro-Prep High Q fractions of hRED1-L and -S (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 number 2 containing pure hRED1-L (lane 3) or hRED1-S (lane 6).

Both forms of hRED1 were capable of converting up to 50% of the adenosines to inosines on extended synthetic dsRNA (Fig. 5A). This result correlates with activities described for DRADA and RED1 on artificial dsRNA substrates (Hough & Bass, 1994; Kim et al., 1994a; O'Connell & Keller, 1994; Melcher et al., 1996b). To investigate the specificity of editing, primer extension assays were performed on the GluR-B minigene B13 for adenosines at the Q/R site and the hotspot 1 (Melcher et al., 1995; O'Connell et al., 1997). The adenosine at the Q/R site was edited up to 60% by either hRED1 protein in the primer extension assay (Fig. 5B). The linear range in these assays only extends to 30–40% editing and the maximum conversion of adenosine to inosine is 50–60%. This effect is probably caused by misfolding of the RNA substrate. Editing at the

hotspot 1 was also investigated, but only low adenosine to inosine conversion could be detected: 4% editing with 50 ng hRED1-S and 2.5% with 50 ng hRED1-L (data not shown). To investigate the R/G site in GluR-B, RT-PCR was performed on products of the editing reaction and sequenced (Fig. 5C,D; O'Connell et al., 1997).

Although there was some variation between the assays, hRED1-S was approximately twice as active as hRED1-L in all cases. Hence, the insertion of the Alu-J cassette in the deaminase motif seems to reduce the catalytic activity, but does not affect the specificity of the editing reaction. Our data concerning the specificity of the editing in GluR-B in vitro strongly correlate with those reported previously for recombinant and purified RED1 and DRADA (Melcher et al., 1996b; O'Connell et al., 1997). In these studies, the hotspot 1
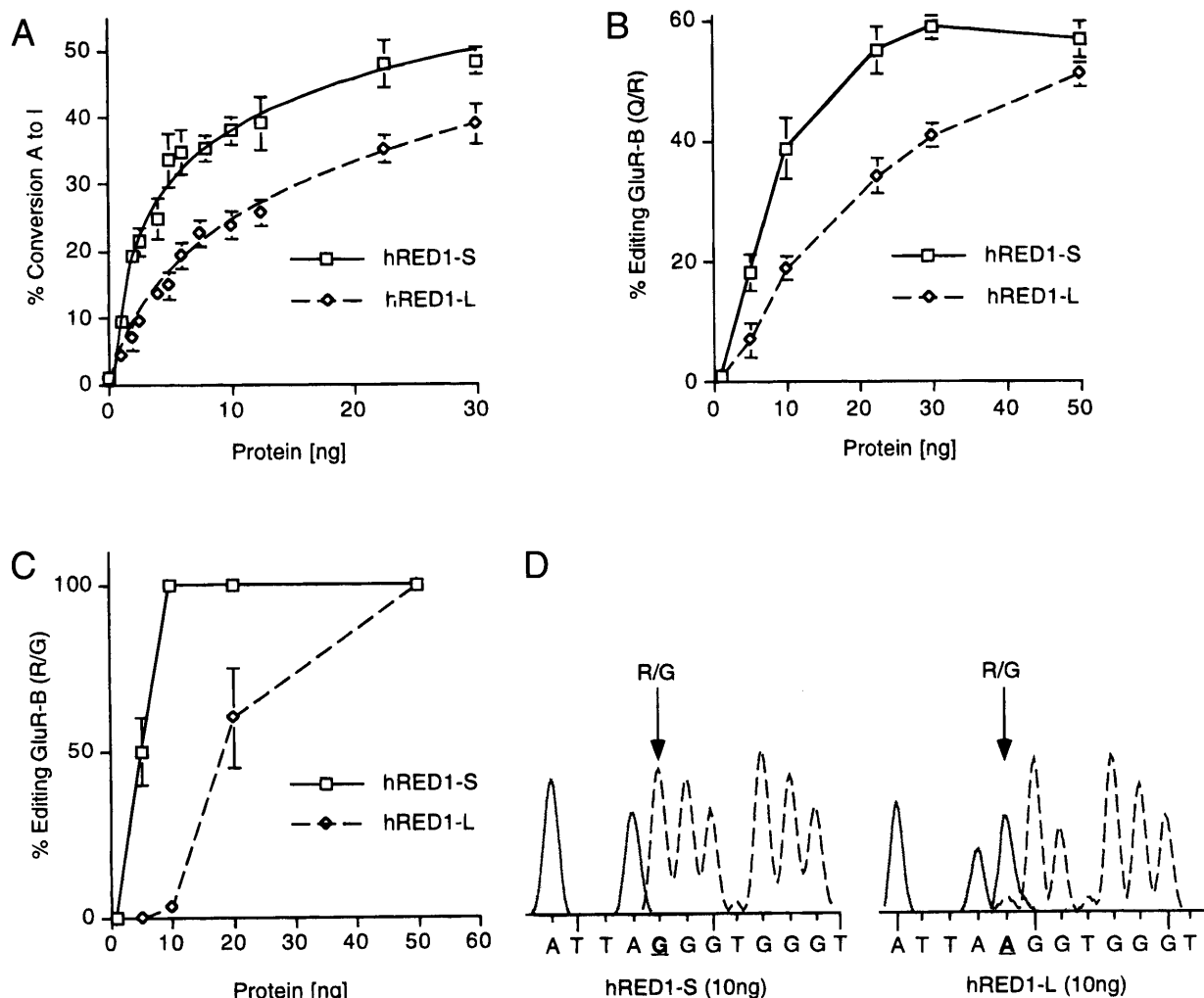

FIGURE 5. Activity assays with purified recombinant hRED1-S and -L. **A:** Conversion rate of adenosine to inosine on extended synthetic dsRNA (O'Connell & Keller, 1994). Two-hundred femtomoles of labeled adenosines in dsRNA were incubated with different amounts of hRED1-S or -L in a 25-μL reaction for 1 h at 30 °C in the presence of 200 μg/mL BSA. **B:** Conversion of adenosine to inosine at the Q/R site in GluR-B measured with a primer extension assay. Ten femtomoles of GluR-B minigene B13 were incubated as described in A. **C:** Sequence analysis of the edited GluR-B at the R/G site. Ten femtomoles GluR-B minigene pBgl was incubated as described in A. RT-PCR was performed and the products sequenced. **D:** Sequence analysis with 10 ng hRED1 as described in C. Only the trace data of the nucleotides adenosine and guanosine surrounding the R/G site are depicted.

was shown to be edited by DRADA, whereas the R/G site was edited by both DRADA and RED1. The Q/R site, which is localized in a perfect dsRNA (Higuchi et al., 1993), was edited specifically only by RED1 (Melcher et al., 1996b; O'Connell et al., 1997).

DISCUSSION

We report the cloning of alternatively spliced cDNAs of human RED1 and the characterization of two clones that differ by the insertion of an Alu cassette in the deaminase domain. We expressed the corresponding two forms of hRED1 in the yeast *P. pastoris* and recombinant proteins were purified to homogeneity. The purified proteins were tested in assays for unspecific dsRNA-dependent adenosine deaminase activity and for specific editing activities in vitro. All assays indicated that hRED1 lacking the Alu cassette (hRED1-S) has a higher catalytic activity than the one including it (hRED1-L).

Analysis of a full-length cDNA (h-1) revealed high homology to the published rat RED1 cDNA (Melcher et al., 1996b) in the coding sequence, as well as in both UTRs (85% identical). The amino acid sequence identity between the human and rat RED1 is 95%. Both contain two dsRBDs (St. Johnston et al., 1992), a deaminase domain, and a bipartite NLS (Dingwall & Laskey, 1991) at their N-terminus (Fig. 1B). This conservation is higher than between human and rat DRADA (79%) (O'Connell et al., 1995). The lower conservation between the latter proteins is mainly due to a diverged N-terminus: only the human clone encodes an RG-enriched domain and contains a duplication of 48 amino acids. The unique human sequence may be modified posttranslationally (O'Connell et al., 1995) and it could play a role in response to interferon (Patterson & Samuel, 1995).

In contrast to DRADA, alternatively spliced variants of hRED1 were found. In addition to the full-length cDNA clone of hRED1-S, we isolated partial cDNA clones that differed in the deaminase domain as well as in their 3'-end sequences (Fig. 1A). Regarding the 3'-end sequences, the alternative forms fail to encode the C-terminal 29 amino acids of hRED1-S and rat RED1, but instead encode two other amino acids, a valine and a histidine, followed by a stop codon and a 3'-UTR (UTR-2), which shows no homology to the 3'-UTRs of rat RED1 or DRADA. Instead, we found more than 20 ESTs whose sequences match to UTR-2. Because the 3'-UTR of the full-length clone (UTR-1) and UTR-2 start to diverge after a potential 5'-splice site, they may originate from alternative splicing. The role of the different UTRs and their origin remains to be elucidated.

We were interested in another putative alternatively spliced region mediated by the insertion of an Alu-like sequence, because this insertion is located in the center of the deaminase motif, the catalytic core of the protein. A comparison between human and rat cDNAs in this region revealed putative exonic splice sites in both the human and the rat clone. Indeed, it has been demonstrated recently that, in the rat, two splice variants can exist in this region that differ by 10 amino acids (Melcher et al., 1996a). In addition, a full-length cDNA sequence coding for hRED1-L followed by UTR-1 has been submitted to the GenBank database by other researchers (accession no. X99227). This demonstrates that the insertion of the Alu-cassette is not necessarily coupled with a C-terminal truncation of the protein, suggesting that both splicing events may occur independently. Therefore, the published rat RED1 sequence (Melcher et al., 1996b) is the homologue of human RED1-L. The corresponding additional 120 bp DNA sequence in the human cDNAs showed significant homology (88%) to a segment of the right part of the Alu-J subfamiliy consensus sequence (Jurka & Smith, 1988). In primates, Alu sequences are composed of two parts, Alu-left and Alu-right, that are both homologous to the 7SL DNA sequence and connected by an adenosine-rich linker (Ullu & Tschudi, 1984). On the other hand, we could not find any homology in the rat cDNA to rodent Alu sequences.

Several other proteins have been described that contain a segment of an Alu sequence (Alu cassette) in their protein- coding region (reviewed in Makalowski et al., 1994). This event could occur by at least two mechanisms; the first is retroposition, by which more recent Alu subfamilies are believed to proliferate in the primate genome (Deininger et al., 1992; Schmid & Maraia, 1992). This process requires reverse transcription of the RNA of a retropositionally active Alu subfamily and its subsequent insertion into the coding region of a gene. Typically, the Alu element is full-length or truncated in the 5' region and is flanked by direct repeats (Makalowski et al., 1994; Margalit et al., 1994). The second and perhaps the more frequent mechanism involves a splice-mediated insertion of intronic Alu sequences into an open reading frame (Makalowski et al., 1994).

To investigate the origin of the Alu cassette, the deaminase domain of the hRED1 gene was sequenced between the two adjacent exons and the exons were mapped (Fig. 2A). We identified both parts of a reverse Alu sequence in the apparent intron that showed 88% identity to the Alu-J consensus sequence (Fig. 2B). This intronic Alu sequence contains the Alu cassette encoded in the cDNA of hRED1-L and is flanked by putative acceptor and donor splice sites. Because these splice sites are poorly conserved in comparison to the consensus sequences of splice sites, the Alu cassette is probably not spliced with high efficiency, and this may lead to two alternatively spliced products of the hRED1 mRNA. This is consistent with our data because we have detected both splice variants in cDNA derived

from HeLa cells and by screening a human brain cDNA library. Furthermore, this result agrees with well-characterized examples of insertions of an antisense Alu cassette into protein-coding sequences, where in most cases the Alu cassette is also not spliced with high efficiency, resulting in alternative splicing (Makalowski et al., 1994). The same segment of the right arm of an Alu sequence as in hRED1 was found to be alternatively spliced in the gene coding for the integrin β_1 subunit (Languino & Ruoslahti, 1992) and is also present in decay-accelerating factor (DAF/CD44), where about 10% of DAF mRNA contain an antisense Alu cassette (Caras et al., 1987). Members of the Alu-J subfamily are thought to be about 55 million years old, dating from the beginning of mammalian radiation and primate divergence (Labuda & Striker, 1989). We can speculate that, at this time of intense amplification of Alu-J (Deininger et al., 1992; Schmid & Maraia, 1992), an Alu-J sequence was inserted retropositionally into the gene of RED1. Further random mutations generated splice sites at the ends of the Alu cassette, leading to alternatively spliced mRNAs of hRED1. This process is believed to play a role in the evolution of proteins in general, because it is a mechanism by which protein diversity is generated. For example, the translation of the alternatively spliced mRNAs of DAF appears to generate two protein products encoding for a membrane bound and a soluble form (Caras et al., 1987). Other examples have been described, but are not as well characterized (Makalowski et al., 1994).

Northern blot analysis revealed high expression of the hRED1 gene in brain and heart and to a lower extent in other tissues, suggesting that the enzyme is involved in the editing of diverse nuclear pre-mRNAs (Fig. 3). Compared with the northern blot analysis of rat RED1 (Melcher et al., 1996b), a stronger signal was detected in human heart and skeletal muscle. One can speculate that the human and rat genes may be regulated differently at the level of transcription, having either different promoters or enhancers.

The EST L25485, which is part of the hRED1 cDNA, was mapped recently to the long arm of chromosome 21, region q22.2–q22.3, by hybridization to a set of HC21 derived YACs and cosmids (Cheng et al., 1994). This genomic region is strongly suspected to contain genes responsible for some major features of Down's syndrome (q22.2), such as mental and growth retardation and congenital heart defects. A type of progressive myoclonus epilepsy (EPM1) is also suspected to be localized in this region (q22.3). It is not known whether hRED1 is involved in the pathogenesis of any of these diseases.

To estimate the influence of the Alu insertion on enzymatic activities, hRED1-S and hRED1-L were over-expressed in the yeast *P. pastoris* and the recombinant proteins were purified to homogeneity (Fig. 4). We found that *P. pastoris* is an excellent organism to ex-

press and purify dsRNA adenosine deaminases because extracts from untransformed cells do not contain any measurable endogenous dsRNA-specific adenosine deaminase activity. Both forms of hRED1 were capable of deaminating up to 50% of the adenosine in extended dsRNA in vitro and edited the adenosine in GluR-B at the Q/R and the R/G site efficiently, but were unable to edit the adenosine at the hotspot 1 (Fig. 5). These results correlate strongly with those obtained from recombinant and purified RED1 and DRADA (Melcher et al., 1996b; O'Connell et al., 1997), and suggest that both enzymes have separate and overlapping substrate specificities. The hotspot 1 was shown to be edited by DRADA, whereas the R/G site was edited by both DRADA and RED1. The Q/R site, which is localized in a perfect dsRNA structure (Higuchi et al., 1993), is edited specifically only by RED1 (Melcher et al., 1996b; O'Connell et al., 1997).

Interestingly, hRED1-L was approximately half as active as hRED1-S in the in vitro assays. Whether this twofold difference is reflected in significant physiological differences in vivo is speculative. The predicted secondary structure of hRED1-L (data not shown; see Materials and Methods) reveals that the Alu-J sequence most likely encodes for an extra loop within two β-strand-rich regions formed by adjacent amino acids. Although no differences in specificity in vitro are observed, it cannot be excluded that the additional loop, if it is exposed at the surface of the protein, may interact with other proteins and thus influence site selectivity in vivo. The distance between the second and third putative Zn^{2+} chelating amino acid in the deaminase domain is increased by the Alu insertion. Therefore, our results suggest that the distance between these residues may influence the rate of the reaction.

Immunoblot analysis with polyclonal anti-hRED1 serum (O'Connell et al., 1997), recognizing both forms of the protein, specifically detected only a single 90-kDa band in heart, brain, and in other tissues (data not shown). In addition, only one form of hRED1 was detected and purified to homogeneity from HeLa nuclear extract (O'Connell et al., 1997). We isolated alternatively spliced cDNAs of hRED1. In apparent contradiction, just one just one form of the protein seems to be present in HeLa nuclear extract and in different human tissues. Unfortunately the HeLa protein migrates aberrantly on SDS-PAGE. Hence, it is not possible to determine from its molecular mass which spliced variant was purified. To elucidate the discrepancy, we want to generate an antibody that recognizes specifically the 40-amino acid insertion in hRED1-L, to enable us to distinguish between the two froms of hRED1. However, a developmentally regulated tissue-specific or even cell-type-specific distribution in the expression levels of the different protein forms might exist. Variations in the amounts of enzymes may help to regulate the specific editing activity in the cell and may also

determine whether a given adenosine is a substrate to be edited or not.

MATERIALS AND METHODS

Oligonucleotides and GluR-B minigenes

The PCR primers used for cloning human ESTs homologous to rat RED1 were R1, 5'-CTACAGGATCCAAATGTATTAA TGG-3'; and R2, 5'-CCACCGTGCAAGCTTGTCACTGC-3', encoding internal cleavage sites for *BamH* 1 and *Hind* III (underlined). The PCR and sequencing primers for the alternatively spliced region of hRED1 were hSV3, 5'-CATCTGT ACATCAGCACCTC-3'; hSV4, 5'-CATGGCTCACTGCAG CCTC-3'; hSR6, 5'-CCAGACTCTATTTTGGTCC-3'; hSR7, 5'-GGTTGAGGTGGAAGGATCGC-3'. PCR primers used for subcloning the coding sequence of hRED1-S into pSK-FLIS$_6$ were RP1, 5'-CGGGATCCGGACTAGTAGATATAGAAGAT GAAGAAAACATG-3' and RPHis, 5'-TGGTCGACTCAAT GGTGATGGTGATGGTGGACTAGTGGCGTGAGTGAGA ACTGGTCCTGC-3' containing *Spe* I restriction sites (underlined). GluR-B minigenes used in this study were B13 (encoding the Q/R and hotspot 1 edited sites; Higuchi et al., 1993), and pBgl (encoding the R/G site; Melcher et al., 1995). These constructs were linearized with *Bgl* I and *Stu* I, and the RNA transcribed as described previously (Melcher et al., 1995). The antisense oligonucleotide used in the primer extension assay was B-RT, 5'-GGCGAAATATCGCATCCTTG-3', which is complementary to the Q/R edited site (Melcher et al., 1995); BHS-RT, 5'-ACCATGAATATCCACTTGAG-3', is the antisense primer used in the primer extension assay at the hotspot 1 in GluR-B intron 11. The PCR, reverse transcription, and sequencing primers were BFFK3, 5'-GACACGGTACCACACAACG GATTGTGAGTTACCTCATATCCG-3', which is the reverse transcriptase primer antisense on minigene pBgl; PCRK3, 5'-GACACGGTACCACACAACGG-3', a PCR primer for cDNA primed with BFFK3; cis55, 5'-CTCTGCGAGCTCAGGTCC AACTGCACCTCGG-3', a vector-specific 5' primer; intB1, 5'-GCGGTACCGTGAGTTACCTCATATCCGTAT-3' (Melcher et al., 1995) and intB2, 5'-ATCCCTCCTAGACAAACCGTT AAGAGTC-3', both of which are antisense on minigene pBgl (Maas et al., 1996).

Cloning and sequencing of human RED1 cDNAs

The human ESTs L25485 and T70335 were used to design PCR primers R1 and R2 with internal restriction sites. PCR with these primers on a HeLa λgt11 cDNA library resulted in two products (575 bp and 455 bp). The 575-bp PCR product was cloned into the *BamH* 1–*Hind* III sites of pBluescript II KS (Stratagene) and sequenced with Sequenase version 2.0 (United States Biochemical Corp). The fragment was ^{32}P-labeled with a random primed DNA Synthesis Kit (Boehringer) and used to screen a human brain λZAP II cDNA library. Plating and duplicate filter preparation was performed according to standard protocols (Sambrook et al., 1989). The nitrocellulose filters were prehybridized at 42 °C for 3 h in hybridization buffer (50% formamide, 5× SSC [0.75 M NaCl, 0.075 M sodium citrate], 5× Denhardt's solution, 1% SDS, 100 μg of sonicated herring sperm DNA per

mL solution). The hybridization buffer was exchanged and 10^6 cpm of denatured probe per milliliter was added and further incubated overnight at 42 °C. The filters were washed at room temperature and at 42 °C in 2× SSC, 0.5% SDS for 1 h with several changes of buffer, 10 min at 65 °C in 0.2× SSC, 0.1% SDS, and then exposed overnight with two intensifying screens. Three positive clones of human homologues of rat RED1 (hRED1) were isolated from 920,000 plaques. Nested deletions on both strands were made with the Erasea-Base System (Promega) and sequenced with a Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) on an Applied Biosystems 373A sequencer according to the manufacturer's instructions.

Northern (RNA) analysis

A human multiple tissue northern blot of poly(A)$^+$ RNA (Clontech) was probed with a ^{32}P-labeled 704 bp *Hind* III/ *Pst* I fragment of the hRED1-S clone (amino acid position 281–515). Hybridization was performed in ExpressHyb Hybridization Solution (Clontech) with 2×10^6 cpm/mL probe as suggested by the manufacturer. The blot was washed for 30 min in 2× SSC, 0.1% SDS at room temperature, 30 min in 0.1× SSC, 0.1% SDS at 65 °C, and exposed with two intensifying screens for 48 h at −70 °C. The blot was reprobed with a β-actin probe to ensure equal amounts of poly(A)$^+$ RNA in all lanes, and a *Hind* III/*Pst* I fragment of the rat RED1 clone (a gift from P. Seeburg) at 55 °C as another control (data not shown).

Immunoblot analysis

A human multiple-tissue western blot (Clontech) was probed with a rabbit polyclonal anti-hRED1 serum (1:1,000; O'Connell et al., 1997). Probing and washing were done as suggested by the manufacturer. Proteins were detected with chemiluminescence staining (Boehringer, Mannheim) as described previously (O'Connell et al., 1995).

Genomic DNA amplification and sequencing

The exon/intron boundaries at the alternatively spliced region were amplified by PCR (Sambrook et al., 1989) with an automated DNA thermal cycler (Crocodile III, Appligene). One-hundred nanograms of genomic human DNA prepared from HeLa cells was used as a template in a 100-μL reaction containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.5, 1.5 mM MgCl$_2$, 200 μM each of dATP, dCTP, dGTP, dTTP), and 100 pmol of each oligonucleotide primer. The reaction mixture was heated to 95 °C for 5 min. After cooling to 80 °C, 2 units of *Taq*-DNA polymerase (Boehringer) were added to each sample and 35 cycles were performed (30 s at 94 °C, 30 s at 52 °C, and 1 min at 72 °C). The last extension cycle at 72 °C was for 5 min. The 2.4-kb PCR product amplified with the primers R1 and hSR6, and the 1.45-kb PCR product amplified with primers hSV3 and hSR6 were purified on an agarose gel (JETsorb, Genomed) and sequenced directly with primers R1, hSV3, hSV4, hSR6, and hSR7 and the Sequenase Reagent Kit (United States Biochemical Corp; Casanova, 1993). In addition, PCR was performed with the same primers on the purified fragments to determine the length of the introns.

Overexpression in the yeast *P. pastoris* and purification of hRED1

The open reading frame of the hRED1-S clone was amplified by PCR with the primers RP1 (sense) and RPHis (antisense), which contain *Spe* I sites, and cloned subsequently into these sites in the polylinker of Bluescript KS (Stratagene). To exclude PCR errors, a *Stu* I–*Mlu* I fragment (1,479 bp) was replaced by cDNA and the flanking regions were sequenced (see Fig. 1A for a restriction map). A full-length hRED1-L expression construct was generated by introducing a *Bst*E II/*Mlu* I fragment of the hRED1-L clone in the respective restriction sites of the hRED1-S construct described above. Both coding sequences were further subcloned into a *P. pastoris* expression vector (pSK-FLIS$_6$) via *Spe* I restriction sites and analyzed for the correct orientation. pSK-FLIS$_6$ is a derivative from pHIL-D2 (Invitrogen), which was engineered to encode a 5'-FLAG- and a 3'-histidine hexamer epitope. A *Spe* I site between both expression tags served as a cloning site for the protein-coding region. The resulting expression vectors pSK-FLIS$_6$-hRED1-S and -L were linearized with *Not* I and electroporated into the *P. pastoris* strain GS115 ($\tau =$ 5, 1.5 kV, 25 μF, 200 Ω). Screening for His$^+$/Mut^S phenotypes and further expression was performed according to the manufacturer's instructions (Invitrogen). Twelve His$^+$/Mut^S transformants were picked as single colonies from YPD (yeast extract peptone dextrose medium) plates and grown in 50-mL pilot cultures at 30 °C, 260 rpm. After two days, the cultures were induced with methanol for another two days. Liquid nitrogen extracts of the cells (see below) were tested for overexpression by immunodetection with polyclonal rabbit anti-hRED1 serum (1:2,500; O'Connell et al., 1997). The extracts were further tested for their ability to convert adenosines to inosines on extended dsRNA (O'Connell & Keller, 1994). One strain was used for large-scale overexpression. Twelve milliliters buffered glycerol-complex medium (BMGY; containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 × 10^{-5}% biotin, 1% glycerol) were inoculated with a single colony and incubated at 30 °C, 260 rpm for 2 days. The entire culture was used to inoculate 300 mL of BMGY in a 2-L Erlenmeyer flask (30 °C, 260 rpm, 2 days). Cells were washed with sterile water and induced in 1 L of buffered methanol-complex medium (BMMY; BMGY containing 0.5% methanol instead of glycerol) and further incubated. Because methanol evaporates from the culture, an additional 5 mL were added after 24 h. After 2 days, the cells were harvested, washed twice with 500 mL sterile water, and 250 mL buffer A/80 mM KCl (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/mL pepstatin, and 0.4 μg/mL leupeptin). Cells were resuspended in 20 mL buffer A/80 mM KCl and broken mechanically in a mortar filled with liquid nitrogen. The suspension was thawed on ice and centrifuged twice at 4 °C, 12,000 rpm for 15 min in a SS34 rotor. All further manipulations were conducted at 4 °C, and aliquots or extracts were frozen in liquid nitrogen. hRED1-S and -L were purified in parallel under the same conditions. The extracts were loaded on a 40-mL Macro-Prep High Q column (Bio-Rad) that had been equilibrated with buffer A containing 80 mM KCl. The column was washed with 10 column volumes buffer B (buffer A without EDTA) containing 80 mM KCl and developed with a 200-mL gradient from 80–500 mM KCl. The fractions were analyzed by western blot with anti-hRED1 serum. The peak fractions were mixed with 2 mL of a 50% slurry of Ni^{2+}-nitrilotriacetic acid agarose (Quiagen) equilibrated in buffer C (buffer B, 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 buffer C containing 20 mM imidazole-HCl, pH 8.0. The protein was eluted in the same buffer containing 250 mM imidazole-HCl. Fractions were dialyzed against buffer C. Protein concentrations were determined according to Bradford (1976) with BSA as a standard. In addition, aliquots of both hRED1 proteins and BSA were titrated and loaded on a 7.5% SDS polyacrylamide gel (Laemmli, 1970) stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

Activity assays

All assays were performed in buffers containing 20% glycerol and incubated at 30 °C for 1 h. The assays were repeated at least twice in duplicates. Unspecific dsRNA adenosine deaminase activity was measured by the conversion of adenosine to inosine on extended dsRNA, as described (O'Connell & Keller, 1994). An analysis of the GluR-B minigene B13 was performed by primer extension assay with the B-RT primer for the Q/R site and the BHS-RT primer for hotspot 1, as described (Melcher et al., 1995; O'Connell et al., 1997). The primer extension products were quantified on a PhosphorImager 425 (Molecular Dynamics). RT-PCR amplification and subsequent sequencing analysis was done for the R/G site in edited GluR-B pre-mRNAs on an Applied Biosystems 373A sequencer, as described (O'Connell et al., 1997).

Sequence analysis

DNA and protein sequences were aligned with the GCG software program BESTFIT with a gap weight of 3.0 (Devereux et al., 1984). The program BLAST (Altschul et al., 1990) was used to search the GenBank database; a protein secondary structure prediction was done at the e-mail server at the EMBL, Heidelberg (predictprotein@embl-heidelberg.de).

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Two forms of human double-stranded RNA-specific editase 1 (hRED1) generated by the insertion of an Alu cassette.

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