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Purification and Characterization of MAR1

A MITOCHONDRIAL ASSOCIATED RIBONUCLEASE FROM *LEISHMANIA TARENTOLAE**

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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 substrate 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 an open reading frame of 579 nucleotides. The 18-amino acid N-terminal sequence shows characteristics of an uncleaved mitochondrial targeting sequence. Data base searching revealed two homologues of MAR1 corresponding to unidentified open reading frames in *Caenorhabditis elegans* (GenBank™ accession number Z69637) and *Archaeoglobus fulgidus* (GenBank™ accession number AE000943). The function of MAR1 in mitochondrial RNA metabolism in *L. tarentolae* remains to be determined.

The processing of mitochondrial RNAs shows great variation between species. In mammalian mitochondria, both DNA strands are completely transcribed, and the transcripts are processed by the excision of interspersed tRNAs (1, 2). In *Saccharomyces cerevisiae* mitochondria, 5′ end maturation of tRNAs is carried out by a mitochondrial RNase P (3), and RNA degradation may involve a 3′-5′ exonuclease activity (4). The 3′ end processing of the cytochrome *b* (Cyb)[1] mRNA in yeast is mediated by the product of the nuclear gene *CBT1* (5). In plant mitochondria, inverted repeats at the 3′ ends of protein-coding genes serve as processing signals for 3′ end maturation (6), and RNase Z has been identified as the nuclease responsible for the specific processing of the 3′ ends of tRNAs (7).

In the kinetoplast mitochondrion of the trypanosomatid protozoa, *Leishmania tarentolae* and *Trypanosoma brucei*, the maxicircle DNA encodes two rRNA genes and 18 potential protein-coding genes, 12 of which are cryptogenes whose transcripts are edited by the insertion and deletion of uridine residues usually within coding regions (8–11). RNA editing reactions appear to be initiated by specific cleavages of the pre-edited mRNAs, mediated by base pairing with specific cognate guide RNAs (gRNAs) (12, 13). Little is known about the processing and turnover of mitochondrial RNAs in these cells, either in terms of specific cis-acting signals or enzymatic activities. Three different endoribonuclease activities, separable by sedimentation or anion exchange chromatography, have been identified in a mitochondrial extract from *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 Cyb mRNAs two nucleotides upstream of the first editing site. However, the *T. brucei* activity had specificity for the pre-edited Cyb 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 polyuridylylation. 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 mitochondrion of *Leishmania*.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF083881.

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[1] The abbreviations used are: Cyb, cytochrome *b*; gRNA, guide RNA; MSB, mitochondrial storage-breakage buffer; CHAPS, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol; RT-PCR, reverse transcription-polymerase chain reaction; slRNA, 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 μM) (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 ($1.5–1.8 \times 10^8$ cells/ml) were harvested by centrifugation at $5000 \times g$ 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-breakage buffer (MSB) containing 50 mM HEPES (pH 7.5), 50 mM KCl, and 10% glycerol. Protein assays were performed using the BCA assay (Pierce).

Extract Preparation—Renografin-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 pepstatin, 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 $12,000 \times g$ 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 $100,000 \times g$ in a Beckman TLA-100.4 fixed angle rotor for 1 h in a Beckman Optima tabletop centrifuge. 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 nuclease activity.

MAR1 Purification—The S-100 supernatant (in MSB buffer) was loaded onto 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 QB buffer (QA buffer containing 1 M 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 nuclease 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. Proteins bound to 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 endoribonuclease 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) equilibrated with MSB buffer and then eluted in MSB buffer containing 10% glycerol and stored at −70 °C. Peak fractions from the different chromatographic steps were electrophoretically separated in 10% Tricine/SDS gels and silver-stained (Bio-Rad).

In Vitro Transcription and Labeling of Synthetic RNA—Cyb (fully edited and pre-edited) and ND7 (pre-edited) mRNAs were transcribed using T7 RNA polymerase and plasmids containing the templates for the various transcripts under the control of a T7 promoter. Plasmids were linearized by restriction digestion, the linearized plasmids were incubated in an *in vitro* transcription mixture containing T7 RNA polymerase, nucleotides, and the appropriate buffer (24). The resulting synthetic RNAs (CybNE, CybFE, and ND7) were 3′ end-labeled by ligation of [α-^{32}P]pCp using T4 RNA ligase (Life Technologies, Inc.). Alternatively, the mRNAs were dephosphorylated with calf intestinal alkaline phosphatase in calf intestinal alkaline phosphatase buffer as described by the manufacturer (Life Technologies, Inc.) and 5′ end-labeled by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase for 1 h at 37 °C, in T4 polynucleotide kinase buffer (Life Technologies, Inc.). Once labeled, the RNAs were gel-purified by electrophoresis in 7 M urea/8% acrylamide, and the bands were visualized by exposure of the gel to x-ray film. The RNA was recovered by elution for 10 h at 4 °C in 400 μl of 300 mM sodium acetate, 1 mM EDTA buffer. The labeled RNA was precipitated with 1.2 ml of ethanol at −20 °C for 15 min. The pellets were washed twice with 70% ethanol, dried under vacuum, and suspended in TE buffer (10 mM Tris, 1 mM EDTA (pH 8.0)).

Enzyme Assays—Either 5′ or 3′-labeled RNAs were incubated at 27 °C for 30 min in 50 mM HEPES (pH 7.5), 1 mM DTT, 20 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA buffer (HKM buffer), in the presence or absence of enzyme. Samples (100 μl) were extracted with 100 μl of Tris/EDTA-saturated phenol/chloroform (pH 8.0). The RNA in the aqueous phase was precipitated with 1/10 sample volume (10 μl) of 3 M sodium acetate (pH 5.2) and 3 volumes (300 μl) of ethanol, with 1 μg of glycogen as carrier. Samples were pelleted by centrifugation in an Eppendorf microcentrifuge at $14,000 \times g$ at 4 °C for 30 min. The pellets were washed with 1 ml of 70% ethanol, dried under vacuum, and resuspended in denaturing 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 M urea/6% (or 10%) acrylamide at 500 V for 2 h. Gels were transferred to 3MM blotting paper (Whatman), dried under vacuum at 85 °C for 45 min, and then exposed to a PhosphorImager screen (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 into 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 by 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 20 × 20 k6F Silica Gel Thin Layer Chromatography Plate (Whatman) and eluted from the gel matrix in 0.3 M sodium acetate at 55 °C, 30 min, followed by ethanol precipitation and resuspension in water. The following oligonucleotides were utilized in this study: S-2255, 5′-CTGIAGTTC(C/I)ACGCC(C/I)AGGAA(C/I)GC(C/I)GTCTT-3′; S-2271, 5′-TTGAATTCGCATTGAGCACCTGCTTTTTTTTTTTTTTTTT-3′; S-2272, 5′-TTGAATTCGCATTGAGCACCTGC-3′; S-2273, 5′-AACTAACGCTATATAAGTATCAGTTTCTGTACTTTATTG-3′; S-2323, 5′-CGG(T/C)CGCGTGAC(T/C)TCCAGGTG-3′; S-2356, 5′-ACTACCGGGAAAATTGTGTCG-3′; S-2357, 5′-CTGGAGTTCCACGCCGAGGAAG-3′; S-2473, 5′-CTTGGAGATCTACGCACAGAAACGCTG-3′; S-2545, 5′-CTGCAGTCTCTAGACTTCACTTGTCATCGTCATCCTTGTAGTCAAGCTT-3′; S-2546, 5′-GCTAGGCATGGATCCATGCCTCGCTTGATGCCGCATTATTCTACG-3′; S-2630, 5′-GATTTCGAAGCTACTGAGGGAGG-3′. The nucleotides in parentheses represent degenerate positions and (I) represents inosine.

Peptide Isolation and N-terminal Sequencing—The purified protein was electrophoretically separated in a 10% acrylamide/Tricine/SDS 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 destaining 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: MPRLMPHYSTSKTAFLGVDLQCAG.

Nested RT-PCR Reactions—Based on the 24 N-terminal amino acids sequence obtained from the purified MAR1 protein, the degenerate oligonucleotides S-2323 (GACQLDV) and S-2255 (QLDVGLFATK), 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 nM S-2323 and the *L. tarentolae* spliced leader RNA (slRNA)-specific primer, S-2273. Amplification of the *MAR1* 5′ sequence was performed in a GeneAmp 9000 thermocycler (Perkin-Elmer) in a reaction mixture containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, and 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 slRNA-specific primer, S-2273, 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 (Fig. 7*A*) (Prime-IT II kit, Stratagene).

DNA Sequencing and Generation of Nested Deletions—DNA sequencing of cloned inserts was performed manually by the dideoxy-termination reaction with 10 pmol of plasmid DNA template and Sequenase V.2.0 (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The sequencing products were then separated in an 8 M 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 *Eco*RI-*Bam*HI sites of the pBluescript II SK(+) vector (Stratagene), generating pB1-MAR1. The pB1-MAR1 plasmid was linearized by digestion with *Kpn*I and *Eco*RI, to generate an Exo-III-resistant restriction site and an Exo-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 using 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 to the *MAR1* gene random-labeled using the Prime-IT kit (Stratagene).

Mapping the 5′ and 3′ Terminus of the Mature MAR1 mRNA—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 mapping of the 3′-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 cDNA mixture was incubated for 30 min at 25 °C and then for 1 h at 45 °C. An aliquot of 5 μl of the cDNA synthesis reaction was PCR-amplified with the primers, S-2272 and S-2356, in a standard PCR reaction mixture (Promega). The sample was subjected to 2-min denaturation at 94 °C followed by 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 2 min and a final step of 72 °C for 10 min. A 5-μl aliquot of the first PCR reaction was subjected to a second PCR amplification in a standard PCR reaction mixture (Promega) with the primers S-2272 and S-2630, using the same conditions as described for the first PCR amplification. The PCR products were separated in 1% agarose/TAE gel and the amplified DNA was cloned into the pCR 2.1-TOPO vector (Invitrogen).

RNA Mapping and Sequencing—5′-Labeled *in vitro* synthesized RNAs (Cyb or ND7) were incubated with sequence-specific nucleases (T1, U2, Phy M, and *Bacillus cereus*), as described in the RNA Sequencing Kit (Amersham Pharmacia Biotech). These reactions were used as sequencing ladders in 10% acrylamide/7 M urea gels together with MAR1 cleavage reactions with the same mRNAs. To assay for gRNA dependence of MAR1 cleavage, reactions were performed in the presence and absence of gRNAs and separated as above.

Sequence Analysis of MAR1—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, *C. elegans*, *Bacillus subtilis*, and *Archaeoglobus fulgidus*. Manual refinements were performed to optimize the alignments, taking into consideration the predicted secondary structure obtained from the individual polypeptides. Predict Protein (*PHDsec*) (http://www.ebi.ac.uk/searches/searches.html) was used to perform the secondary structure predictions. The Monte Carlo algorithm implemented by the RDF2 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 amphipathic 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 coding sequence of the *MAR1* gene was cloned into the pQE-31 expression 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 antiserum 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 pX vector (29). Prior to ligation, a PCR reaction was carried out with oligonucleotides S-2545 and S-2546, which are complementary to the 5′ and 3′ end, respectively, of the MAR1 coding region. These oligonucleotides were designed to insert a *Bam*HI site at the 5′ end and an *Xba*I 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 *Bam*HI and *Xba*I and cloned between the *Bam*HI and *Xba*I 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 200 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.

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. 1*A*, 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 extraneous factor.

MAR1 has an absolute requirement for divalent cations (Fig. 1*B*). 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.

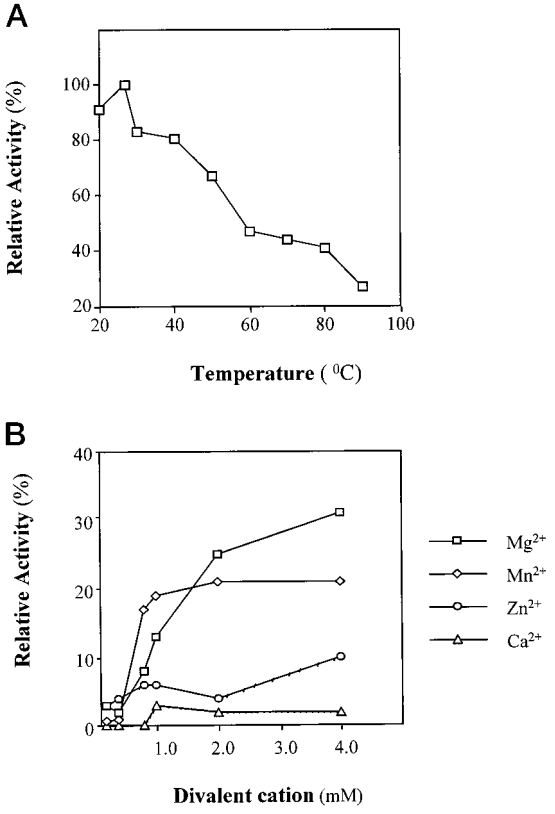

FIG. 1. **Effect of preincubation at different temperatures on the MAR1 reaction and divalent cation requirements.** *A*, constant amounts of unclarified mitochondrial detergent lysates were heated in buffer for 20 min at different temperatures. Samples were quenched in ice, radioactively labeled substrate was added, and the samples were incubated at 27 °C 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
Purification of MAR1

Crude indicates the 12,000 × *g* − 20 min clarified supernatant of the CHAPS lysate (as described under "Experimental Procedures"). S-100 is the supernatant after centrifugation of the crude fraction at 100,000 × *g* for 1 h. Mono Q, Superose 12, and Mono P represent the pooled peaks of nuclease activity from each chromatography step. A unit is defined as 1 nmol of substrate RNA cleaved per min. N/A, not available.

Fraction	Total protein	Total activity	Specific activity	-Fold purification	Yield
	mg	*units*	*units/mg*		%
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

Purification of MAR1—Due to the relative thermal stability of MAR1, heating of a 12,000 × *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 × *g* for 1 h. Chromatography of the resulting S-100 supernatant through Superose 12, Mono Q (Fig. 2*A*), and Mono P (Fig. 2*B*) yielded a 16,000-fold enrichment of specific activity (Table I). Analysis of the peak fractions by SDS-acrylamide gel electrophoresis and silver staining is shown in Fig. 2*C*. The Mono P peak showed a

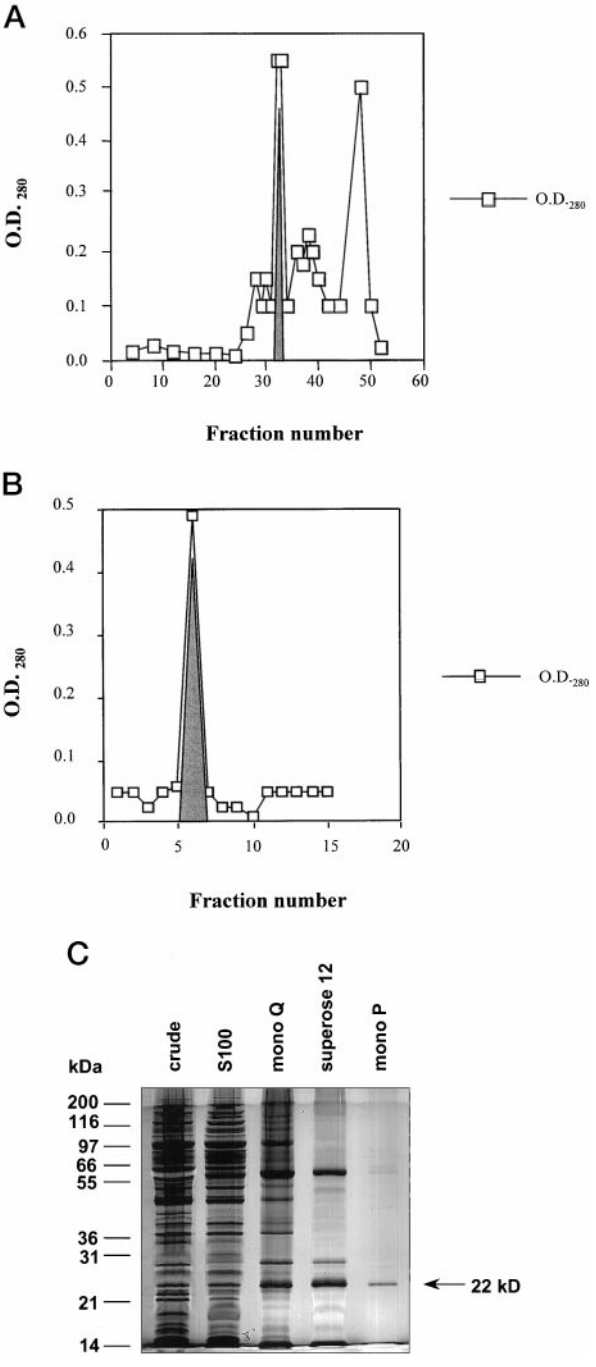

FIG. 2. **Chromatography of MAR1.** *A*, Mono Q anion exchange chromatography of the heated S-100 supernatant. The column was equilibrated with QA buffer and developed with a linear gradient of QB buffer. The *shaded area* represents the elution of the peak of cleavage activity. *B*, chromatogram of 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.

major 22-kDa band that represented 97% of the protein in this fraction. No changes in substrate cleavage patterns were observed between the enzyme activity in the crude extract or the Mono P fraction (data not shown).

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

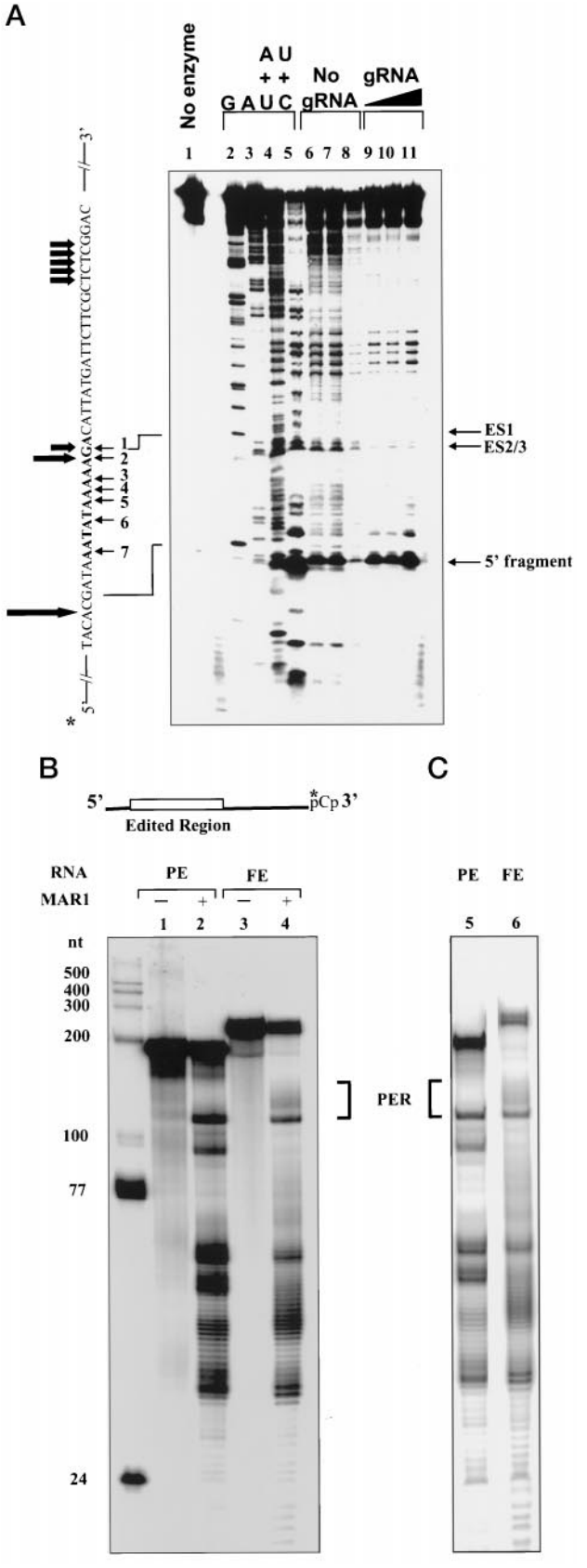

FIG. 3. **Mapping of the MAR1 cleavage sites on pre-edited ND7 and pre-edited and fully edited Cyb mRNA substrates.** *A*, synthetic ND7 pre-edited mRNA was 5′ end-labeled with [γ-^{32}P]ATP. The labeled RNA was used as a substrate for MAR1 cleavage in the presence (*lanes 9–11*) or absence of gRNA (*lanes 6–8*), and in the presence (*lanes 6–11*) or absence of MAR1 (*lane 1*). *Lanes 2–5* show the product of a sequencing ladder generated with the sequence-specific nucleases T1 RNase (G), U2 RNase (A), Phy M RNase (A + U), and *B. cereus* RNase (U + C). "*ES1*" and "*ES2*" refer to the relative migration of fragments cleaved at editing sites 1 and 2. *B*, 3′ end-labeled synthetic pre-edited Cyb (*PE*) mRNA (*lanes 1* and *2*) and fully edited Cyb (*FE*) mRNA (*lanes 3* and *4*) were incubated in the presence (*lanes 2* and 4) and absence (*lanes 1* and *3*) of MAR1. (Similar results were obtained using 5′ end-labeled substrates.) *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*. The reactions were separated on a 7 M urea/10% acrylamide gel. *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*.

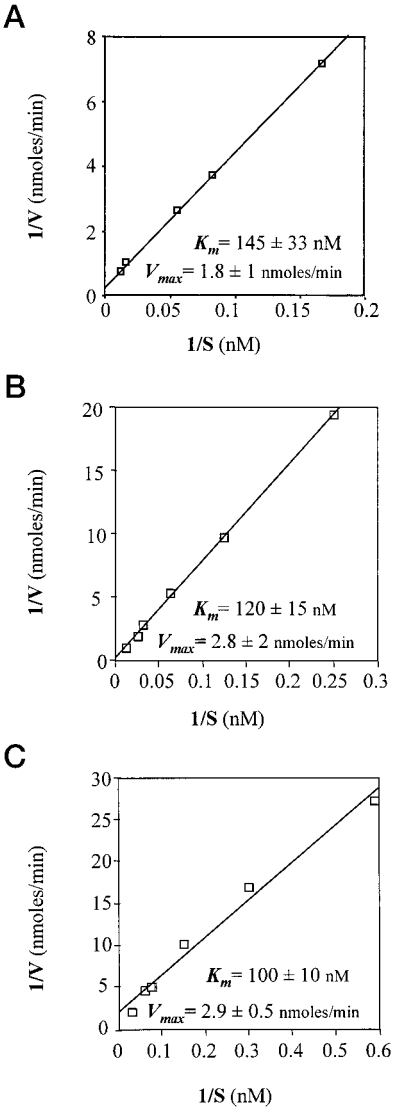

FIG. 4. **Kinetic analysis of the MAR1 cleavage reaction.** Increasing concentrations (6–80 nM) of 5′-labeled pre-edited ND7 mRNA (*A*), pre-edited Cyb (*B*), and fully edited Cyb mRNA (*C*) were incubated with 10 ng (9.2 μM) MAR enzyme at 27 °C for 30 min. The fraction of the various concentrations 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.

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

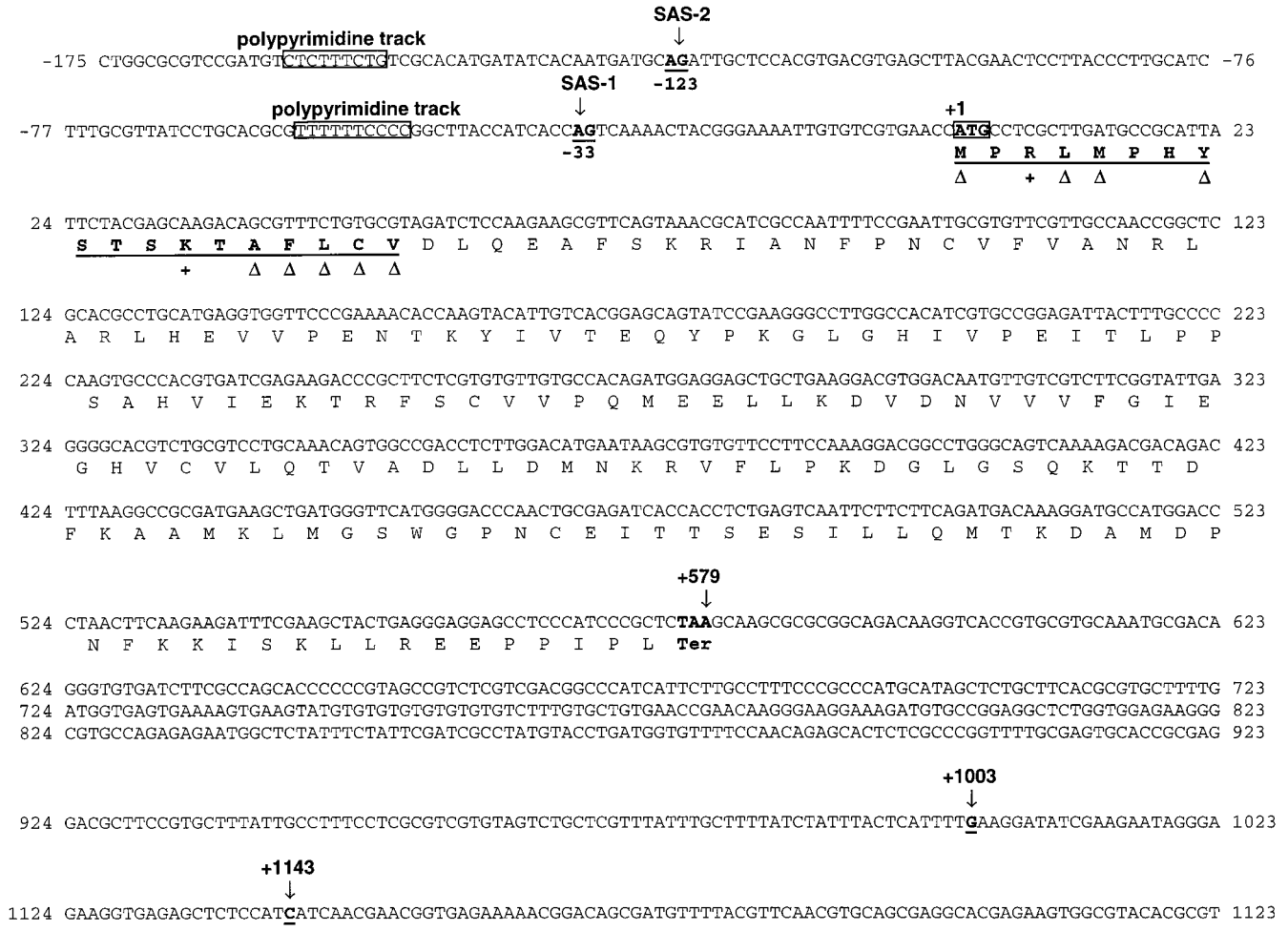

FIG. 5. **Nucleotide sequence and predicted amino acid sequence of the *MAR1* gene from *L. tarentolae*.** The 3-kb *Eco*RI-*Bam*HI cloned genomic fragment was sequenced. A segment of 1298 bp of this fragment is shown. Nucleotides within the protein coding region of *MAR1* are *numbered positively* from the Met start codon (*boxed ATG*), and the predicted amino acid residues of MAR1 are *numbered positively* starting from the first Met. A termination codon (TAA) is indicated in *bold* at position +579. The predicted mitochondria importation signal sequence is *underlined*. Hydrophobic amino acids are indicated by an △, and positively charged amino acids are indicated by a +. Nucleotides within the 5′-untranslated region are *numbered negatively* starting from the initiation ATG codon from *MAR1*. The nucleotides that correspond to the splice acceptor sites of *MAR1* transcript are indicated (SAS-1 and SAS-2). Polypyrimidine tracks 5′ to each splice acceptor site are *boxed*. The two polyadenylation sites identified in the *MAR1* transcript are indicated at positions +1003 and +1143.

enzyme and 5′ or 3′ end-labeled substrates. As shown in Fig. 3*A*, 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. 3*A*, 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. 3*B*). 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 ± 2 nmol/min for the pre-edited Cyb mRNA (Fig. 4*B*) 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. 4*C*). Similar values for K_m and V_{max} were obtained (Fig. 4*A*) 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-2323 primer was used to synthesize the first-strand cDNA. This cDNA was then subjected to RT-PCR amplification utilizing the S-2323 3′ primer and the slRNA-specific S-2273 5′ primer. The product of the first PCR reaction was subsequently re-amplified in a nested PCR reaction with the degenerate S-2255 3′ primer and the 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 (MPRLMPHYSTSKTAFL**C**VDLQ**EAF**)

(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 (STSKTA) confirmed the identity of the cloned fragment (Fig. 5).

The 130-bp PCR product hybridized with a 3-kb *Eco*RI-*Bam*HI genomic fragment. The 3-kb region of *Eco*RI-*Bam*HI-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. 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 slRNA (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 polypyrimidine 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 slRNA 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).

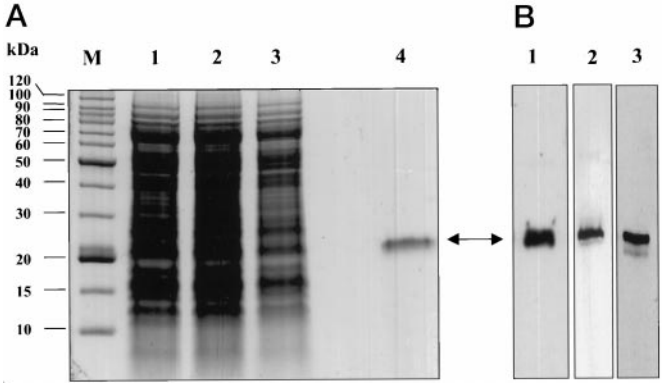

FIG. 6. **Expression of the FLAG-tagged MAR1 in *L. tarentolae* cells.** *A*, electrophoretic separation, on a 10% acrylamide/Tricine-SDS gel, of various fractions from an anti-FLAG affinity column. The gel was stained with Coomassie Brilliant Blue. *Lane 1*, cell-free extract prior to loading on the affinity column. *Lane 2*, the eluant fraction from the column loading. *Lane 3*, the column was washed with "low salt" buffer 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 antiserum raised against the *E. coli*-expressed protein (*lane 1*) or with 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*.

Recombinant MAR1 Shows Identical Specific Activity and Cleavage Specificity as Native MAR1—The *MAR1* gene was expressed in *E. coli* cells 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. 6*A*), as well as by Western analysis using either anti-MAR1 antiserum or anti-FLAG antibody (Kodak) (Fig. 6*B*). 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. 6*B*, *lane 3*).

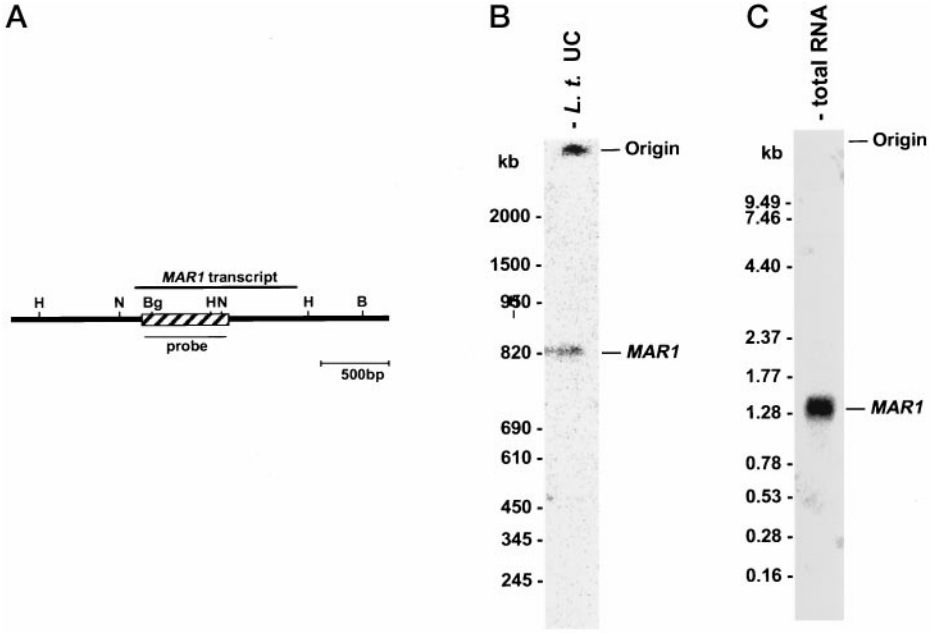

FIG. 7. **Molecular characterization of the *MAR1* gene from *L. tarentolae*.** *A*, schematic representation of the *MAR1* locus restriction fragment map. The probe used in the Southern and Northern blots, as well as in the CHEF gel hybridization experiments, is indicated. *B*, Southern blot of a CHEF gel electrophoresis of *L. tarentolae* chromosomal DNA. A band of approximately 820 kb hybridized with the *MAR1* probe. The size markers are *S. cerevisiae* chromosomes (Promega). *C*, Northern blot hybridization of *L. tarentolae* total RNA with the probe indicated in *A*. A single transcript of approximately 1.3 kb is detected. RNA size standards are indicated on the *left*.

A

```
                1.........+ .........+ .........+ .........+ .........50 .........+ .........+ .........+ .........+
MAR             ..MPRLMPHY STSKTAFLCV DLQEAFSKRI ANFPNCVFVA NRLARLHEVV PENTKYIVTE QYPKGLGHIV PEI..TLPPS AHVIEKTRFS
C. elegans      MAARKLIARI NPTNSALFVC DLQEKFASNI KYFPEIITTS RRLIDAARIL SIPT..IVTE QYPKGLGHTV PTLKEGLAEN TPIFDKTKFS
A. fulgidus     .......... ....MALVVV DVQEKLAPHI EGIEEMLKRT VKLVKA..IV ALKQPVIFTE QIK..LGKTV DELAEYAEEP ...IVKSTFS
Consensus                     *:.   *:** :: .*    : : :  :  :*      :: .     *.** *    **: *  :            : *: **
Fold                           HHHHHHHHH     HHHHHHH HHHHHHHHH   LLL BBBBB B LL  LL   HHHH  LLLL LL

                .......100 .........+ .........+ .........+ .........+ .......150 .........+ .........+ .........+
MAR             CV....VPQM EELLKDVDNV VVFGIEGHVC VLQTVADLLD MNKRVFLPKD GLGSQKTTDF KAAMKLMGSW GPNCEITTSE SILLQMTKDA
C. elegans      MC....IPPT EDTLKKVQNV ILVGIEAHVC VLQTTYDLLE RGLNVHVVVD AVSSRSHTDR HFAFKQME.. QAGAILTTSE ATILGLVGGS
A. fulgidus     CYKDENFRRK IEELK.PEKV ILAGIEAHIC VLQTAIDMKR AGYDVYVVAD CTGSRKAEDK EIALMRLA.. GEGIKLTTWE SIVYELLESA
Consensus                 . : **  ::* :: ***.*:* ****. *:    .  *.:  *   .*:.  *  . *:  :      .  :** * : :  :  .:
Fold                    HHH HHHH     B BBBBBBBBBB B   HHHHHH  LLLBBBBB  LLLLLL HHH HHHHHHHH             HHHHHH

                .........+ .......200 .......
MAR             MDPNFKKISK LLREEPP... .IPL...
C. elegans      DHPKFKEVQK LILTSAPDTG LVPLSKL
A. fulgidus     EHPAFREILQ IVKN...... .......
Consensus        .* *::: : ::
Fold               HHHHHHH HH
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B

```
                ..110 .........+ .........+ .......140
MAR          ...EELLKDVDNV VVFGIEGHVC VLQTVADLLD MNKRVFLPKD...
C. elegans   ...EDTLKKVQNV ILVGIEAHVC VLQTTYDLLE RGLNVHVVVD...
A. fulgidus  ...IEELK.PEKV ILAGIEAHIC VLQTAIDMKR AGYDVYVVAD...
B. subtilis  ...LTELQ.VRHI IITGIAGNIC VLFTANDAYM REYSITIPKD...
                *:   :: :: ** .::* ** *. *       : :  *
```

C

	B. subtilis	*A. fulgidus*	*C. elegans*
MAR	8.2	16.1	37.8
C. elegans	8.8	26.0	
A. fulgidus	11.0		

FIG. 8. **Alignment of MAR1 amino acid sequence with data base sequences.** *A*, the predicted polypeptide sequence of MAR1 is aligned with the *C. elegans* (GenBank™ accession number Z69637) and *A. fulgidus* (GenBank™ accession number AE000943) sequences. The predicted N terminus signal sequences of MAR1 and the *C. elegans* sequence are *underlined*. The consensus of the alignment is indicated. "*" indicates amino acid identities; ":" and "." indicate conserved substitutions. The predicted secondary structure fold (above 90% certainty) utilized as a guide to refine the sequence alignment is shown: H, α-helices; L, loops, and B, β-sheets. *B*, domain of the multiple sequence alignment, including the isochorismatase sequence from *B. subtilis* (GenBank™ accession number P37532) (*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).

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. 3*C*), confirming that the MAR1 gene encodes the MAR1 nuclease.

MAR1 Is a Single Copy Gene That Maps to an 820-kb Chromosome and Has a Transcript of 1.3 kb—The *MAR1* gene was analyzed by Southern blot hybridization of digested genomic DNA. *L. tarentolae* DNA was digested with a variety of restriction enzymes and the blots hybridized with the full-length *MAR1* gene (data not shown). The enzymes, *Eco*RI, *Bam*HI, and *Hin*dIII, which do not cleave within *MAR1*, generate a single DNA fragment that hybridizes with the *MAR1* probe. The enzymes, *Bgl*II, *Hin*fI, and *Nco*I, which cleave once within the *MAR1* open reading frame, generated two DNA bands with the *MAR1* probe. *Bgl*II also cleaves at the 5′ end of the gene leaving a short fragment that was not detected. Double digestions also generated patterns consistent with *MAR1* being a single copy gene. The restriction map of *MAR1* is shown in Fig. 7*A*.

The *MAR1* gene was localized to a 820-kb chromosome of *L. tarentolae* by CHEF gel electrophoresis and Southern analysis (Fig. 7*C*). A transcript of 1.3 kb is shown by the Northern analysis in Fig. 7*C*.

MAR1 Does Not Contain a Cleavable Mitochondrial Targeting Signal—Analysis of the first 18 amino acids at the N-terminal of MAR1 revealed characteristics compatible with other signal sequences reported in the literature. The presence of Arg, Leu, Ser, and Ala, the presence of at least one Thr and one Lys, and the absence of Glu and Asp residues are all consistent with this serving as a mitochondrial targeting sequence (Fig. 5). Moreover, the N-terminal 18 amino acids of MAR1 show 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 alignments to the MAR1 sequence are shown in Fig. 8, *A* and *B*. The amino acid alignments showed a high degree of similarity of the MAR1 sequence with the *C. elegans* and *A. fulgidus* sequences (Fig. 8*A*). Moreover, the presence of an amphipathic helix at the N terminus of *C. elegans* and its absence in the *A. fulgidus* is consistent with this N terminus sequence representing a mitochondrial importation signal in *C. elegans* as well. The statistical significance of the alignments are shown in Fig. 8*C*; these values suggest that the alignments of the MAR1 sequence with the *C. elegans* and the *A. fulgidus* are highly significant. The alignment of the MAR1 sequence with the *B. subtilis* sequence is limited to the short region shown *boxed* in Fig. 8*B*, but the statistical significance of this alignment is high. The *B. subtilis* sequence is a homologue of the isochorismatase gene family, but a comparison of the MAR1, *C. elegans,* and *A. fulgidus* polypeptide sequences with several members of the isochorismatase gene family did not provide evidence that these genes belong to that family (data not shown).

DISCUSSION

MAR1 is the first mitochondrial endoribonuclease 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 nuclease 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 c_1 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 archaebacterium, and a conserved motif was found in a eubacterial protein. The homologies, 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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