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An *in vitro* system for the editing of ATP synthase subunit 9 mRNA using wheat mitochondrial extracts

(plant mitochondrial genes/RNA editing)

ALEJANDRO ARAYA*, CHRISTINE DOMEC, DOMINIQUE BEGU, AND SIMON LITVAK

Laboratoire de Biologie Moléculaire Végétale, Institut de Biochimie Cellulaire et Neurochimie–Centre National de la Recherche Scientifique, 1 rue Camille Saint Saëns, 33077 Bordeaux, France

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ABSTRACT **A posttranscriptional modification (C-to-U) at specific positions of plant mitochondrial mRNA leads to changes in the amino acid sequence as well as to the emergence of novel initiation or termination sites. This phenomenon, named RNA editing, has been described for several mitochondrial genes from different plant sources. We have found recently that RNA editing of the ATP synthase subunit 9 (*atp9*) mRNA involves eight changes including the creation of a new stop codon. In this article, we describe an *in vitro* system devised to follow the editing of wheat mitochondrial *atp9* mRNA. Nonedited mRNA was obtained to serve as substrate for this reaction by *in vitro* transcription of the corresponding gene with T7 RNA polymerase. The source of conversion factor(s) was a soluble fraction obtained from purified wheat mitochondria lysed with salt and detergent. Edited RNA molecules were detected by hybridization with an end-labeled synthetic oligodeoxynucleotide probe complementary to a short region containing four editing events. Optimal conditions for the *in vitro* RNA editing reaction were determined. The reaction is sensitive to high temperature and protease digestion. Pretreatment with micrococcal nuclease decreased RNA editing activity in the mitochondrial extract, suggesting that a nucleic acid is necessary for the enzymatic reactions. Analysis of the edited mRNA showed that the *in vitro* reaction led to the same products as those observed *in vivo*.**

Until recently the protein-coding sequence of a mRNA appeared to be inviolable. However, recent data show that in several instances disparate types of RNA modification can change the coding capacity of mRNA (1). Thus, the mitochondrial genes of a variety of plants undergo posttranscriptional changes at multiple sites creating a U residue in the RNA where a C was found in the genome (2–4). Editing of plant mitochondrial RNA alters the coding sequence in mRNAs, leading to amino acid changes as well as to the emergence of termination or initiation codons (5).

Plant mitochondrial RNA editing seems to be somewhat simpler than the phenomenon observed in trypanosome mitochondria (6) but is remarkably similar to the editing of a single residue in the apolipoprotein B mRNA observed in some animal tissues (7, 8). The mechanism by which the C-to-U change operates in plant mitochondria is unknown, although an *in vitro* system has been described in the case of apolipoprotein B mRNA modification (9). A model has been proposed for RNA editing in kinetoplastid mitochondria involving "guide" RNA molecules transcribed from maxicircle or minicircle mitochondrial DNA (10).

In studying the organization and expression of protein genes essential for wheat mitochondrial function, our laboratory has mapped and sequenced several wheat mitochondrial genes (11–14). We showed by cDNA sequencing that eight C-to-U changes occurred in wheat mitochondrial *atp9* mRNA (15, 16). Minor forms of *atp9* cDNA with partial or overedited sequences were also found. Partial protein sequencing showed that editing events were reflected at the protein level, with the emergence of a stop codon six residues before the position predicted from the gene sequence. The large number of editing events in the transcript of a short gene such as *atp9* makes it a very appropriate model for the *in vitro* study of plant mitochondrial RNA editing. Our previous experience in the study of wheat mitochondrial DNA replication and transcription allowed us to prepare an active wheat mitochondrial extract able to modify a nonedited form of *atp9* mRNA synthesized with the T7 RNA polymerase system. Here we present an *in vitro* system able to edit faithfully the expected C-to-U residues of wheat mitochondrial *atp9* mRNA. We describe optimal conditions and requirements of this *in vitro* system that lead to an mRNA carrying the same modifications as those obtained *in vivo*.

MATERIALS AND METHODS

Materials. Seeds of an alloplasmic line of wheat (*Triticum aestivum* cv. Marius) were obtained from Benoist (Orgerus, France). All enzymes and other proteins were purchased from Sigma, Boehringer, Genofit (Geneva), and Pharmacia. Radioactive precursors and cDNA synthesis kits were from Amersham. DNA sequencing kits were from Pharmacia LKB.

Mitochondrial Extract. Wheat embryo mitochondria were prepared after 20 hr of imbibition (17) and mitochondrial lysate was prepared essentially as described (18). Sucrose gradient-purified mitochondria (4 mg of protein) were resuspended in 2.84 ml of 10 mM Tris·HCl, pH 8.0/1 mM EDTA. Lysis was carried out by addition of 0.96 ml of 4 M $(NH_4)_2SO_4$ in the presence of 0.2% (vol/vol) Triton X-100. After centrifugation at 100,000 × *g* for 60 min at 4°C, supernatant was diluted with 6 ml of 10 mM Tris·HCl, pH 8.0/1.5 mM $MgCl_2$/15 mM KCl/0.1 mM EDTA/0.5 mM dithiothreitol. The protein solution was applied to a 2.5-ml DEAE-cellulose column as described (18). The excluded fraction was pooled and precipitated by addition of $(NH_4)_2SO_4$ to 0.227 g/ml. The protein pellet was dissolved in 10 mM Tris·HCl, pH 8.0/0.1 mM EDTA/1 mM dithiothreitol and dialyzed against the same buffer containing 50% (vol/vol) glycerol. Aliquots (50 μl) were stored at −20°C until use.

RNA Synthesis. One microgram of a genomic clone containing the coding region of *atp9* was linearized with *Pst* I endonuclease, and *in vitro* transcription was carried out with 20 units of T7 RNA polymerase at 37°C for 30 min. Then, 140 units of DNase I (RNase-free) and 20 units of human placental RNase inhibitor were added, and incubation proceeded for an additional 10 min at 37°C. RNA was extracted

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*To whom reprint requests should be addressed.

twice with phenol/chloroform (1:1, vol/vol) and three times with diethyl ether and precipitated with ethanol.

RNA Editing Assay. *In vitro* RNA editing was measured in a total volume of 50 μl containing 50 mM Tris acetate (pH 8.5), 10 mM magnesium acetate, 0.2 μg of nonedited RNA, and 20 units of human placental RNase inhibitor. The reaction was started by addition of 2 μg of mitochondrial lysate proteins. Incubation was at 30°C for 60 min. After the addition of 150 μl of 1× TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA), the reaction mixture was extracted twice with phenol:chloroform (1:1) and three times with diethyl ether. After precipitation with ethanol, the RNA was dissolved in 20 μl of sterile water.

cDNA Synthesis and PCR Amplification. *T. aestivum atp9* cDNA synthesis of lysate-treated RNA and polymerase chain reaction (PCR) were performed as described (16). The 50-μl PCR mixture contained 10 mM Tris·HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each dATP, dTTP, dGTP, and dCTP, 50 pmol (1 μM) of each primer, and 1.2 units of *Taq* DNA polymerase (Promega). The first cycle included incubation at 95°C for 5 min before the addition of the enzyme, then 2 min at 55°C and 2 min at 70°C. This step was followed by 30 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 70°C. PCR products were analyzed by agarose gel electrophoresis, phenol-extracted, and precipitated three times with ethanol after the solutions were adjusted to 2.5 M ammonium acetate.

Amplification products were blotted from gel or spotted onto nylon membranes (Amersham) after denaturation with 0.5 M NaOH/1.5 M NaCl. Membranes were dried in an oven at 80°C for 2 hr. Hybridizations were carried out overnight at 45°C in 6× standard saline citrate (SSC) containing 5× Denhardt's solution and 0.5% SDS. Subsequently, the filters were washed in 2× SSC twice for 15 min at room temperature and then in 0.2× SSC/0.1% SDS once for 10 min at room temperature and once for 10 min at 50°C. The filters were autoradiographed on Hyperfilm-MP (Amersham). The films were scanned with an Ultroscan XL laser densitometer (LKB).

RNA Editing Probe. A 39-mer extending from codons 63 to 76 (13) was polymerized in an Applied Biosystems 381A DNA synthesizer. The probe covers the last four editing sites of the coding region of *atp9* (codons 64, 69, 71, and 75). The oligonucleotide was labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP.

cDNA Cloning and Sequencing. The amplified cDNA was digested with *Sal* I and *Xho* I endonucleases and ligated to a *Sal* I-linearized Bluescribe vector. Transformation of *Escherichia coli* DH5α cells was carried out as described (19). Positive clones were screened by blue/white color selection on ampicillin plates. Recombinant plasmids were prepared by the boiling method (20). cDNA sequencing reactions were carried out on recombinant plasmids by T7 or T3 priming using a T7 DNA polymerase sequencing kit (Pharmacia) and [α-[^{35}S]thio]dATP (600 Ci/mmol) (Amersham) as labeled precursor. Reaction products were electrophoresed in 6% acrylamide gels and visualized by autoradiography.

RESULTS

Experimental Strategy. Previous studies in our laboratory have shown that eight C-to-U changes are involved in the editing of the coding region of *atp9* mRNA (13, 14). The high concentration of editing events in this short RNA prompted us to use the nonedited *atp9* transcription product as a tool to develop an *in vitro* system to study this posttranscriptional process. Fig. 1 represents the experimental strategy used for this purpose: a genomic clone containing the coding region of wheat mitochondrial *atp9* was inserted in a plasmid, downstream of the promoter region of the bacteriophage T7 RNA polymerase. This particular *atp9* clone carried a G residue

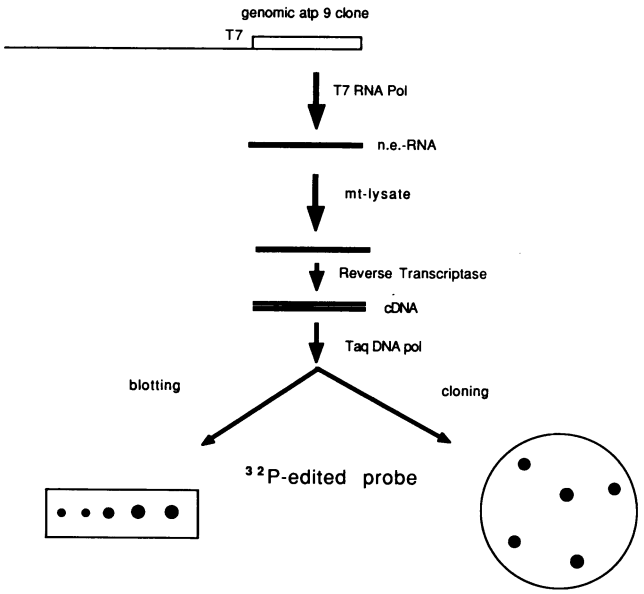

FIG. 1. Diagram of the experimental strategy used for *in vitro* editing of *atp9* RNA. Pol, polymerase; n.e., nonedited; mt, mitochondrial.

instead of A at position 117 and allowed us to distinguish the edited products obtained *in vitro* from possible endogenous sequences contaminating the lysate. Transcription of this plasmid construct with T7 RNA polymerase enabled preparation of the "nonedited" *atp9* mRNA substrate for the *in vitro* assay. After incubation with a wheat mitochondrial extract, the "edited" mRNA product was reverse transcribed, and the cDNA product was amplified by PCR. The PCR product was used for molecular hybridization with ^{32}P-labeled synthetic oligodeoxynucleotide probes or for cloning and further DNA sequencing.

Detection of "Edited" *atp9* mRNA. To detect *in vitro* C-to-U modifications catalyzed by wheat mitochondrial extracts, a complementary synthetic probe was synthesized that could distinguish between the edited and nonedited forms of *atp9* mRNA. A region suitable for this purpose is the 3′ end of this gene, where four C-to-U editing events occur: at codon 64, a proline-to-leucine change; at position 69, a "silent" editing corresponding to a leucine residue; at position 71, a serine-to-leucine change; and at position 75, the creation of a stop codon from an arginine triplet. Thus, the synthetic 39-mer oligonucleotide overlapping the 3′ end of the coding region of *atp9* (Fig. 2*A*) was used to probe the *in vitro* products obtained after incubation of the nonedited *atp9* mRNA with the mitochondrial extracts. Incubation of nonedited *atp9* RNA in the presence or absence of wheat mitochondrial lysate led to a 350-base-pair (bp) amplified DNA fragment detected by ethidium bromide staining after agarose gel electrophoresis (Fig. 2*B*, lanes 1 and 2). Southern hybridization of the amplified product with the 5′-end-labeled probe allowed detection of a complementary sequence only in the case of the cDNA coming from the mRNA incubated with the mitochondrial lysate (lane 3). To confirm the nature of the edited product, cDNA synthesized from RNA edited *in vitro* was amplified by PCR and cloned into Bluescribe plasmid. Colony lifts were screened by differential hybridization. Three out of 50 colonies hybridized to the 3′ *atp9*-specific probe. All positive clones showed, after DNA sequencing, the edited residues (T) at the expected positions in codons 64, 69, 71, and 75. Codons 7, 27, 28, and 45 were also edited (results not shown).

Requirements for *in Vitro* C-to-U Conversion. Fig. 3 shows different properties of the *in vitro* RNA editing system using

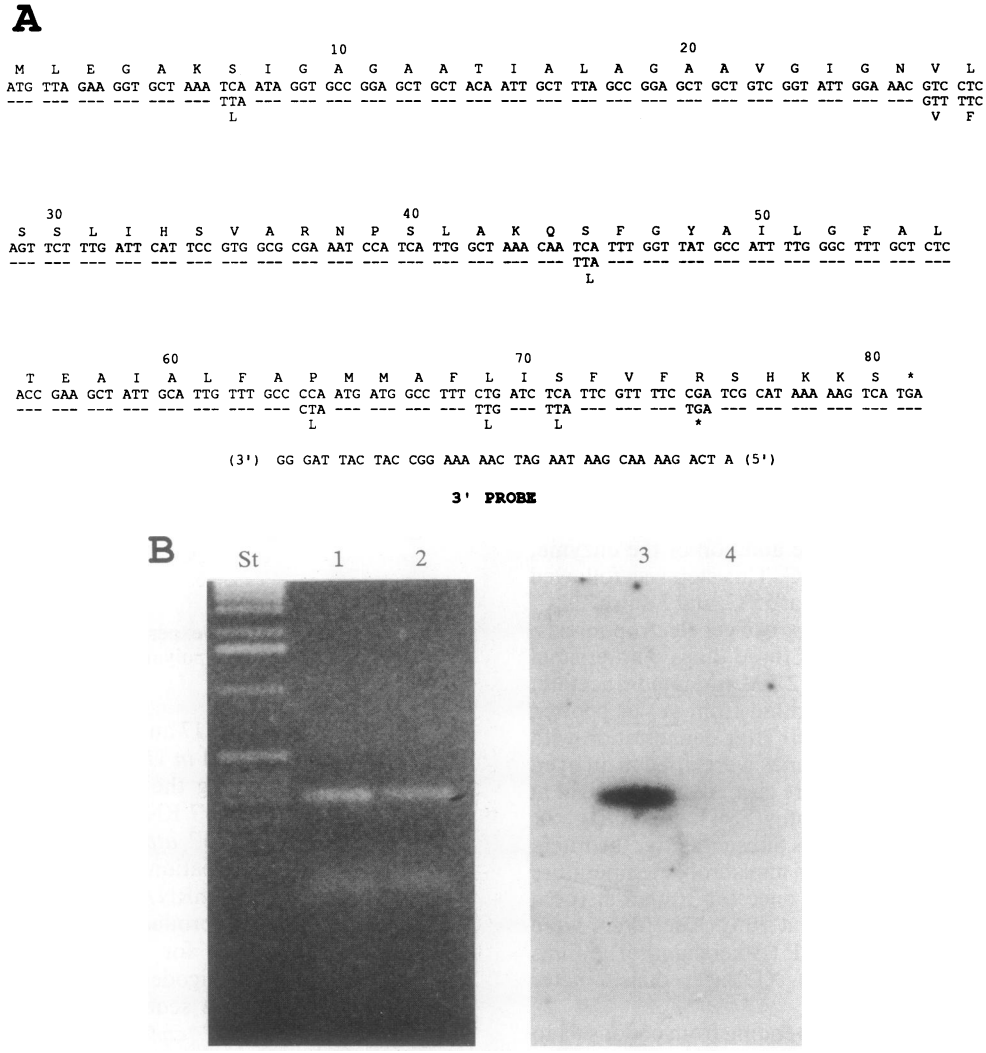

FIG. 2. (*A*) Sequence of the *atp9* coding region. Amino acid residues are indicated by the one-letter code; numbers indicate every 10 codons. The lower line (dashed) indicates the edited sequence, with only the edited codons indicated. The 39-mer oligonucleotide sequence (3′ probe), overlapping the 3′ end of the coding region of the edited *atp9* mRNA used in this work, is shown. (*B*) Southern blot analysis of PCR-amplified cDNA hybridized with the ^{32}P-labeled 3′-probe. Lanes 1 and 2, ethidium bromide staining of PCR products after and before incubation, respectively. Lanes 3 and 4, hybridization with 3′ probe after and before incubation with the mitochondrial lysate. In the experiment where the lysate was omitted during the incubation, the lysate was added at the end of the incubation period prior to the phenol extraction step. Lane St, molecular size standards.

nonedited *atp9* mRNA as substrate and wheat mitochondrial lysate as source of the RNA-converting activity. The reaction is dependent on the presence of a divalent cation, since EDTA completely inhibited editing (Fig. 3*A*) Mg^{2+} ions between 5 and 10 mM seemed to be optimal. Mn^{2+} could not replace Mg^{2+} ions; the stimulatory effect of 5 mM Mg^{2+} was clearly inhibited by 0.1 mM Mn^{2+}. KCl was not necessary, nor did it inhibit the reaction (Fig. 3*A*). When the pH dependence of the editing reaction was investigated, a pH of 8.5 was found to give the best results (Fig. 3*B*). The C-to-U conversion was dependent on mitochondrial lysate protein concentration up to 80 μg/ml; higher protein concentrations were inhibitory (Fig. 3*C*). The decrease of signal observed with 120 μg/ml seems due to trace amounts of nucleases impairing the PCR amplification step, as observed by ethidium bromide staining of the products (data not shown). The reaction kinetics were linear up to 90 min of incubation at 30°C (Fig. 3*D*). Temperature-dependence studies showed that maximal activity was observed at 30°C, while a minimal response was obtained at 0°C or with a heat-denatured lysate (Fig. 3*E*). The latter results, as well as the inhibitory effect of trypsin (results not shown), strongly suggest that protein(s) may play a crucial role in the *in vitro* RNA editing reaction.

An interesting result was obtained when the possible role of a nucleic acid in the *in vitro* RNA conversion reaction was investigated. For this, we used micrococcal nuclease, which is a Ca^{2+}-dependent enzyme. Thus, this nuclease can be inactivated by addition of EGTA after preincubation with the mitochondrial lysate, prior to the *in vitro* incubation with the nonedited RNA substrate. A significant part of the wheat mitochondrial lysate's ability to produce an edited form of *atp9* mRNA was lost after preincubation with nuclease (Fig. 4, lane 6), suggesting that a nucleic acid could be involved in the reaction. Pretreatment of the lysate with either $CaCl_2$ alone or $CaCl_2$ plus EGTA (lanes 3 and 7) showed that the effect observed was due to micrococcal nuclease. In fact, nuclease treatment of the lysate in the absence of $CaCl_2$ gave no inhibition of the editing reaction (lane 5). Nuclease inactivation by EGTA treatment was complete, since the signal obtained when the lysate was preincubated with EGTA-inactivated nuclease, which remains during the editing assay (lane 7), was essentially the same as the one obtained in the absence of micrococcal nuclease (lane 1). When the nuclease was not inactivated with EGTA (lane 2), no signal was observed. In this case no PCR amplification product was

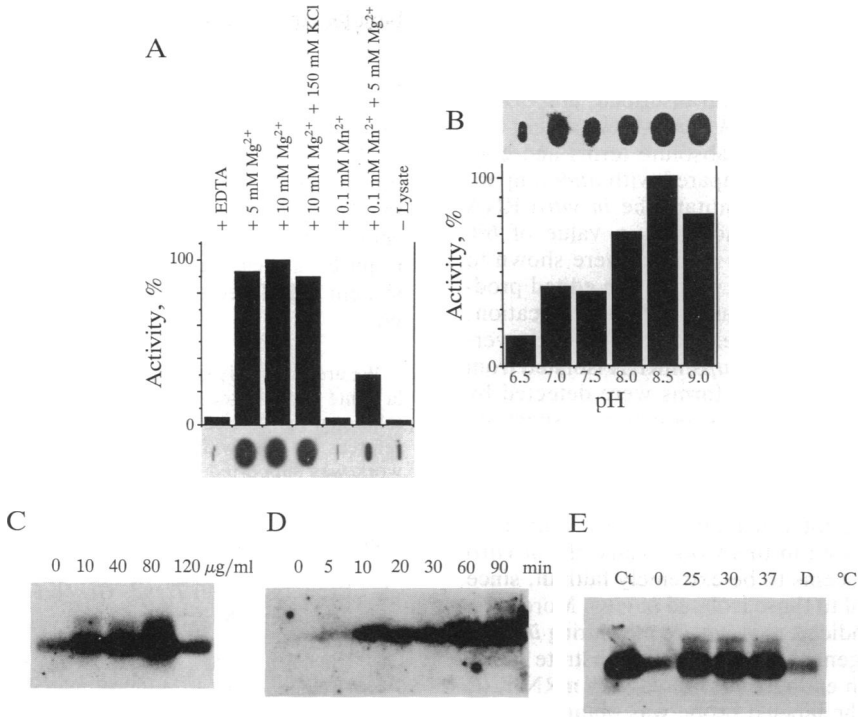

FIG. 3. Optimization of the *in vitro* editing assay. (*A*) Divalent-ion dependence. Synthetic *atp9* RNA corresponding to the coding region was incubated with 2 μg of mitochondrial lysate. After incubation at 30°C for 1 hr, RNA was extracted, cDNA was synthesized and PCR was performed. The amplified products were spotted on nylon membranes and hybridized with the edited 3′ probe. (*B*) pH dependence. Conditions were the same as in *A*, except that the assay mixture contained 10 mM $MgCl_2$. Buffer used was Tris acetate at pH ranging from 6.5 to 9.0. (*C*) Protein concentration dependence. Conditions were the same as in *A*, except that all reaction mixtures contained 10 mM $MgCl_2$. The PCR-amplified cDNA was loaded on a 1.2% agarose gel. After electrophoresis, the cDNA was transferred to nylon membrane and hybridized with the 3′ probe. (*D*) Time dependence. Conditions were the same as in *C*, except that all reaction mixtures contained 2 μg of protein lysate. (*E*) Temperature dependence. Reactions were carried out as described in *Materials and Methods*. Ct, control with edited RNA; D, heat-denatured lysate proteins. Autoradiograms were scanned in an Ultroscan XL laser densitometer. Activity is expressed as percentage of the maximal absorbance obtained by densitometry.

detected by ethidium bromide staining, due to enzymatic hydrolysis of the RNA substrate (data not shown).

DISCUSSION

RNA editing was discovered in the mitochondria of several kinetoplastid protozoa, where the sequences of some mature mRNAs were found to differ from those of the corresponding genes by insertion or deletion of U residues at specific sites (for reviews see refs. 1 and 21). It was found that RNA "guide" molecules coded in maxi- or minicircle DNA could serve as the templates for this process (10). The molecular mechanisms by which RNA editing operates in other systems seem to be simpler than those in trypanosome organelles. In this work, we deal with a posttranscriptional RNA conversion process taking place in wheat mitochondria, involving the specific conversion of some C residues to U residues. Although the enzymology of the overall process may seem quite simple, since the C-to-U change could be catalyzed by deaminating enzymes, the functional relevance of the plant mitochondrial RNA editing process is crucial. Thus, in the absence of mRNA editing the mature protein would be, at least in some cases, quite different in size and amino acid composition compared with that predicted from the genomic sequence and could have an altered function (22). The C-to-U conversion, which characterizes RNA editing in plant mitochondria, is more similar to the animal apolipoprotein B situation than to the drastic RNA changes observed in trypanosome mitochondrial RNA editing.

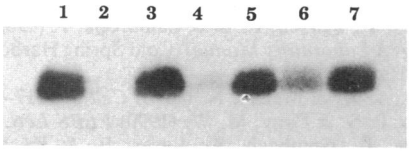

FIG. 4. Effect of micrococcal nuclease on the *in vitro* RNA editing assay. Lysate proteins (20 μl) were incubated in the presence of 1 mM $CaCl_2$ and 100 units of micrococcal nuclease at 20°C for 30 min. Reaction was terminated by addition of 5 mM EGTA. Four microliters (2 μg of protein) of the incubated lysate was used for the editing reaction. Edited products were detected as described in *Materials and Methods*. Lanes: 1, control without lysate; 2, nuclease-treated lysate, without EGTA; 3, lysate incubated in the presence of $CaCl_2$, without nuclease; 4, nonedited RNA alone; 5, lysate preincubated in the absence of nuclease and $CaCl_2$; 6, Nuclease-treated lysate; 7, Lysate incubated in the presence of $CaCl_2$ and EGTA.

In this work we have described an *in vitro* system for the specific editing of *atp9* mRNA. These results and the previous finding of partial and overedited forms of *atp9* mRNA (16) confirm that this process is a posttranscriptional event. The requirements of the wheat mitochondrial lysate *in vitro* assay show some differences from those of a nuclear lysate system as described for the editing of rat liver apolipoprotein B mRNA (9). Thus, the plant mitochondrial *in vitro* system seems to be dependent on Mg^{2+} ions and inhibited by EDTA, whereas EDTA was shown to stimulate the animal RNA editing system. As in the case of apolipoprotein B mRNA, the addition of nucleoside triphosphate to the wheat organelle lysate did not affect RNA editing (results not shown).

The nonedited *atp9* mRNA used as substrate for the RNA editing reaction by the wheat mitochondrial lysate is the uncapped T7 RNA polymerase transcription product, spanning the entire protein-coding region. The study of gene

organization in wheat mitochondrial DNA has shown that the genes for subunits 9 (*atp9*) and α (*atpA*) of the wheat mitochondrial ATP synthase are always adjoining (13, 14, 23). Moreover, the two genes are cotranscribed, and cDNA sequence studies of the *atpA* mRNA have shown a very low number of editing events, both in absolute terms and considering the larger size of *atpA* compared with *atp9* (unpublished work). It is difficult to quantitate the *in vitro* RNA editing reaction described here; however, a value of 6% seems reasonable since 3 out of 50 colonies were shown to hybridize with the 3′ wholly edited probe. The edited product, after reverse transcription and PCR amplification, showed the presence of the eight expected C-to-U conversions at the specific sites found for *atp9* mRNA isolated from purified organelles. No overedited forms were detected by hybridization, although 11 additional potential C substrate residues are present in the *atp9* mRNA region around the 3′ end. The presence of partially edited forms cannot be discounted and a larger number of clones would have to be sequenced to find such intermediates, as they cannot be detected by the probe used in this work. Thus, the *in vitro* system described here seems to be extremely faithful, since its products are identical to those isolated *in vivo*. Moreover, three results strongly indicate that we are measuring *in vitro* RNA editing of the exogenously added RNA substrate rather than contamination with endogenous edited *atp9* mRNA: (*i*) no hybridization with the labeled probe was obtained when amplification was carried out with nucleic acids isolated from a reaction mixture prior to the incubation with the lysate (Fig. 2*B*, lane 4); (*ii*) PCR amplification of the lysate gives neither ethidium bromide-detectable product nor hybridization signal (data not shown); and (*iii*) the clone used for synthesis of the nonedited *atp9* mRNA substrate had a G residue at position 117, whereas normally *atp9* carries an A residue at this position.

The temperature dependence of the reaction, as well as the effect of protease, indicates that a protein factor is essential for the reaction. It will be interesting to search for plant mitochondrial protein(s) able to bind specifically to the editing region, as described for a 40-kDa protein in the rat liver apolipoprotein B system (24). It is also possible that the specificity of the system is provided by the nucleic acid partner that seems to be involved in the *in vitro* RNA editing reaction, as deduced from our experiments with micrococcal nuclease. Wang and Gegenheimer (25) reported that EGTA-inactivated micrococcal nuclease may induce the apparent inhibition of RNA processing reactions by binding of the inactivated enzyme to the RNA substrate, thus masking the substrate from the enzyme. We have tried to solve this problem by performing competition assays with heterologous RNA (*E. coli* tRNA or yeast rRNA) as described by those authors. This approach seems unsuitable for our system, since control experiments showed a significant degree of inhibition when the *in vitro* editing activity was followed in the presence of exogenous RNA. However, EGTA-inactivated nuclease added to the RNA substrate before the incubation with the lysate, or pretreatment of the lysate with inactivated nuclease, gives no inhibition. These results argue against a substrate masking effect, although further work will be necessary to answer this question.

The discovery of RNA editing, in addition to the rearrangement at the DNA level, or splicing of pre-mRNAs from split genes, indicates that the basic principles of molecular biology are less simple and straightforward than initially thought. Thus, in some biological systems, the prediction of protein primary sequence from the coding region at the genomic level may lead to incorrect conclusions concerning the mature product. In plant mitochondria, RNA editing could play a crucial role in controlling mitochondrial function, since the C-to-U mRNA conversion is responsible for the production of the functional proteins indispensable for cellular energy production. The importance of this process requires, as described here, the characterization of an *in vitro* system able to edit mtRNA in an identical way as found *in vivo*.

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