**ARD-1 cDNA from Human Cells Encodes a Site-specific Single-strand Endoribonuclease That Functionally Resembles *Escherichia coli* RNase E**

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The human **ARD-1** (activator of RNA decay) cDNA sequence can rescue mutations in the *Escherichia coli rne* gene, which specifies the essential endoribonuclease RNase E, resulting in RNase E-like cleavages in vivo in *rne*-defective bacteria and in *vitro* in extracts isolated from these cells (Wang, M., and Cohen, S. N. (1994) _Proc. Natl. Acad. Sci. U. S. A._ 91, 10591–10595). Recent studies indicate that the 13.3-kDa protein encoded by **ARD-1** cDNA is almost identical to the carboxyl-terminal end of the bovine protein NIPP-1, a nuclear inhibitor of protein phosphatase 1; separate transcripts formed by alternative splicing are proposed to encode the discrete ARD-1 and combined ARD-1/NIPP-1 products (Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W., and Bollen, M. (1995) _J. Biol. Chem._ 270, 28068–28074). Here we show that affinity column-purified protein encoded by human **ARD-1** cDNA in *E. coli* is a site-specific Mg²⁺-dependent endoribonuclease that binds in *vitro* to RNase E substrates, cleaves RNA at the same sites as RNase E, and, like RNase E, generates 5′-phosphate termini at sites of cleavage. Our results indicate that the ARD-1 peptide can function as a ribonucleolytic analog of *E. coli* RNase E as well as a domain of the protein phosphatase inhibitor, NIPP-1.

RNA degradation in eukaryotes, as in bacteria, appears to be a regulated process that involves multiple enzymatic steps (for reviews, see Refs. 3–5). Although a variety of ribonucleases have been identified in eukaryotes, with few exceptions little or no information is available about the specific biological role of these enzymes or the genes that encode them (for reviews, see Refs. 3–5). Although a variety of ribonucleases have been identified in eukaryotes, with few exceptions little or no information is available about the specific biological role of these enzymes or the genes that encode them (for reviews, see Refs. 3–5). Although a variety of ribonucleases have been identified in eukaryotes, with few exceptions little or no information is available about the specific biological role of these enzymes or the genes that encode them (for reviews, see Refs. 3–5). Although a variety of ribonucleases have been identified in eukaryotes, with few exceptions little or no information is available about the specific biological role of these enzymes or the genes that encode them (for reviews, see Refs. 3–5).

**ARD-1** cDNA, a single-strand-specific Mg²⁺-dependent endoribonuclease that binds to RNase E substrates, cleaves short oligoribonucleotides and complex substrates in A+U-rich regions at the sites cut by RNase E, and generates 5′-phosphate termini. Thus, the amino acid residues comprising the ARD-1 sequence appear to function both as a domain of the protein phosphatase inhibitor NIPP-1 and a human analog of *E. coli* ribonuclease E.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmid and Host Strain**—For expression of the histidine-tagged ARD-1 protein in *E. coli*, two oligonucleotides, 5′-ACGTG-ACTCATATGTTGCAAATCGAGTGTGTC3′ (primer 1) and 5′-TCAC-GTGCTGGATCCTCAAATCAGCAAGGTG-3′ (primer 2), correspond to the 5′-end and 3′-end of **ARD-1** cDNA sequence, respectively, were synthesized for PCR amplification of the **ARD-1** cDNA sequence. An NdeI site and a BamHI site were added to the 5′-ends of primers 1 and 2, respectively. The PCR product, after digestion with **NdeI** and **BamHI**, was ligated to the **NdeI**- and **BamHI**-cleaved pET16 expression vector (Novagen, WI), which contains a polyhistidine region and Factor Xa protease-cleavable region, and introduced into the protease-deficient **DE3** lysogenic strain BL21, which expresses T7 RNA polymerase.

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polymerase under control of an IPTG (isopropyl-1-thio-β-D-galactopyranoside)-inducible lacUV5 promoter (15, 16).

**RESULTS**

**Purification of the ARD-1 Protein**—The histidine-tagged fusion protein was purified by binding to divalent cations (Ni²⁺) immobilized on His-Bind (Novagen) metal chelation resin at 0 °C. The column was washed at 0 °C in 0.5M NaCl, 20 mM Tris-HCl, pH 7.9, containing 60 mM His-Bind (Novagen) metal chelation resin at 0 °C. The column was filtered through a 0.45-μm filter before applying to a column.

**Cleavage Assays**—**Polyacrylamide gels** with a cross-linking ratio of 37.5:1 in 100 mM Tris-HCl (pH 8.0), 50 mM glycerine buffer. Electrophoresis was carried out at 4 °C at 240 V for 3 h. The gels were dried and then exposed to x-ray film (DuPont) with an intensifier screen at −70 °C.

**Analysis of 5’ Termini Generated by ARD-1 Cleavage**—Unlabeled GGGRNA-I was synthesized and purified as described for 32P-labeled GGGRNA-I but in the absence of [α-32P]GTP. GGGRNA-I (1 μg) was incubated at 30 °C with ARD-1 (2.5 μg), full-length RNase E (0.5 μg), or RNase A (2 μg) in 50-μl reactions for 3 h, 10 min, and 15 min, respectively. The ARD-1 and RNase A reactions contained 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, and 10 mM MgCl₂. The RNase E reaction contained 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 100 mM NaCl, 5% glycerol, 0.1% Triton X-100, and 0.1 mM dithiothreitol. The ARD-1 and RNase E reactions contained 100 units of ribonuclease inhibitor. After incubation, samples were extracted with phenol/chloroform, precipitated with ethanol, and split into two aliquots. One aliquot from each sample was treated with calf intestine alkaline phosphatase (Boehringer Mannheim) to dephosphorylate the 5’ termini, extracted with phenol/chloroform, and precipitated with ethanol. The other aliquots were incubated with T4 polynucleotide kinase (the vendor). After phenol/chloroform extraction and ethanol precipitation, the RNA was resuspended in 50 μl of diethyl pyrocarbonate-treated H₂O and passed through a MicroSpin S-200 HR column (Pharmacia) to remove unincorporated radiolabeled oligonucleotides. Samples (5 μl) were mixed with formamide sequencing loading buffer (5 μl), heated for 3 min at 85 °C, and loaded in 6% sequencing gels.

**Polyacrylamide gels** with a cross-linking ratio of 37.5:1 in 100 mM Tris-HCl (pH 8.0), 50 mM glycerine buffer. Electrophoresis was carried out at 4 °C at 240 V for 3 h. The gels were dried and then exposed to x-ray film (DuPont) with an intensifier screen at −70 °C.

For RNA competition, radiolabeled RNA was first mixed with varying amounts of unlabeled RNA competitor and the samples were then centrifuged at 10,000 × g for 10 min to remove debris. The lysate was filtered through a 0.45-μm filter before applying to a column.
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a product migrating at 17 kDa, which was slower than expected from the calculated molecular mass of the 13.3-kDa protein encoded by the ARD-1 open reading frame. The observed retarded migration in gels of ARD-1, which has a pI of 10.43 and contains highly charged regions at both the NH2-terminal and COOH-terminal ends (pI = 10.43) (1), parallels the anomalous migration properties of Rne, which is a highly charged 118-kDa protein that migrates in SDS-polyacrylamide gels at the 180-kDa position (12, 24).

**Binding of ARD-1 to RNase E Substrates**—Both Northwestern and gel mobility shift assays showed that ARD-1 is an RNA-binding protein. In initial Northwestern blots using crude extracts from *E. coli* overexpressing the His/ARD-1 fusion (data not shown), a 19-kDa protein band was observed to bind to the RNase E substrates 9 S RNA, GGG-RNA I, and the BR13 oligonucleotide. Fig. 3 (*lanes 1 and 4*) shows binding of the affinity-purified His/ARD-1 fusion protein to 9 S RNA and RNA I. *E. coli* extracts of cells expressing RNase E but lacking ARD-1 cDNA contained bands that in Northwestern blots were shown to indicate the absence of a detectable contaminating band in a lane containing >100 times the amount of protein required for detection of ARD-1. The positions of protein molecular weight markers are indicated.

9 S RNA (*lanes 3 and 4*), total B cell RNA (*lanes 5 and 6*), which consists largely of ribosomal RNA, and poly(A) mRNA isolated from B cells (*lanes 10 and 11*) efficiently competed with the 32P-labeled 9 S RNA probe for binding to ARD-1. While the presence of unlabeled polyadenylic acid (poly(A)) in 10-fold excess (*lanes 7 and 8*) or tRNA in 50-fold excess (*lane 9*) did not prevent binding of ARD-1 to 9 S RNA, both of these RNAs reduced 9 S-specific binding of ARD-1 when added in still greater excess. As shown in Fig. 4A (*bottom*), the RNA-binding specificity of ARD-1 was identical to that seen for RNase E.

**ARD-1 Cleaves Oligoribonucleotides and Complex RNAs at the Same Sites as RNase E**—ARD-1 protein purified under nondenaturing conditions by affinity chromatography and shown to migrate as a single band in silver nitrate-stained gels (see above) was assayed for endoribonuclease activity on 9 S RNA, GGG-RNA I, and the BR13 and BR10 oligoribonucleotides, all of which are cleaved by purified RNase E at highly specific sites (18, 19, 22, 27–29). Control reactions that show the cleavages produced by RNase A, a common eukaryotic single-strand-specific endoribonuclease, were included for comparison; these controls demonstrated that the cleavage patterns of ARD-1 and RNase A are different, and also indicated the absence of RNase A activity in our purified ARD-1 preparations.

As shown in Fig. 5A, the affinity-purified His/ARD-1 fusion protein produced the same cleavages in 9 S RNA as RNase E,
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**FIG. 5.** Cleavage of native and synthetic RNAs by ARD-1. A. $^{32}$P-labeled 9 S RNA (30,000 cpm, 75 ng) in 20-μl reactions was incubated at 30 °C with buffer (lane 1), with 0.5 μg of purified His/ARD-1 fusion protein for 0, 1, 2, 3, 4, and 5 h (lanes 2–7, respectively) or with 100 ng of NH$_2$-terminal domain of RNase E (lane 8), or 5 pg of RNase A (lane 9) for 10 min. Samples were extracted with phenol/chloroform, mixed separately with 20 μl of sequencing loading buffer, denatured by heating at 85 °C for 3 min, and run on 6% polyacrylamide sequencing gels. The RNA products generated by ARD-1 and RNase E cleavage are shown at the right of the figure. The diagram at the bottom shows the 9 S RNA substrate, the RNase E cleavage sites, and the lengths of fragments generated by cleavage. B, radiolabeled GGG-RNA I in 20-μl reactions was incubated for 1 h with buffer at 4 °C (lane 1), buffer at 30 °C (lane 2), 0.25, 0.5, and 1 μg of ARD-1 (lanes 3, 4, and 5, respectively), 100 ng of NH$_2$-terminal domain of RNase E (lane 6), and 5 pg of RNase A (lane 7). The arrow indicates the RNA product generated by RNase E and ARD-1. After the cleavage, samples were treated as in the legend of Fig. 5, and run on 6% sequencing gels. C, BR10 substrate, 5′-ACAGUAUUUG-3′, 32P-labeled at the 5′-end (see “Experimental Procedures”) was incubated with the following: lane 1, buffer only at 4 °C for 1 h; lane 2, buffer only at 30 °C for 1 h; lanes 3–5, 0.25, 0.5, and 1 μg of ARD-1, respectively, at 30 °C for 1 h; lane 6, 100 ng of NH$_2$-terminal domain of RNase E; lane 7, 5 pg of RNase A. Lane 7 contains a ladder generated by partial digestion with mung bean nuclease, which produces 5′-monophosphate termini on products 3′ to the cleavage site (48). Plasmid RNase inhibitor, which inhibits RNase A but has no effect on cleavages produced by either ARD-1 or RNase E, was included in all ARD-1 and RNase E reaction mixtures as a precaution against possible adventitious contamination of assays by RNase A.

Although cleavage at the “a” site (30, 31) was relatively more prominent for ARD-1; an identical cleavage pattern was observed for the discrete ARD-1 peptide generated by Factor Xa treatment of the fusion protein (results not shown). GGG-RNA I was cleaved by ARD-1 at a site located 8 nt from the 5′ end, leaving a product of 103 nt (Fig. 5B), as has been observed for RNase E (18, 26, 32). While the major product resulting from RNase A digestion of GGG-RNA I was also a 103-nt species, RNase A additionally cleaved this substrate at other sites (Fig. 5B).

Analysis of ARD-1 and RNase E cleavages of two synthetic oligoribonucleotides, BR10 and BR13, containing the same sequence as the 5′ single-strand region of RNA I and GGG-RNA I, respectively (18) provided further evidence of the identical cleavage specificity of the two enzymes. McDowall et al. (18) have shown that BR10 and BR13 are each cleaved by RNase E at the intranucleotide bonds cleaved in RNA I; as seen in Fig. 5 (C and D, lane 6 of both), purified ARD-1 cleaved these oligoribonucleotides at the same sites as E. coli RNase E. However, differences in relative amounts of the products generated by the two enzymes, as indicated by the intensity of gel bands corresponding to individual RNA species, were observed. Cleavage of BR10 by ARD-1 occurred approximately equally at phosphodiester bonds located 5 and 6 nt from the 5′ end, whereas this oligonucleotide was cleaved by RNase E predominantly at a site 5 nt from the 5′ end (Fig. 5C; see also Ref. 18).

RNase A cleaved oligoribonucleotides BR10 and BR13 at different locations than either ARD-1 or RNase E (Fig. 5, C and D). Additionally, RNase A generated 5′-OH termini that could be phosphorylated without further treatment using radioactively labeled ATP (Fig. 6, lane 7), as has been shown previously (33, 49); in contrast, both RNase E (cf. Refs. 29 and 34) and ARD-1 generate 5′-phosphate termini that cannot be phosphorylated in vitro unless treated with alkaline phosphatase (Fig. 6, lanes 3 and 5). Cleavages by ARD-1 and RNase E did not occur in reaction mixtures that contain 0.01 M EDTA and lack Mg$^{2+}$, whereas cleavages by RNase A do not require divalent cations (35).

**DISCUSSION**

The human gene ARD-I was identified by the ability of ARD-I cDNA to rescue temperature-sensitive and deletion mutants of the E. coli rne gene (1), which is essential for bacterial viability as well as for RNA decay and processing (36, 37). rne-defective mutants expressing ARD-I can carry out RNAse E-like cleavages in vivo, and extracts of cells that express ARD-I but are deleted for the rne gene segment encoding the catalytic domain of RNase E (22), were observed to cleave 9 S RNA in vivo (1). Like Rne, ARD-I was inferred by DNA sequence analysis to be a highly proline-rich peptide that has similarity to segments of the small ribonucleoprotein RNA-
binding proteins (38) and to dynamin, an eukaryotic protein implicated in endocytosis, membrane trafficking, and microtubule-based organelle transport (39, 40). Analogous features of the 13.3-kDa ARD-1 and 118-kDa Rne proteins are further evident from our demonstration that ARD-1 has the same RNA binding properties as Rnase E. However, antibodies raised against Rnase E did not cross-react with ARD-1 (data not shown), consistent with the observation that these two proteins do not show regions of amino acid sequence homology (1, 12, 24).

The studies reported here show that highly purified ARD-1 protein is an endoribonuclease that, like Rnase E (19, 28, 41), cleaves single-strand RNA segments in A+U-rich regions. ARD-1 cleavage of native substrates and synthetic oligonucleotides containing Rnase E cleavage sites occurs at the same phosphodiester bonds cleaved by E. coli Rnase E. However, the relative rate of ARD-1 cleavage and Rnase E cleavage differed at different sites within some substrates. These differences provide additional evidence that the activity we assayed for affinity-purified ARD-1 preparations, which had been synthesized in E. coli and were >99% pure by silver stain analysis (Fig. 2), does not result from contamination by Rnase E.

No ARD-1 activity was detected in reaction mixtures that lack Mg²⁺ and contain 0.01 mM EDTA, indicating that ARD-1 activity, like Rnase E activity, requires divalent cations. Neither ARD-1 nor Rnase E was inhibited by placental Rnase inhibitor, which inhibits Rnase A and its analogs (35). Both Rnase E (18, 31, 32, 41) and ARD-1 (this paper) cleave single-strand segments of RNA 3’ to both purines and pyrimidines in A+U-rich regions, whereas Rnase A cuts single-stranded RNAs predominantly 3’ to pyrimidine residues (C or U nucleotides) (33).

Cleavage of RNA I by ARD-1 generates 5’-phosphate termini on the product 3’ to the cleavage site (Fig. 6), as does cleavage of RNA I and 9 S RNA by Rnase E (Refs. 29 and 34; also Fig. 6). Other endonucleases generating 5’-phosphate ends include the bacterial enzymes Rnase III and Rnase P (42, 43). In contrast, the mammalian ribonuclease, Rnase A, and analogous endoribonucleases of E. coli that degrade substrates by cleavage at a large number of sites (i.e. Rnase M, I, I*, and R) generate products that contain 5’-OH ends (33, 44).

During the course of these experiments, we observed that a small amount of ARD-1 was produced in the absence of IPTG treatment; presumably, this was due to incomplete repression. During the course of these experiments, we observed that a small amount of ARD-1 was produced in the absence of IPTG treatment; presumably, this was due to incomplete repression. We minimized these effects, and upon addition of IPTG, routinely achieved a 20–30-fold induction of ARD-1 expression.

Recent studies have shown that three regions of homology exist between the cDNA sequences of ARD-1 and NIPP-1, a nuclear inhibitor of protein phosphatase 1 (2). Two of these regions are located in a segment that corresponds to the 5’-untranslated region of ARD-1 mRNA, and are separated by a 470-bp fragment that is not present in mRNA encoding NIPP-1 (Fig. 7). On the other hand, NIPP-1 cDNA contains a specific 220-bp segment not found in ARD-1 cDNA. The remaining sequences of both cDNAs suggest that the ARD-1 polypeptide is identical to the COOH terminus of NIPP-1 (2). Chromosome mapping studies (45) have shown that the common 3’-untranslated regions of the gene(s) encoding ARD-1 and NIPP-1 are located on human chromosome 1. The sequences of the ARD-1 (1), NIPP-1 (2), and Rnase E (13, 12) proteins all encode regions that resemble the highly conserved 70-kDa RNA binding component of the U1 small ribonucleoprotein complex, which is involved in mRNA splicing in eukaryotes. Moreover, PP-1, which interacts with NIPP-1 and is the target of NIPP-1 inhibition, has been shown to have a role in both in the regulation of spliceosome assembly and in the splicing process itself (46). Thus, it is tempting to speculate that ARD-1, which we have now shown to be a site-specific single-strand endonuclease having the cleavage specificity of Rnase E, may be implicated in mRNA splicing.

Recently, a ~65-kDa enzyme having Rnase E-like cleavage specificity in vitro has been identified and partially purified from human cells (47). This enzyme and its E. coli counterpart were both shown to cleave in vitro the pentanucleotide motif (i.e. AUUU), which has been proposed as a determinant of c-myc mRNA stability in mammalian cells. The relationship of this enzyme to ARD-1 is unknown.

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FIG. 7. Schematic representation of mRNAs encoding human ARD-1 and bovine NIPP-1 proteins. See also Van Eynde et al. (2). The diagram is based on a dot blot hybridization experiment with a 125-I-labeled cDNA probe for ARD-1 and a cDNA probe for NIPP-1. The solid bars under each mRNA indicate the size of ARD-1 and NIPP-1 polypeptides.
