Purification and Characterization of MAR1

A MITOCOCHONDRIAL ASSOCIATED RIBONUCLEASE FROM LEISHMANIA TARENTOLOAE*

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A relatively thermostable 22-kDa endoribonuclease (MAR1) was purified more than 10,000-fold from a mitochondrial extract of Leishmania tarentolae and the gene cloned. The purified nuclease has a $K_m$ of 100–145 ± 33 nm and a $V_{max}$ of 1.8–2.9 ± 2 nmol/min, depending on the RNA substrate, and yields a 3′-OH and a 5′-phosphate. Cleavage was limited to several specific sites in the sub- sonate RNAs tested, but cleavage of pre-edited RNAs was generally independent of the addition of cognate guide RNA. The MAR1 gene was expressed in Escherichia coli or in L. tarentolae cells, and the recombinant protein was affinity-purified. The cleavage specificity of the recombinant enzyme from L. tarentolae was identical to that of the native enzyme. The single copy MAR1 gene maps to an 820-kilobase pair chromosome and contains one upstream of a duplex RNA region (14–16), precisely as predicted by the enzyme cascade model for RNA editing (12). Another activity, which sedimented at 15 S and was independent of added gRNA for cleavage, might correspond to an endoribonuclease activity that has been described previously in T. brucei (14). One of these activities, which sedimented at 20 S in glycerol gradients, exhibited a gRNA-dependent cleavage at the first mismatch upstream of a duplex RNA region (14–16), precisely as predicted by the enzyme cascade model for RNA editing (12). Another activity, which sedimented at 15 S and was independent of added gRNA for cleavage, might correspond to an endoribonuclease activity that has been described previously in crude mitochondrial extracts from both L. tarentolae and T. brucei (17, 18). The endoribonuclease activities in the crude extracts were both shown to cleave pre-edited Cytb mRNAs two nucleotides upstream of the first editing site. However, the T. brucei activity had specificity for the pre-edited Cytb sequence and did not to cleave the mature edited sequence.

In addition to a gRNA-dependent editing endoribonuclease, additional nucleases are presumably necessary for kinetoplastid mitochondrial mRNA maturation. There is some evidence for polycistronic transcription of the maxicircle (16, 19–22). Primary transcripts are then subjected to 5′ processing and 3′ end cleavage, followed by 3′ polyadenylation and polyuridylation. In addition, many of the mitochondrial protein-coding genes have overlapping 5′ and 3′ ends, and the maturation of such transcripts could represent an additional level of gene regulation (19). Clearly, multiple specific ribonucleases must be required for the processing and turnover of rRNAs, mRNAs, gRNAs, and tRNAs in the mitochondria of these organisms. However, to date, the only ribonuclease purified to homogeneity from kinetoplastid mitochondria is RNase H (23, 24).

In this paper we describe the isolation and characterization of MAR1 (for Mitochondrial Associated Ribonuclease) from a mitochondrial extract of L. tarentolae. MAR1 was purified to homogeneity and the MAR1 gene cloned and expressed. Further biochemical characterization and genetic analysis should help elucidate the role of this nuclease in the processing of RNAs within the mitochondrial of Leishmania.

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1 The abbreviations used are: Cytb, cytochrome b; gRNA, guide RNA; MSB, mitochondrial storage-breakage buffer; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; Tricine, N-[2-hydroxyethyl]2-hydroxyethyl]glycine; DTT, dithiothreitol; RT-PCR, reverse transcription-polymerase chain reaction; sRNA, spliced leader RNA; bp, base pair(s); kb, kilobase(s); CHEF, contour-clamped homogeneous field electrophoresis; PER, pre-edited region.
**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—** *L. tarentolae* UC strain cells were grown in brain heart infusion (Difco) supplemented with hemin (1.6 μg/ml) (Sigma). For small-scale mitochondrial preparations (1–2 liters of culture or less), cells were grown with rotation in a Cell Production Roller Apparatus (Bellco) at 27 °C. For large-scale mitochondrial preparations (15 liters), cells were grown in a BioFlo IV fermentor (New Brunswick Scientific). The cells were harvested with a Masterflex tangential filter apparatus (Millipore). For purification of mitochondria, cells (1.5–1.8 × 10^6 cells/ml) were harvested by centrifugation at 5000 × g for 10 min at 4 °C. Cells were lysed in hypotonic Tris-EDTA, and the mitochondria were purified by isopycnic flotation in Renografin gradients as described previously (25). The mitochondrial fractions had less than 5% cytosolic contamination. Mitochondria were stored at −70 °C in mitochondrial storage buffer (MSB) containing 50 mM HEPES (pH 7.5), 50 mM KCl, and 10% glycerol. Protein assays were performed using the BCA assay (Pierce).

**Extraction Procedure—** Renograin-purified mitochondria (1.0 g/ml, wet weight) were thawed on ice and solubilized by adding MSB buffer containing CHAPS and ammonium acetate (10 and 500 mM final concentration, respectively). A protease inhibitor mixture (0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 0.01 μg/ml chymostatin, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride) was also added to the mitochondrial extract, which was kept at 4 °C for 30 min and then clarified at 12,000 × g for 20 min. The extract was then heated at 55 °C for 20 min. This heating step causes a large percentage of the proteins to coagulate while MAR1 remains in solution. The heated extract was further clarified by centrifugation at 100,000 × g in a Beckman TLA-100.1 rotor at 100,000 × g for 1 h in a Beckman Optima tabletop ultracentrifuge. The supernatant (S-100) was saved and the pellet discarded. The S-100 (100 ml) was dialyzed against 4 liters of MSB buffer and either stored at −70 °C or immediately used for MAR1 purification and analysis. Storage at −70 °C for long periods of time (1–3 months) produced no loss of nucleic activity.

**MAR1 Purification—** The S-100 supernatant (in MSB buffer) was loaded to a Mono Q HR5/5 (anion exchange) column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min at 4 °C. After loading, the column was extensively washed with 30 ml of MSB containing 1 mM CHAPS (QA buffer) until no more detectable protein eluted. The column was developed with a linear gradient of 20 ml of QA buffer (QA buffer containing 1 mM KCl). Individual fractions (0.5 ml) were assayed for endoribonuclease activity. Fractions containing the peak of nuclease activity were pooled, concentrated with an Ultra-Free 15 concentrator (Millipore), and loaded onto a Superose 12 column (Amersham Pharmacia Biotech) (equilibrated with 0.1 M triethanolamine (pH 8.0)). The Superose 12 column was developed with 30 ml of 0.1 M triethanolamine (pH 8.0) at a flow rate of 0.3 ml/min. Fractions containing the peak of nucleic activity were pooled and loaded onto a Mono P HR5/5 column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min at 25 °C. Protein bands on the Mono P column were eluted with 20 ml of Polybuffer 74 (Amersham Pharmacia Biotech) (pH 5.0), allowing a pH gradient to form in the column. Individual fractions (0.5 ml) were assayed for endonuclease activity and peak fractions pooled and concentrated with an Ultra-Free 15 concentrator. The Mono P-pure MAR1 was gravity-loaded onto a PD-10 column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min at 25 °C. Protein bands on the PD-10 column were eluted with 20 ml of Polybuffer 74 (Amersham Pharmacia Biotech) (pH 7.0). The samples were pelleted at 100,000 × g for 1 h at 4 °C for 30 min. The pellets were washed with 1 ml of 70% ethanol, dried under vacuum, and resuspended in desaturing buffer (8 M urea/0.5% SDS, 0.3% xylene cyanol, 0.3% bromphenol blue, 2 mM EDTA, 10 mM Tris-HCl (pH 8)). The samples were heated at 95 °C for 5 min and electrophoretically separated in 7% urea/6% (or 10%) acrylamide gel at 250 V. The band was visualized by UV (256 nm) shadowing on a Molecular Dynamics. For analysis of the initial velocity kinetic constants, increasing concentrations of 5'-labeled synthetic mRNA substrates were incubated as above with constant concentration of enzyme (10 ng). Reactions were carried out at 27 °C for 30 min, and samples were processed as above. The velocity of conversion of full-length mRNA to cleaved products was calculated and plotted as a function of substrate concentration. These data were plotted as double-reciprocal plots, allowing for calculation of the first order rate constants (K_m and V_max) for the various substrates.

**Oligodeoxynucleotides—** Oligodeoxynucleotide primers for PCR amplification, RT-PCR, hybridization, and primer extension assays were synthesized with standard phosphoramidite methods (Life Technologies, Inc.) and purified by electrophoresis in an 8 M urea/8% polyacrylamide gel at 250 V. The band was visualized by UV (256 nm) shadowing on a Molecular Dynamics. For analysis of the initial velocity kinetic constants, increasing concentrations of 5'-labeled synthetic mRNA substrates were incubated as above with constant concentration of enzyme (10 ng). Reactions were carried out at 27 °C for 30 min, and samples were processed as above. The velocity of conversion of full-length mRNA to cleaved products was calculated and plotted as a function of substrate concentration. These data were plotted as double-reciprocal plots, allowing for calculation of the first order rate constants (K_m and V_max) for the various substrates.

**Peptide Isolation and N-terminal Sequencing—** The purified protein was electrophoretically separated on a 10% acrylamide gel (Bio-Rad). The purified protein was electroblotted onto a polyvinylidene difluoride membrane and visualized by staining for 1 min with 0.1% Coomassie Blue R-250 in 50% methanol, followed by 2-min washing with 50% methanol. The portion of the membrane containing the stained band was excised and subjected to N-terminal sequencing on an Applied Biosystem 477A automated sequencer. The following sequence was obtained: MPRLMHPYSTSKATFLGVDLQCAG.

**Nested RT-PCR Reactions—** Based on the 24 N-terminal amino acid sequence obtained from the purified MAR1 protein, the degenerate oligonucleotides S-2323 (GACQLDV) and S-2255 (QLDVGFLATK), which were complementary to MAR1 mRNA, were used in a nested RT-PCR reaction to obtain the 5' sequence. S-2323 was used as primer for the synthesis of the first-strand cDNA in a reaction containing 1 μg of poly(A)^+ RNA from *L. tarentolae*; 120 pmol of S-2323 and 200 units of Superscript RT-II (Life Technologies, Inc.) in a 20-μl reaction volume, according to the manufacturer's instructions. A 2-μl aliquot of the cDNA reaction was used as template in a PCR reaction containing 100 ng S-2323 and the *L. tarentolae* spliced leader RNA (sRNA)-specific primer, S-2273. Amplification of the MAR1 5' sequence was performed in a 100 μM final concentration of dNTPs, 2.5 units of Taq polymerase, in a final volume of 100 μl. The sample was denatured for 2 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 40 °C for 0.5 min, and extension at 72 °C for 1 min. An aliquot of the first PCR reaction (5 μl) was used as a template for a second PCR reaction containing the degenerate primer S-2255 and the sRNA-specific primer, S-2257, as
described above. The PCR products were separated in a 2% agarose-TAE (0.04 M Tris acetate, 0.001 M EDTA (pH 8.0)) gel containing 0.5 μg/ml ethidium bromide and visualized by UV light. A 130-bp DNA fragment was cloned into the pGEM-T Easy vector (Promega), according to the manufacturer’s instructions, and transformed into Escherichia coli DH5α competent cells. Insert-containing plasmids from several E. coli clones were sequenced.

**DNA Ligation and Colony Hybridization**—DNA ligations were performed as described (Invitrogen) using the pCR 2.1-TOPO cloning kit (Invitrogen). Colony hybridization experiments were performed as described (26).

**Northern Analysis**—A Northern blot of 6 μg of total RNA and 2 μg of poly(A)^+ RNA isolated from *L. tarentolae* cells was performed in a 18% formaldehyde/1.5% agarose gel (16 h, 1.7 V/cm). The RNA was transferred to a Zeta-Probe membrane (Bio-Rad), which was hybridized with either end-labeled oligonucleotide primers or the random-primed MAR1 probe (7A) (Prime-IT II kit, Stratagene).

**DNA Sequencing and Generation of Nested Deletions**—DNA sequencing of cloned inserts was performed manually by the dideoxy-terminating reaction with 10 pmol of plasmid DNA template and Sequenase V2.0 (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. The sequencing products were then separated in an 8 % urea/6% polyacrylamide gel and visualized by autoradiography. The *L. tarentolae* 3 kilobase pair genomic fragment containing the MAR1 gene sequence was cloned into the EcoRI site of the SK (+) vector (Stratagene), generating pBI-MAR1. The pBI-MAR1 plasmid was linearized by digestion with KpnI and EcoRI, to generate an Eco-III-resistant restriction site and an Eco-III-sensitive site. Ten 400-bp nested deletion fragments were generated with the Erase-A-Base kit (Promega), according to the manufacturer’s instructions. Five clones from each deletion time point were sequenced by the Applied Biosystems-Perkin-Elmer automated sequencer (Applied Biosystems-Perkin-Elmer). This approach ensured the sequencing of each nucleotide from at least three independent clones in both directions.

**Contour-clamped Homogeneous Field Electrophoresis (CHEF) — *L. tarentolae* genomic DNA blocks were prepared according to Van der Ploeg (27) and separated in 1% agarose-1/2 TBE in a CHEF apparatus (28) at 200 V for 15 h with 1-min pulses, followed by electrophoresis for 9 h with 90-s pulses. The chromosomal bands were visualized by ethidium bromide staining and the gel photographed using a C-80 Epi-illumination UV Darkroom (Ultraviolet Products). The DNA was transferred to a Zeta-Probe membrane (Bio-Rad), according to the manufacturer’s instructions and hybridized by the MAR1 gene random-labeled using the Prime-IT kit (Stratagene).

**Mapping the 5’ and 3’ Termi- nus of the Mixture** The 5’-terminus of the MAR1 mRNA was defined by RT-PCR in a reaction containing the oligonucleotides S-2474, which is antisense to the MAR1 mRNA, and S-2273, which corresponds to a part of the spliced leader sequence. The PCR products were separated in 2% agarose-TAE and cloned into pCR 2.1-TOPO (Invitrogen), and transformed into competent E. coli DH5α cells. The 5’-untranslated sequence of the MAR1 transcript was performed by a modification of the procedure described above.

cDNA synthesis from poly(A)^+ RNA was performed using an oligo(dT) primer, S-2271, and Superscript II RT (Life Technologies, Inc.). The PCR products were separated in 2% agarose-TAE and cloned into pCR 2.1-TOPO (Invitrogen), and transformed into competent E. coli DH5α cells. The 5’-untranslated sequence of the MAR1 transcript was performed by a modification of the procedure described above.

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**Results**

**Optimum Temperature and Divalent Cation Requirements**—In crude mitochondrial extracts the optimal temperature for the MAR1 reaction is 27 °C (data not shown). However, the activity was relatively resistant to heating at 55 °C for 20 min, and the thermal stability was independent of the presence of RNA substrate. As shown in Fig. 1A, 70% of the activity in the crude extract remained in the supernatant after the 55 °C heating step. A similar stability against thermal denaturation was observed with purified MAR1 (data not shown), indicating that this is an intrinsic characteristic of the enzyme and not due to some unidentified factor.

**MAR1 has an absolute requirement for divalent cations (Fig. 1B).** When the chloride salts of Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+} were used in the cleavage reaction, Mg^{2+} (up to 10 mM) was the preferred cation, but Mn^{2+} also could satisfy this requirement, while Zn^{2+} and Ca^{2+} worked to a much lesser extent.

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**L. tarentolae Mitochondrial Ribonuclease**

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**Sequencing**—The available sequence data bases (GenBank™ nonredundant data base and Swissprot) were searched using the predicted polypeptide deduced from the MAR1 open reading frame. The search programs employed were BLASTP (National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/index.html), using a Blosum62 data matrix, and BLITZ (European Bioinformatics Institute at http://www.ebi.ac.uk/searches/searches.html). The program CLUSTALX was used to align the amino acid sequence of *L. tarentolae* MAR1 with those of *Caenorhabditis elegans* and *Arabidopsis fulgidus*. Manual refinements were performed to optimize the alignments, taking into consideration the predicted secondary structure obtained from the individual polypeptides. Predictprot Protein (PHDsec) (http://www.ebi.ac.uk/searches/searches.html) was used to perform the secondary structure predictions. The Monte Carlo algorithm implemented in the PHDsec program (GCG package) on a VAX 4000 computer was used to evaluate the statistical significance of the alignments. The result is shown as S.D. values (standard deviations from the mean of randomized sequences) of 200 rounds of randomization with a ktuple of 2. The isoelectric point of MAR1 was determined with the programs ISOELECTRIC (GCG package) and MultiIdent (http://expasy.hcuge.ch/www/EXPASY-TOP.html). The N terminus amphiphilic nature of MAR1 and C. elegans sequences was determined with the program HELICAL-WHEEL (GCG package).

**Expression and Purification of the Recombinant MAR1 Protein and Generation of Polyclonal Antiserum**—The MAR1 gene was used to express a histidine-tagged (His-tagged) version of the MAR1 protein in E. coli cells or an epitope-tagged version in *L. tarentolae* cells. For E. coli expression, the plasmid was transformed into competent E. coli cells and the MAR1 gene was cloned into a pET-31 vector (Qiagen), which places the gene under the control of an isopropyl-β-D-thiogalactopyranoside-responsive promoter. E. coli BL21 cells were grown in 2 × YT medium, and recombinant protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (2 mM final concentration) as described in the Qiagen manual. The His-tagged recombinant MAR1 protein was purified by metal-chelate chromatography (Qiagen).

Recombinant MAR1 protein expressed in E. coli was used for the production of polyclonal antisera in rabbits (Animal Pharm Services, Inc.). A 1:10,000 dilution of serum, from the seventh cycle of immunization, was used for Western analysis.

For expression in *L. tarentolae*, the MAR1 gene was ligated into the pIMARFLAG vector, a PCR reaction is performed with primers, S-2271 and S-2272, in a standard PCR amplification which are complementary to the 5’ and 3’ end, respectively, of the MAR1 coding region. These oligonucleotides were designed to insert a BamHI site at the 5’ end and an XbaI site at the 3’ end of the MAR1 coding sequence. In addition, the 3’ oligomer, S-2545, carries the sequence coding for the FLAG-epitope (N-Asp Tyr Lys Asp Asp Asp Asp Lys-C) (Eastman Kodak Co.). The PCR product generated by amplification of MAR1 with oligonucleotides, S-2545 and S-2546, was digested with BamHI and XbaI and cloned between the BamHI and XbaI sites of pX, generating the plasmid pXMARFLAG. The pXMARFLAG plasmid was used to transfect *L. tarentolae* cells. Transformants were selected by the G418-resistant phenotype provided by the plasmid-derived marker (29). Two liters of pXMARFLAG-transformed *L. tarentolae* cells were grown in brain heart infusion medium containing 500 μg/ml of G418 for 14 h at 27 °C and harvested as described above. The pellet was suspended in 50 mL of MSB and sonicated at medium power. The extract was centrifuged at 100,000 × g and loaded onto a 1-ml FLAG-affinity column (Kodak). The cleavage activity and specificity of the recombinant MAR1 were determined as described above.
Purification of MAR1—Due to the relative thermal stability of MAR1, heating of a 12,000 $\times g$ for 20 min clarified CHAPS lysate at 55 °C was used as the initial step in purification (Table I), followed by centrifugation at 100,000 $\times g$ for 1 h. The activity at 27 °C was taken as the 100% value. B, divalent cation requirement for the MAR1 reaction. Reactions were performed with constant concentrations of MAR1 (50 ng), saturating concentrations of substrate (500 nM), and increasing concentrations of the chloride salts of Mg$^{2+}$ (□), Mn$^{2+}$ (○), Zn$^{2+}$ (○), and Ca$^{2+}$ (△). Relative activity, expressed as the percentage of the substrate cleaved in 1 h at 27 °C, was plotted versus the different cation concentrations.

**TABLE I**

| Fraction       | Total protein | Total activity | Specific activity | Fold purification | Yield % |
|----------------|---------------|----------------|-------------------|-------------------|---------|
| Crude          | 1425.0        | 21090.0        | 0.01              | N/A               | 100     |
| S-100          | 43.0          | 465.7          | 0.2               | 25                | 2.0     |
| Mono Q         | 2.0           | 250.0          | 125.0             | 12500             | 1.2     |
| Superose 12    | 0.6           | 85.0           | 141.0             | 14100             | 0.4     |
| Mono P         | 0.4           | 66.7           | 166.7             | 16675             | 0.3     |

**Kinetic Analysis of MAR1 and Cleavage Specificity**—To make an initial analysis of the cleavage specificity of MAR1 and also to examine the possibility that MAR1 is involved in the cleavage activity of the heated S-100 supernatant. The column was equilibrated with QA buffer and developed with a linear gradient of QB buffer. The shaded area represents the elution of the peak of cleavage activity. B, chromatogram of the Superose 12 fraction on a Mono P chromatofocusing column. The shaded area represents the peak of cleavage activity. The $A_{280}$ of each fraction is plotted. The peak of activity corresponds to pH 6.5–6.8. C, silver-stained 10% SDS-acrylamide gel of various fractions in the purification of MAR1. S-100 refers to the heat-treated supernatant. Mono Q, Superose 12, and Mono P refer to the peak fractions at each chromatographic step. The arrow (−) denotes the position of the 22-kDa MAR1 band.
RNA editing, cleavage sites were mapped on two different pre-edited mRNA substrates (ND7 and Cyb) and on one fully edited mRNA substrate (Cyb), using the biochemically purified FIG. 3.

**Mapping of the MAR1 cleavage sites on pre-edited ND7 and pre-edited and fully edited Cyb mRNA substrates.**

A, synthetic ND7 pre-edited mRNA was 5' end-labeled with \([\gamma-32P]\)ATP. The labeled RNA was used as a substrate for MAR1 cleavage in the presence (lanes 9–11) or absence of gRNA (lanes 6–8), and in the presence (lanes 6–11) or absence of MAR1 (lane 1).

B, 3' end-labeled synthetic pre-edited Cyb mRNA (lanes 1 and 2) and fully edited Cyb mRNA (lanes 3 and 4) were incubated in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of MAR1. Arrows indicate major cleavage sites. The length in nucleotides (nt) of various markers is shown on the left. An asterisk denotes the position of the radioactive label on the various substrates. The locations of the PER and the cleavages within the 3'-unedited region are indicated by brackets.

C, FLAG-tagged recombinant MAR1 enzyme, affinity-purified from transfected *L. tarentolae* cells, was used with the identical PE and FE RNA substrates as in B.

**Fig. 4. Kinetic analysis of the MAR1 cleavage reaction.** Increasing concentrations (6–80 nM) of 5'-labeled pre-edited ND7 mRNA (A), pre-edited Cyb (B), and fully edited Cyb mRNA (C) were incubated with 10 ng (9.2 μM) MAR enzyme at 27 °C for 30 min. The fraction of the various concentrations of input RNA cleaved was determined by PhosphorImager analysis of the dried gels. Fraction cleaved (cleaved product/cleaved product + uncleaved × 100/reaction time) was used to calculate the amounts of cleaved product made per min. Double-reciprocal plots of velocity versus substrate concentration were used to calculate the *K_m* and *V_max* for the MAR1 enzyme under initial velocity conditions.
enzyme and 5’ or 3’ end-labeled substrates. As shown in Fig. 3A, two major cleavage sites were located at editing site 2 (ES2) and editing site 3 (ES3) of pre-edited ND7 mRNA. Another major cleavage site was located just upstream of editing site 7 (ES7), and five additional cleavage sites were located downstream of the pre-edited region (PER). The locations of the major cleavage sites provided no evidence for sequence specificity, but it is also apparent that the digestion is not random. Digestion with a large excess of enzyme for extended periods led to complete cleavage of the substrate to short oligomers (8–10 nucleotides) (data not shown).

Also shown in Fig. 3A, the addition of excess cognate gRNA for block I of ND7 did not induce a cleavage at ES1 as would be predicted for a nuclease involved in the initial step of gRNA-mediated editing. Instead, the cleavages at ES2 and ES3 were inhibited and the major cleavage upstream of ES7 was unaffected, as were the downstream cleavages.

In the case of the pre-edited Cyb substrate, the precise cleavage sites were not determined. However, major cleavages located at or near ES1 and at several sites within the 3’ region of the RNA downstream of the ER were observed (Fig. 3B). A 5’ end-labeled substrate yielded identical results as the 3’ end-labeled substrate shown (data not shown). Use of a fully edited Cyb substrate led to the disappearance of one of the 3’ cleavages, but there was no effect on the ES1 cleavage. To test the possibility that there could still be a substrate preference for pre-edited mRNAs, a kinetic analysis was carried out under initial velocity conditions. With all of the substrates tested, MAR1 cleavage activity followed Michaelis-Menten kinetics with a $K_m$ of 120 ± 15 nM and a $V_{max}$ of 2.8 ± 0.56 nmol/min for the pre-edited Cyb mRNA (Fig. 4B) and a $K_m$ of 100 ± 10 nM and $V_{max}$ of 2.9 ± 0.5 nmol/min for the fully edited version of the same mRNA (Fig. 4C). Similar values for $K_m$ and $V_{max}$ were obtained (Fig. 4A) with the pre-edited ND7 substrate. We conclude that both pre-edited and fully edited RNAs were cleaved by MAR1 with comparable efficiency.

Cloning and Sequencing of the MAR1 Gene—A 24-amino acid N-terminal sequence (MPRLMPHYSTSKTAFLGVDLQCAG) was obtained by microsequence analysis of the purified MAR1 protein. Based on this sequence, the degenerate S-2255 3’ primer and the slRNA-specific S-2273 5’ primer. A 130-bp DNA fragment was cloned and sequenced. The sequence of the 130-bp fragment revealed three amino acid differences (MPRLMPHYSTSKTAFLGVDLQCAG)
(shown in boldface letters) from that of the N-terminal sequence obtained from microsequencing. These amino acid differences are toward the C terminus of the peptide and represented ambiguous amino acid assignments. However, the internal deduced amino acid sequence upstream of primer S-2255 (STS6KTA) confirmed the identity of the cloned fragment (Fig. 5).

The 130-bp PCR product hybridized with a 3-kb EcoRI-BamHI genomic fragment. The 3-kb region of EcoRI-BamHI-digested genomic DNA was cloned into the pGEM-7Zf(+) vector. The MAR1-containing plasmid was identified by colony hybridization with the 5′-labeled MAR1-specific S-2356 oligonucleotide and the sequence of the insert DNA determined.

**Fig. 6. Expression of the FLAG-tagged MAR1 in L. tarentolae cells.** A, electrophoretic separation, on a 10% acrylamide/Trice-SDS gel, of various fractions from an anti-FLAG affinity column. The gel was stained with Coomasie Brilliant Blue. Lane 1, cell-free extract prior to loading on the affinity column. Lane 2, the eluant fraction from the column loading. Lane 3, the column was washed with “low salt” buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl. Lane 4, “high salt” wash with 50 mM Tris (pH 8.0), 400 mM NaCl. Lane 5, 0.1 M glycine (pH 4.0) elution. M denotes the size markers used during electrophoresis. B, Western blot of the recombinant MAR1 expressed in L. tarentolae cells and the native MAR1. In lanes 1 and 2, recombinant MAR1 was electrophoretically separated on 10% tricine-SDS acrylamide gels, and the gels were electroblotted onto nitrocellulose membranes and probed with polyclonal MAR1 antiserum. Lane 1, cell-free extract prior to electrophoresis. Lane 2, monoclonal antibody against the FLAG epitope (Kodak) (lane 2). An identical Western analysis of total cell extract from L. tarentolae using the polyclonal MAR1 antiserum is shown in lane 3. The position of the MAR1 protein is indicated by a double arrow.

This sequence contains a single open reading frame of 579 nucleotides that encodes for a protein of 192 amino acids with a molecular mass of 21.6 kDa and a predicted isoelectric point of 7.38 (Fig. 5). RT-PCR of the 5′ region of the transcript indicated the presence of two alternative splice acceptor sites for the trans-spliced 39-nt sRNA (data not shown). One site (SAS-1) is located at position −33 from the adenylate residue of the predicted methionine translation initiation codon (boxed in Fig. 6) and is preceded by a 10 nucleotide polypyrimidine track at positions −57 to −48. The second splice acceptor site (SAS-2) is located at position −123, with an upstream polyuridylic track at positions −157 to −149 (Fig. 5). Primer extension assays of total RNA using the 5′ end-labeled S-2474 primer revealed that SAS-1 is used for splicing of the sRNA in 60% of the transcripts, and SAS-2 is used in the remaining 40% (data not shown). RT-PCR analysis of the 3′ end of MAR1 revealed two polyadenylation sites, one at position +1003 and another at position +1143, 424 and 564 nucleotides, respectively, from the second adenylate residue of the predicted TAA termination codon. These results would predict a transcript ranging from a maximum of 1305 nucleotides to a minimum of 1075 nucleotides (not including the length of the poly(A) tail).

**Recombinant MAR1 Shows Identical Specific Activity and Cleavage Specificity as Native MAR1—**The MAR1 gene was expressed in E. coli as a His-tagged protein and purified by affinity chromatography. The specific activity of the E. coli-expressed MAR1 was 200-fold lower than that of the native enzyme (data not shown). However, expression in E. coli allowed the production of sufficient recombinant MAR1 protein to generate anti-MAR1 polyclonal antibodies in rabbits.

To circumvent the low specific activity of the bacterial expressed MAR1, an epitope-tagged (FLAG tag) version of the MAR1 protein was expressed in L. tarentolae cells (see “Experimental Procedures”). The recombinant protein was purified using an antibody-affinity column (Kodak). This expression approach yielded apparently homogeneous MAR1 protein as determined by SDS-polyacrylamide gel electrophoresis (Fig. 6A), as well as by Western analysis using either anti-MAR1 antiserum or anti-FLAG antibody (Kodak) (Fig. 6B). The anti-MAR1 antiserum recognized in a total cell lysate of L. tarentolae a single band of the identical gel mobility as the biochemically purified MAR1 and the recombinant MAR1 proteins (Fig. 6B, lane 3).
The recombinant epitope-tagged protein expressed in *L. tarentolae* had a specific activity (160 ± 1.7 nmol/min/mg) and a cleavage specificity that was indistinguishable from the native enzyme (Fig. 3C), confirming that the MAR1 gene encodes the *MAR1* nuclease.

**MAR1 Is a Single Copy Gene That Maps to an 820-kb Chromosome.** *L. tarentolae* DNA was digested with a variety of restriction enzymes and the blots hybridized with the full-length MAR1 probe. The enzymes, *Bgl II*, *Hin fI*, and *Nco I*, which cleave once within the *MAR1* gene (data not shown). The enzymes, *Bgl II*, *Hin fI*, and *Nco I*, generate a mitochondrial importation signal in *C. elegans* (Fig. 6), and its absence in the *A. fulgidus* sequence is consistent with this N terminus sequence representing the characteristic amphipathic pattern of known importation signal sequences (data not shown).

Three genes were identified as possible homologues of MAR1: *C. elegans* (GenBank™ accession number Z69637), *B. subtilis* (GenBank™ accession number P37532), and *Archaeoglobus fulgidus* (GenBank™ accession number AE000943). These sequences are unidentified open reading frames. The amino acid alignments showed a high degree of similarity to the MAR1 sequence with the *B. subtilis* sequence limited to the short region shown boxed region in A). C. Table of S.D. values resulting from a Monte Carlo statistical analysis of the different sequences (>5 S.D. units is considered a significant match).

**DISCUSSION**

*MAR1* is the first mitochondrial endonuclease purified from a kinetoplastid protozoan. Isolation of this enzyme was facilitated by its high relative thermal stability, which allowed the use of an initial heating step to remove a large amount of protein prior to chromatography on three columns. The puri-
fied MAR1 has an absolute requirement for divalent cations and migrated in an SDS acrylamide gel as a 22-kDa band. This enzyme may correspond to previously described mitochondrial endoribonucleases from *L. tarentolae* and *T. brucei* (17, 18). The *L. tarentolae* nuclease activity had an estimated molecular mass between 10 and 30 kDa, had an absolute divalent cation requirement, and showed a major cleavage site of pre-edited Cyb mRNA 2 nucleotides upstream of editing site 1 in addition to several other cleavages throughout the PER (18). However, the *L. tarentolae* activity also showed a stimulation by heparin or by digestion of the crude lysate with proteinase K and an inhibition by adenylate nucleotides or GTP. The *T. brucei* nuclease activity showed a relatively high thermal stability, had a specificity for sites within the PER’s of Cyb, cytochrome oxidase II, and cytochrome oxidase III mRNA substrates, and did not cleave within the fully edited regions of the same RNAs (17). Both activities were only characterized from crude mitochondrial lysates.

Purified MAR1 cleaved a synthetic pre-edited Cyb substrate at approximately the same location as the previously described activities, but showed additional cleavages 5’ and 3’ of the PER. The MAR1 enzyme also did not distinguish between pre-edited and fully edited Cyb mRNA substrates and was unaffected by the addition of heparin and was sensitive to proteinase K (data not shown). Although these differences could mean that MAR1 and the previously described nucleases are different enzymes, they could also reflect structural differences between the Cyb RNA substrates used in the cleavage reactions and could be due to the fact that the previous nuclease activities were detected and characterized in crude mitochondrial lysates, whereas this study was performed both with a highly purified and a recombinant enzyme.

The demonstration that the affinity-purified recombinant FLAG-tagged MAR1 protein expressed in *L. tarentolae* exhibited nearly identical specific activity and cleavage specificity to that of the native enzyme provides definitive evidence that the MAR1 gene encodes the MAR1 enzyme.

The cleavage of the Cyb and ND7 pre-edited mRNA substrates by MAR1 occurred in a gRNA-independent fashion. Interestingly, when increasing concentrations of a cognate gRNA were annealed to the ND7 substrate mRNA prior to the cleavage reaction, an inhibition of the cleavage adjacent to the first editing site was observed. The presence of several gRNA-independent endoribonucleases in a mitochondrial extract from *T. brucei* has been described previously (14, 15, 31). One of these activities co-sedimented with other editing activities in glycerol gradients, but was separable from the gRNA-dependent cleavage believed to be the editing-specific nuclease (14). This activity required DTT to function in vitro and cleaved pre-edited mRNAs in the PER. MAR1 shows a similar cleavage and fractionation behavior as the DTT-requiring enzyme, but has no requirement for DTT (data not shown).

The lack of specificity for pre-edited mRNAs and the lack of a gRNA-dependent cleavage at ES1 both suggest that MAR1 may not be involved in RNA editing. However, it should be pointed out that additional, yet undetermined, specificity factors could confer gRNA-dependence to MAR1 and this must remain an open question. Also, an *in vitro* RNA editing-like activity independent of gRNA but dependent on the secondary structure of the mRNA substrate has been described in mitochondrial extracts from *L. tarentolae* (32, 33), and an involvement of MAR1 in this process still remains a possibility.

MAR1 is a mitochondrial protein that lacks a cleavable N-terminal mitochondrial targeting sequence. We presented evidence that the 18 N-terminal amino acids represent a non-cleaved signal sequence, but this must be confirmed by direct experimentation. Cytochrome c1 is the only other known example of a trypanosome protein targeted to the mitochondrion without a cleavable presequence (34). The MAR1 protein sequence is fairly conserved in evolution, as homologues were found in a eukaryote and an archaeabacterium, and a conserved motif was found in a eubacterial protein. The homologues, however, could not be used to determine a function, as the sequences are unidentified reading frames.

As stated above, the possibility of MAR1 being involved in RNA editing remains open, but it is equally likely that MAR1 could be involved in mitochondrial RNA turnover. The expression of an epitope-tagged MAR1 in *E. coli* and in *L. tarentolae* has permitted the generation of an anti-MAR1 antiserum. Use of this immune reagent together with a detailed kinetic characterization of the cleavage reaction should help answer further questions about the specificity, substrate recognition, and mechanism of action of this nuclease.

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