The chloroplast genome of a dinoflagellate consists of a group of small circular DNA molecules (minicircles), most of which carry a single gene. With RT-PCR, primer extension, and Northern analyses, we show that the entire minicircle is transcribed and that some minicircles can produce RNAs larger than themselves. Using an RNA ligase-mediated rapid amplification of cDNA ends method, we were able to detect large processed precursors that are generated by endonucleolytic cleavage of an even longer molecule. This cleavage produces the mature mRNA 3′-end and at the same time the 5′-end of the precursor. The tRNAs encoded by the petD and psbE minicircles appear to be processed in the same way. We propose a “rolling circle” model for chloroplast transcription in which transcription would proceed continuously around the minicircular DNA to produce transcripts larger than the minicircle itself. These transcripts would be further processed into discrete mature mRNAs and tRNAs.

Dinoflagellates are a diverse group of protists. About one-half of dinoflagellate species are photosynthetic and serve as important primary marine producers, although they are best known as culprits of red tides. Most photosynthetic dinoflagellates have chloroplasts derived from a red alga via secondary endosymbiosis (1, 2), which feature three envelope membranes and a unique light-harvesting pigment, peridinin. The chloroplast genomes are also unique in that the genes, instead of being located on a large circular DNA of 100–200 kb, are individually harbored in small circular DNAs of about 2–3 kb (3, 4). A few minicircles are larger (up to 10 kb) and carry 2–4 genes, but genes found together are not in the same clusters as in other chloroplast or cyanobacterial genomes (5–8). So far, only 17 genes found together are not in the same clusters as in other chloroplast genomes, the dinoflagellate chloroplast genome is the smallest (11). A number of missing chloroplast genes have been relocated to the nuclear genome, partially explaining this severe reduction of the chloroplast genome size (12, 13).

Although dinoflagellates are important members of the marine ecosystem, the nature of the dinoflagellate cell has posed obstacles for biochemical research. The cell is encased by a exterior covering (thecal plates) and interior protective layers (pellicle) (14), making it difficult to disrupt the cell without damaging organelles, particularly the large complex lobed chloroplasts (15). Sexual reproduction is not well studied, and there are no mutant collections. Genome sequencing is likely to be far in the future, because the smallest dinoflagellate genomes are as large as the human genome (16).

Despite these difficulties, the transcription of minicircle genes has been studied in a variety of dinoflagellate species (3, 5, 10, 17–19). For any protein-coding gene, the most abundant type of transcript features a poly(U) tail at the 3′-end and is believed to be the mature mRNA (8, 19). In addition, some low abundance species of long RNAs are occasionally detected. In Amphidinium carterae, several genes have large transcripts with sizes comparable with the minicircles (20). RT-PCR results showed that the 5′- and 3′-ends of these transcripts border each end of a conserved core sequence in the non-coding region. Nisbet et al. proposed that a conserved motif GAAACGACA in the core may serve as the promoter, from which a long precursor RNA is transcribed and subsequently processed to a short mature one (20).

In H. triquetra, polycistronic transcripts of the petD and psbE minicircles can be detected, where the protein-coding gene is co-transcribed with the downstream tRNA genes (8). In the present study, we used Northern analyses, primer extension and RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE)2 to show that some minicircle transcripts of H. triquetra are larger than their templates (minicircles), suggesting a unique “rolling circle” transcription mechanism. Moreover, by using an RLM-RACE method, we provide evidence to show how the long transcripts are processed into mature mRNAs.

**EXPERIMENTAL PROCEDURES**

Algal Cultures—H. triquetra (CCMP 449) was obtained from the Provasoli-Guillard Culture Center for Marine Phytoplankton (Boothbay Harbor, ME) and grown in f/2-Si medium at 18 °C on a 12-h light/12-h dark cycle at 50 μmol m−2 s−1 light intensity.
Rolling Circle Transcription

transcriptase (Invitrogen) and random primers (hexamer, Invitrogen). Primer sequences and primer combinations are listed in supplemental Table S1. PCR products were directly sequenced so that the results represent the population of cDNAs.

Mapping of Transcriptional Initiation Sites—The method takes advantage of a modified procedure for RLM-RACE (22). In this study, we used the FirstChoice RLM-RACE kit (Ambion) and followed the manufacturer’s instructions with minor modifications. First, no calf intestinal phosphatase treatment was used for the total RNA. To prepare the tobacco acid pyrophosphatase (TAP)-treated (T+)-cDNA sample, about 10 μg of total RNA was treated with TAP to remove the 5'-triphosphate group. One-half of the reaction product (about 5 μg of treated total RNA) was then used in an RNA ligation reaction that enzymatically added a synthesized RNA linker (provided in the kit) to the 5'-end of the RNA. A fraction of the linker-ligated RNA (2 μg) was made into cDNA with random primers. For the TAP-untreated (T−)-cDNA sample, 5 μg of total RNA, without any TAP treatment, was directly used in the RNA ligation reaction and the subsequent cDNA synthesis. For each minicircle transcript, two rounds of PCR were performed. The first round PCR used either T+ or TAP-untreated (T−)-cDNAs as template, with the 5'-RACE outer adaptor primer and an outer gene-specific primer (inside or close to the coding region). Each PCR product was diluted 200–1000 times to serve as the template for a second round of PCR. PCR products from T+ and T−samples were separated side by side on agarose gels. The bands were purified and sequenced to determine the precise 5'-end position. Reverse primers used for the 5'-RACE of each gene are listed in supplemental Table S2.

RESULTS

Detection of Multicistronic Minicircle Transcripts—In our previous study, the 5'-UTRs of mature chloroplast mRNAs of *H. triqueta* were determined to be 40 nucleotides on average (23). However, the 5'-ends of these mRNAs are unlikely to represent the transcription initiation sites because the simplified RACE method used in that study can only detect processed 5'-ends carrying a monophosphate group (22). Moreover, analysis of the genomic sequences immediately upstream of the 5'-ends of these mature mRNAs showed no consensus promoter elements such as −35 and −10 motifs (data not shown). To determine the 5'-UTR of the *psbB* precursors, we used RT-PCR with the reverse primer inside the coding region and the forward primers in various locations (Fig. 1). Surprisingly, each

FIGURE 1. Transcription of the *psbB* minicircle non-coding region tested with RT-PCR. Top, structure of *psbB* minicircle with the approximate positions of primers (arrowheads), open reading frame (heavy arrow), and non-coding region (NC, thin line). Bottom, RT-PCR results with different combinations of primers. The size marker is a 1-kb DNA ladder (Invitrogen). The cDNA was made with total RNA and random primers. Total RNA was used as control to test the residual DNA in the RNA sample. All PCR products were purified and sequenced.
Rolling Circle Transcription

A primer pair gave a product of the estimated size and sequence, even the primer pair psbBr/psbBf. In other words, the 5'-UTR of psbB transcripts can extend across the full non-coding region and well into the 3'-end of the coding region. Therefore, the RNA detected using psbBr/psbBf primers could represent a long multicistronic RNA containing at least two open reading frames (ORFs), provided that the transcription is not started and terminated inside the ORF.

We expanded our test to seven other protein-coding minicircles (psbA, psbC, psbD, psaA, psaB, petD, and atpA) and found that all of them can produce some transcripts whose 5'-UTR can extend into the 3'-end of the preceding ORF. Therefore, it is possible that a minicircle can produce some partial non-coding region (0.4 kb).

Since the long transcripts are in low abundance, it is possible that the labeled primers could exceed the available binding sites and hybridize with other RNAs carrying similar sequences, leading to false signals (22). To eliminate this possibility, we performed RNA blotting for these four minicircles to confirm that minicircles can produce transcripts longer than themselves. To assess the difference of expression, we used total RNA purified from cells in the light or dark phase for Northern blotting. As shown in Fig. 2B, the pattern of hybridization is similar in both cases, except that the overall expression is slightly stronger in the light phase than the dark phase.

FIGURE 2. Detection of long minicircle transcripts. A, primer extension analyses of psbB, psbC, psaA, and psaB. For each sample, 30 μg of total RNA was used for cDNA synthesis with labeled gene-specific primers; reaction products were resolved on a 3.5% denaturing polyacrylamide gel containing 7 M urea. Interpretation of the results is shown beside the gel image. Table, lengths of minicircle, coding, and non-coding regions (in bp) and the names and positions of primers (first nucleotide of the start codon is +1). B, RNA analysis of psbB, psaA, psbC, and psaB minicircle transcripts. Total RNA was purified from cells growing in mid-light phase (L) or mid-dark phase (D). Total RNA (25 μg) was separated on a 1% formaldehyde agarose gel with Riboruler (Fermentas) as a size marker. The labeled probes for each gene (about 0.5 kb) were complementary to part of the protein-coding region.

B

prima species of RNA carrying more than one repeat of the DNA template.

To further confirm that minicircles can produce long precursor RNAs, we picked four minicircles (psbB, psbC, psaA, and psaB) for primer extension experiments and chose gene-specific reverse primers located about 300 bp downstream of the start codons (Figs. 1 and 2A). Each lane contains multiple bands, suggesting that a minicircle could produce more than one species of RNA with a UTR larger than that of the mature mRNA (40 nucleotides on average). By referring to the DNA sequence of the minicircle templates (Fig. 2A, Table), we reasoned that the 5'-UTR of these RNA species can be divided into two categories. In one type, the UTR only covers the non-coding regions. In some cases, a very small portion of the ORF upstream of the non-coding region may also be included. This type was found in all tested samples. The other type is a transcript carrying an additional intact ORF upstream of the non-coding region. This type was only found in the psbB and psaA lanes. In the psbB lane, four bands of about 1, 1.1, 1.3, and 3 kb can be detected. Because the primer is 341 nucleotides downstream of the start codon, these RNA species should carry 5'-UTRs of about 0.66, 0.76, 0.96, and 2.6 kb, respectively. Because the non-coding region of psbB is 840 bp, the 5'-end of the two larger RNA could span the entire non-coding region. As for the largest band (3 kb), its 5'-UTR (2.6 kb) could contain one full non-coding region (0.8 kb), one full ORF (1.4 kb), and a preceding
Under low exposure conditions, only one strong band could be seen in each sample lane (data not shown). These bands represented the mature RNAs because the sizes are similar to the corresponding ORFs. By extending film exposure time, some weak bands of higher molecular weight could be detected in psbB, psbC, and psaA lanes (Fig. 2B, arrowheads). The psbB lane has one weak band (2.7 kb) and two faint bands (3.2 and 4.5 kb) above the main band (1.5 kb). The psbC lane has two faint bands (3.2 and 4.2 kb) above the main band (1.5 kb). These weak bands in the psbB and psbC lanes are longer than the corresponding minicircles (2.3 and 2.2 kb, respectively). The psaA lane has a weak band of about 3 kb, larger than the main band (2.5 kb) and comparable with the size of the minicircle. Based on the evidence from RT-PCR, primer extension, and Northern blotting, it is clear that minicircles of H. triquetra chloroplasts can produce some transcripts that are longer than the minicircles themselves. No clear band representing long transcripts was detected for psaB, although the RT-PCR and primer extension results show that there are transcripts that span the non-coding region.

Long Precursor RNAs Have Processed 5'-Ends—To characterize the 5'-ends of the long RNAs, we used a modified RLM-RACE method that is able to distinguish between primary and processed transcripts (22). In chloroplasts or mitochondria, newly initiated transcripts carry a triphosphate group at the 5'-end, whereas processed transcripts only carry a monophosphate group (24). Since the T4 RNA ligase can only catalyze the ligation reaction between a 3'-hydroxyl group and a 5'-monophosphate group, a synthetic oligoribonucleotide can only be ligated with processed RNAs. Therefore, RLM-RACE can only provide the 5'-end sequence of processed RNAs (Fig. 3A). To distinguish between the primary and processed 5'-end, TAP is

**FIGURE 3.** Determination of the 5'-end of atpA long transcripts. A, schematic of atpA minicircle and experimental strategy. Two sets of cDNA were made with the RLM-RACE method, using either T+ or T- total RNA. PCRs were carried out with various combinations of gene-specific reverse primers and forward primers complementary to the RNA linker (5'-RACE outer adaptor primer (5OP) and 5'-RACE inner adaptor primer (5IP)). Because the 5'-end of newly initiated RNAs carries triphosphates, it can only be detected in T+ samples. B, first and second round PCR. To detect the low abundant pre-RNAs, nesting PCRs were carried out with conditions shown below each lane. The first round PCR products (1st PCR) were diluted 200–500 times with distilled water and served as the templates for a second round of PCR (2nd PCR) with 5'-RACE inner adaptor primer and nesting reverse primers (atpAnr1, -2, or -3). All products were sequenced; correct products are outlined. The length of the long precursor determined by RLM-RACE is indicated by a thin arc in A. Size marker is a 1-kb DNA ladder (Invitrogen).
TABLE 1
Cleavage sites identified with comparative RACE method

|        | psaA | psaB | psbB | psbE | petB | petD | atpA |
|--------|------|------|------|------|------|------|------|
| 5′-UTR length (bp) determined by RACE | 803  | 790  | 838  | 1757 and 1548 | 1091 | 1294 and 1168 | 943  |
| Non-coding region length (bp) | 806  | 888  | 840  | 1961 | 1545 | 1712 | 1086 |

FIGURE 4. Detecting the 3′-end cleavage site of the psbB minicircle transcript. The 5′-end of the long psbB precursor was determined by RLM-RACE as in Fig. 3 A, schematic of psbB minicircle with positions of primers used for 5′- and 3′-RACE (arrowheads). The black and grey arcs show the approximate span of 5′- and 3′-RACE (psbBf) relative to the DNA template. B, second round PCR with psbBnr2 and 5′-RACE inner adaptor (SIP) primers. The first round PCR used psbBr or psbNr1 primer. Both T+ and T− gave a single product. C, alignment showing the sequences of the long precursor RNA detected by RT-PCR (with psbBr and psbBr) compared with 3′-RACE and 5′-RACE results. The sequences of the poly(U) tail and the RNA linker (enzymatically added in 5′-RACE) are indicated by filled boxes. The open box indicates the stop codon of the psbB ORF.

FIGURE 4C. DNA/polycistronic RNA

A

B

C

DNA/polycistronic RNA

3′ RACE

5′ RACE

ATCTATTTCAATAAATACCTCCATTCTAATAG

ATCGTCTCTTAAAATAACCTCCATTCTAATAG

RNA Linker

Poly(U) tail

atpA, psaA, and psaB minicircles. Each of these minicircles could produce at least one species of precursor with a large 5′-UTR (Table 1). Because all of the results can be obtained from both T+ and T− cDNAs, these data represent the sites where precursor RNAs are cleaved from primary transcripts initiated at unknown sites.

Single-step Cleavage Produces the 5′-End of the Processed Precursor at the Same Time as the Mature 3′-End—The RLM-RACE data in Table 1 suggest that the 5′-ends of the psaA and psbB long precursors are only a few bases from the 3′-ends of the ORFs. For example, 5′-RACE of the psbB minicircle transcripts gave just one band, which represents the processed 5′-end because it is found in both the T+ and T− lanes (Fig. 4B). The 3′-end of the mature mRNA was determined by taking advantage of the 3′ poly(U) tail, which is a hallmark of mature chloroplast mRNAs (8, 19). Alignment of sequence data from both the 5′-RACE and 3′-RACE against the DNA sequence (3) clearly showed that the 3′-end of the mature mRNA is at the same position as the 5′-end of the precursor RNA (Fig. 4B). Combined with the evidence that psaA and psbB minicircles can produce some precursor RNAs longer than the DNA template, it therefore appears that the mature 3′-end and the precursor 5′-end are generated by a single endonucleolytic cleavage of a polycistronic RNA. The precursor must then be cleaved again to give the mature 5′-end and a putative uridylytransferase will add the poly(U) tail to the 3′-end.
A previous study showed that petD and psbE minicircles carry several tRNA genes that are co-transcribed with the upstream protein-coding gene (8). As with all of the other genes investigated here (e.g. Fig. 1), outwardly directed RT-PCR showed that transcripts of these minicircles could span the entire non-coding region, including the tRNA genes, and extend into both ends of the protein-coding gene (data not shown). RLM-RACE analysis of petD minicircle transcripts gave two species of precursor RNA (Fig. 5, left). Because the bands appeared in both the T+ and T− lanes, the 5′-ends of these two precursor RNAs were also generated from RNA cleavage rather than transcription initiation. Alignment of the 5′-RACE sequencing data with the petD minicircle sequence showed that one of the processed long RNA 5′-ends is next to the predicted 3′-end of trnW and that the other is next to that of trnP (Fig. 5, right). Therefore, the first step of tRNA maturation should be an endonucleolytic cleavage that produces the mature 3′-end of the tRNA. In the case of trnP, this leaves a prolonged 5′-end that ends at the 3′-end of the previous gene (trnW). In the case of the psbE minicircle, two cleavage sites were identified (Table 1). One is 1757 bp upstream of the psbE start codon and right at the 3′-end of the predicted trnP gene. The other is 1548 bp upstream and inside the conserved non-coding region but not near any recognizable ORF.

**DISCUSSION**

Transcription Initiation and Termination—Long chloroplast transcripts, almost the same size as the minicircles, have previously been reported in *A. carterae* (17). However, no transcripts covering the entire non-coding region of the minicircle were detected. In this study, we show that in another dinoflagellate, *H. triquetra*, minicircle transcripts can extend across the non-coding region (Fig. 1) and can be even longer than the DNA template (Fig. 2). The sizes of the high molecular weight bands in the psbB and psbC lanes on RNA blots imply that the transcripts could carry two complete ORFs separated by a non-coding region (Fig. 2B). The psaA band of about 3 kb is the same size as the minicircle, but primer extension shows a faint band of about 4 kb, which would imply the existence of a transcript with a second partial ORF.

Chloroplast mRNAs in plants usually require 5′ processing because the 5′-UTRs of the primary transcripts are often larger than those of the mature mRNAs. This means that two 5′-ends can be detected: one corresponding to the transcription initiation site and the other to the processed (mature) end (24, 25). In this study, we were unable to find the transcription initiation site(s) for any tested minicircle. However, using RLM-RACE, we were able to detect long precursor molecules with 5′-monophosphate rather than 5′-triphosphate ends (Fig. 3), which must therefore have been processed from even longer transcripts. The 5′-end of the mature mRNA was generated by a second endonucleolytic cleavage (Fig. 2). In some cases (psaA and psbB minicircle transcripts), the 3′-end of the mature mRNA exactly meets the 5′-end of the processed precursor RNA (Fig. 4), suggesting that a single nucleolytic cleavage creates the mature 3′-end and the precursor's 5′-end. This implies that the initial transcript (so far undetected) was longer than the DNA minicircle.

In plants and algae, almost all of the chloroplast mRNAs have a small inverted repeat (hairpin) in the 3′-UTR (26, 27). In *Escherichia coli*, this stem-loop structure can function as a Rho-independent transcription terminator. However, in plant and algal chloroplasts, it mainly functions in mRNA stability and is very inefficient for transcription termination, which results in 3′-end read-through (28). Transcription termination in chloroplasts is not well understood (26), but may involve protein factors, as shown in mammalian mitochondria (reviewed in Ref. 29). In the case of *H. triquetra* it is unlikely that stem-loop structures are required, because most minicircles do not contain any recognizable inverted repeats (30).

**Rolling Circle Transcription Model of H. triquetra Minicircles**—Based on the data presented in this study, we put forward a model to describe the transcription mechanism of *H. triquetra* minicircles (Fig. 6). Because the templates are closed DNA (minicircles), we hypothesize that the transcription of a minicircle, once initiated, goes along the circular DNA for many rounds to produce polycistronic precursor RNAs. These polycistronic precursors are then cleaved to give processed pre-mRNA precursors that have a long 5′-UTR but the mature 3′-end sequence. The formation of the mature mRNA involves further 5′ cleavage to produce a molecule with a short 5′-UTR (about 40 nucleotides) and the addition of the 3′ poly(U) tail.
We term this unique mechanism “rolling circle” transcription because it is reminiscent of the replication of bacteriophages and dinoflagellate minicircles (31). In this model, due to the physical characteristic of the minicircle, the high accumulation of minicircle gene transcripts could be realized with a weak promoter, because the minicircle can theoretically produce numerous repeats of itself with a single initiation. In fact, transcription could be initiated at random sites rather than downstream of a strong promoter.

In nature, small circular DNAs (e.g. plasmids, virus genomes, and mitochondrial minicircles of trypanosomes) are common in many organisms. These DNAs harbor recognizable promoters and terminators to define the size of primary transcripts, and there is no reported case where the primary transcript is longer than the circumference of the template. The only case similar to the transcription pattern of dinoflagellate chloroplast minicircles is an artificial transcription system utilizing a single-stranded circular DNA template (32, 33). Transcription by either E. coli or T7 RNA polymerases initiates randomly and produces transcripts that can be several times longer than the DNA circle.

In prokaryotes, pre-tRNA usually needs several ribonucleases (RNase E and several exonucleases) to trim the protruding 3′-end to expose the CCA arm (34). Most tRNA genes in chloroplasts and mitochondria do not encode CCA at the 3′-end, but an endonucleolytic cleavage is required before the CCA can be added by tRNA nucleotidyltransferase (34). The endonuclease for processing the 3′-end of plant chloroplast tRNAs has been identified as a ribonuclease Z (35–37). Upon cleavage, it leaves a hydroxyl group at the 3′-end of the tRNA and a phosphate group at the 5′-end of the remaining transcript (35). In H. triquetra, the trnW, trnM, and trnP genes in psbE and petD minicircles do not encode a 3′-CCA. Moreover, our data show that the endonucleolytic cleavages at the 3′-ends of trnW and trnP leave a monophosphate group at the 5′-end of the remaining transcript, because the 5′-end phosphate group is the prerequisite for the ligation of the RNA linker. Taken together, these suggest that the processing of tRNA 3′-ends in dinoflagellate chloroplasts is also likely to be carried out by an enzyme with the characteristics of RNase Z.

In plant chloroplasts, mRNA maturation requires removal of the read-through sequence after the 3′-end stem-loop structure with the aid of an exonuclease (28, 38). In H. triquetra, the 3′-ends of psbB and psaA mRNAs are solely generated by endonucleolytic cleavage. Because the biochemistry of the cleavage is similar to that of the tRNAs (products carry a 5′-phosphate group), we hypothesize that the 3′-end of mRNAs could be generated by the same endonuclease. Canino et al. (37) suggested that tRNAase Z might control the 3′-end maturation of mRNAs as well as tRNAs, because a deletion mutant of chloroplast tRNase Z in Arabidopsis causes a lethal effect. If this is the case in dinoflagellate chloroplasts, RNase Z could also be the endonuclease responsible for mRNA processing (Fig. 6).

In summary, we used several different methods to prove that minicircles could produce large mRNA precursors and identified the cleavage sites in some precursors. These data suggest that the transcription of dinoflagellate minicircles follows a unique rolling circle manner. Moreover, the processing of the 3′-end of the mRNAs is very similar to that of the tRNAs, suggesting that the single-step endonucleolytic cleavage for
mRNAs and tRNAs might be carried out by the same ribonuclease.

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