

# Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in erythrocyte culture

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Received March 14, 1991; Revised and Accepted May 28, 1991 EMBL accession nos X59604, X59605 and X59607

## ABSTRACT

Three distinct ribosomal RNA (rRNA) transcription units (rDNA units), designated A, B and C, were identified in the intraerythrocytic protozoan parasite, *Babesia bigemina*. These rDNA units were cloned, and restriction maps were constructed showing the approximate location of the small and large rRNA coding regions. The arrangement of the genes in the genome and copy number analysis suggests the presence of a single copy of each rDNA unit per haploid genome. The complete nucleotide sequence of the small subunit rRNA coding region (1693 bp) and parts of the 5' and 3' flanking regions were determined for all three units. Units A and B have identical sequences, but unit C differs from units A and B at ten nucleotide positions, two in the small subunit rRNA coding region and four each in the adjacent 5' and 3' flanking regions. The differences in the coding region are confirmed in genomic DNA and RNA from two different isolates of *B.bigemina*. The RNA of both sequence types is transcribed in parasites from erythrocyte culture, however, the products of gene units A + B accumulate at a ratio of approximately 4:1 compared with the product of unit C.

## INTRODUCTION

Ribosomal RNA genes (rDNA units) of most eukaryotes are arranged as multiple copies of nearly identical genes located in tandem arrays (1). The malaria parasite is a notable exception having only four to eight non-tandem repeated rDNA units located on several chromosomes (2–4). In addition, stage-specific expression of structurally distinct rDNA units occurs in *Plasmodium* species (5–7), which is characterized by high sequence divergence (3.5–17%) among the small subunit rRNAs (SSrRNA) (8,9). It is proposed that this unique phenomenon may be a function of the dual host life cycle of the parasite (5,10), and it may be a common feature of related parasites that infect both an arthropod and a vertebrate host. In order to test this hypothesis we have analyzed the arrangement and transcription

of rDNA units from *Babesia bigemina*, a tick-borne Apicomplexan hemoparasite related to *Plasmodium* spp. Our results demonstrate the presence of three different rDNA units in *B.bigemina*, which is similar to another *Babesia* species, *B.bovis* (11). Restriction maps of the rDNA units of both species indicate that they are non-identical having restriction site differences in both coding and flanking regions. The nucleotide sequence of the SSrRNA coding regions, and parts of the 5' and 3' flanking regions, are reported from all three rDNA units of *B.bigemina*. The RNA from blood-stage parasites cultured *in vitro* was analyzed to determine which of the three genes were expressed in this parasitic stage.

## MATERIALS AND METHODS

### *In vitro* cultivation of *B.bigemina*

A Puerto Rico isolate (PR), a Mexico isolate (M) and JG29 (a clone of the Mexico isolate prepared by limiting dilution), were continuously cultivated *in vitro* in fresh bovine erythrocytes using a modified microaerophilous stationary phase (MASP) culturing system (12). Infected bovine erythrocytes were concentrated by percoll gradient (13) when the parasitemia reached 3–5%.

### Extraction and agarose gel analysis of nucleic acids

Bacteriophage, plasmid and genomic DNAs were isolated, restriction enzyme-digested, separated by agarose gel electrophoresis, and blotted as described (14). RNA was isolated by the single step method of acid guanidinium thiocyanate-phenol-chloroform extraction as described (15). Nucleic acids isolated from JG29 were used unless otherwise noted. DNA was radiolabelled for use as a probe by random primer labelling using  $\alpha$ -<sup>32</sup>P-dCTP (16).

### Preparation of *B.bigemina* genomic libraries in lambda bacteriophage and screening for rDNA clones

Genomic libraries of 9–23 kb fragments were constructed in  $\lambda$ GEM11 as described (17). The library of the Mexico isolate was prepared using a Sau3A I partial digest ligated into BamH I-digested phage arms, while that of JG29 was prepared from

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DNA digested to completion with EcoR I and ligated into EcoR I-digested phage arms. The libraries were screened with  $^{32}\text{P}$ -end-labeled *B. bigemina* total RNA probe (18).

#### Determination of the rDNA as a percentage of the genome

Increasing amounts of genomic DNA (90–720 ng) and cloned DNA from unit A (0.13–2.08 ng) were separated on a 0.8% agarose gel, blotted, and hybridized to radiolabelled probe from unit A. The hybridized blot was scanned in an AMBIS radioanalytic imaging system, and the percentage of the genome comprised by the fragment of unit A was calculated by comparing radioactivity present in the genomic rDNA and the corresponding band in unit A.

#### Synthetic oligonucleotides

Oligonucleotides prepared specifically for this study were: 6-1R11, 5'ACGAGGACAACATCCAAGCA3', located at positions 1269–1250 on the (–) strand of units A and B with a  $T_m = 60^\circ\text{C}$ ; 6-1R14, 5'ACGAGGACACAATCCAAGCA3', located at positions 1269–1250 on the (–) strand of unit C with a  $T_m = 60^\circ\text{C}$ ; BbgUS1-1, 5'GGCGGCGTTTATTAGTTCG-TTAACCAC3', located at positions 183–209 on the (+) strand of all units with a  $T_m = 78^\circ\text{C}$ ; and BbgUS8-1R, 5'GGACAG-GACAACTCGATGGATGC3', located at positions 1399–1376 on the (–) strand of all units with a  $T_m = 76^\circ\text{C}$ . All positions are as reported in Figure 2, and oligonucleotides used for sequencing are described in the legend of Figure 2. All oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer at the ICBR DNA Synthesis Core at the University of Florida.

#### Sequencing of nucleic acids

DNA sequencing was performed by the dideoxy nucleotide chain termination method (19) using either Sequenase (US Biochemicals) or a modified T7 DNA polymerase (Pharmacia). RNA sequencing was performed using AMV reverse transcriptase (Promega) and  $^{32}\text{P}$ -end labelled primers as described previously (14,20). Sequences were analyzed using the University of Wisconsin Genetics Computer Group programs package (21).

#### Reverse transcription and polymerase chain reaction (PCR)

Complementary DNA was prepared from rRNA using AMV reverse transcriptase to extend complementary anti-SSrRNA primers as described previously (14). Complementary DNA or cloned rDNA was amplified *in vitro* by the polymerase chain reaction (PCR) technique (22) employing BbgUS1-1 and BbgUS8-1R as primers and using Taq DNA polymerase (Perkin-Elmer-Cetus). Thermal cycling was performed in a Coy Model 60 cyclotherm. The target DNA was denatured by incubation at  $92^\circ\text{C}$  for 10 min followed by 35 cycles of denaturation ( $92^\circ\text{C}$  for 1 min), primer annealing ( $55^\circ\text{C}$  for 3 min) and primer extension ( $72^\circ\text{C}$  for 3 min). At the end of cycling the reaction was held at  $72^\circ\text{C}$  for 10 min and cooled to  $4^\circ\text{C}$ .

## RESULTS

#### Evidence for the presence of three rDNA units

Probe prepared from *B. bigemina* total RNA and heterologous probes encoding different regions of the rDNA of *Plasmodium berghei* (23) were used to analyze the rRNA genes present in *B. bigemina* genomic DNA digests. Southern blots of *B. bigemina*

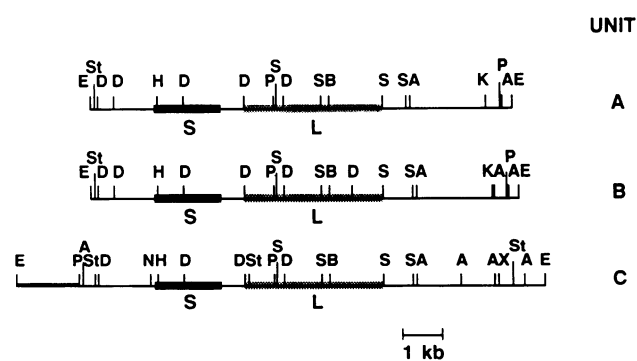
genomic DNA digested with EcoR I displayed the same two bands corresponding to approximately 13.5 kb and 11 kb with all probes (Data not shown) suggesting that the 13.5 and 11 kb fragments each contain the complete coding sequences of small and large rRNAs. Also, in each case the autoradiographic signal from the 11 kb fragment was approximately twice as intense as from the 13.5 kb fragment, suggesting twice as many copies of the 11 kb fragment compared to that of 13.5 kb fragment. Analysis of blots of genomic DNA digested with BamH I + EcoR I, BamH I + Pst I, and Pst I + EcoR I probed with heterologous probes from *P. berghei* and *B. bigemina* total RNA indicated the presence of three different rDNA units with approximate lengths of 10.65, 10.80 and 13.35 kb (not shown).

#### Cloning and mapping of *B. bigemina* rDNA units

Units A and B were cloned from the genomic DNA library made from Sau3A I partial digest and were subcloned as EcoR I fragments into plasmid Bluescript (SK+). Unit C was cloned from EcoR I complete digested genomic DNA library and was subcloned as 3 parts using Pst I, BamH I, and EcoR I restriction sites. Attempts to subclone the 5' end 1.6 kb EcoR I-Pst I fragment of unit C were unsuccessful.

Detailed restriction maps of all three units are presented in Figure 1. The region encoding the mature SSrRNA was localized on the map of each unit based on sequence analysis, whereas the large rRNA coding region was localized by Southern blot analysis. The lengths of the mature rRNA molecules were measured using formaldehyde gel electrophoresis which gave sizes of 1.7 and 2.9 kb for the small and large rRNAs, respectively (data not shown).

Units A and B have nearly identical restriction maps. Exceptions are the presence of one additional site for each of Dra II and Acc I in the 3' half of unit B and an additional ~0.15 kb in the 3' flanking region of unit B between the Sma I sites at the 3'-end of large rRNA coding sequences. In contrast, unit C differs from units A and B in several restriction site positions. In the region which appears to encode the large rRNA, unit C differs from units A and B with an additional Sst I site at the 5' end, and unit B differs from units A and C with an extra Dra II site near the 3'-end.



**Figure 1.** Restriction maps of the three ribosomal RNA gene units of *B. bigemina*. Maps of units A, B and C are presented. A, Acc I; B, BamH I; D, Dra II; E, EcoR I; H, Hind III; K, Kpn I; N, Not I; P, Pst I; S, Sma I; St, Sst I; X, Xho I. Maps are drawn left to right 5' to 3' with respect to the RNA transcript. Coding regions for small and large ribosomal RNAs are identified by thick solid and stippled lines, respectively. This localization was based on the sequence data and DNA filter hybridization data of restricted fragments using total RNA as probe. In unit C the region defined by a double line was restriction mapped only with EcoR I and Pst I.

**Analysis of rDNA as a percentage of the genome**

Analysis of the percentage of the genome comprised of rDNA is consistent with the data from genomic analysis describing the presence of 3 different single copy rDNA units. This analysis also supports the conclusion that the ratio of the co-migrating bands of units A and B to that of unit C is approximately 2:1. The 10.65/10.8 kb-EcoR I-rDNA band in 1 µg of genomic DNA is equivalent to 0.9 ng of the cloned DNA of unit A, and the average ratio of cpm in the 13.35 kb band compared to the 10.65 + 10.80 kb band was approximately 1:2 (Data not shown). The exact genome size of *B. bigemina* is not known, but assuming a genome size of  $2.5 \times 10^7$  bp (24–27), these results indicate that each of the units A, B and C are present as a single copy.

**Sequence analysis of SSrRNA coding regions**

The complete coding sequence of the SSrRNA and parts of the 5' and 3' flanking sequences were determined from both strands for units A, B and C (Figure 2). Comparison of the sequence data to consensus SSrRNA 5' and 3' end sequences (28) defined a 1693 bp sequence for the *B. bigemina* SSrRNA in all three units. This was nearly identical to the length of the mature SSrRNA measured using formaldehyde agarose gel electrophoresis, 1.7 kb (data not shown). In addition, the 5' end of the coding region was defined by primer extension sequencing of the RNA from

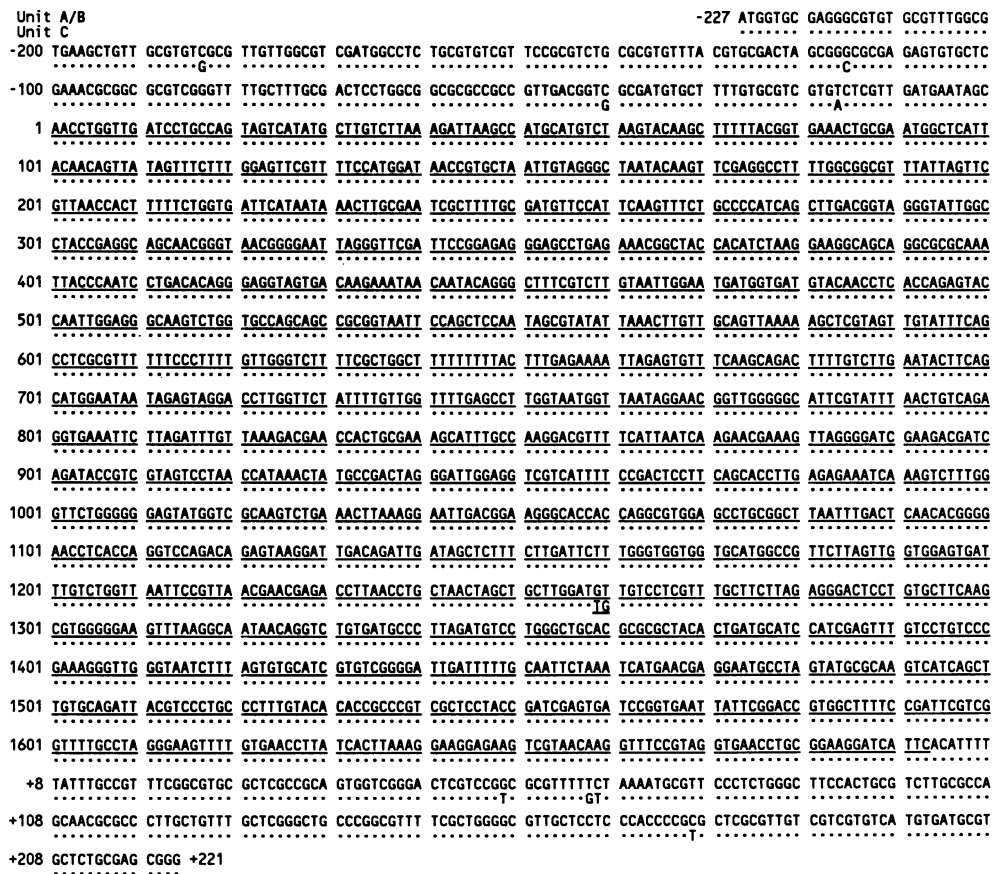
cultured parasites and was consistent with the consensus 5' end (data not shown).

The nucleotide sequence of units A and B were identical in the region examined, whereas unit C differed from A and B by ten nucleotides, four each in the 5' and 3' flanking sequences and two in the coding region at positions 1259 and 1260. In units A and B the bases at positions 1259 and 1260 were guanine and thymine (GT) whereas in unit C they were thymine and guanine (TG), respectively. Each of the 10 polymorphisms resulted in the gain or loss of a restriction endonuclease site, and 2 of these were confirmed by restriction endonuclease mapping. The paired transversions (GT to TG) in the coding region eliminated a Fok I restriction site in unit C. The G to C transversion at nucleotide position -116 created a Not I site in unit C.

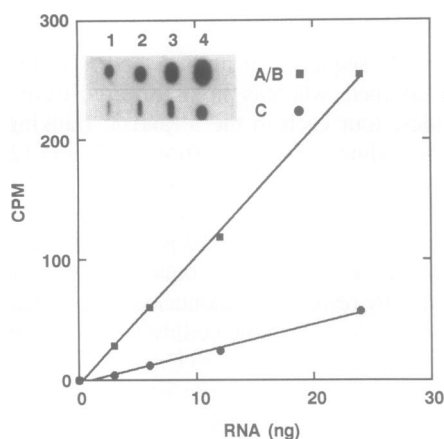
The *B. bigemina* SSrRNA genes were 366–455 nucleotides smaller than those of *Plasmodium* species (8,9). When compared with *P. berghei* unit A, the SSrRNA of *B. bigemina* was 366 nucleotides smaller. The reduced size was predominantly due to shorter variable regions (29).

**Verification of sequence differences in the SSrRNA genes**

The transversions at positions 1259 and 1260 detected in cloned DNA were verified in the genome by hybridizing synthetic oligonucleotides specific for units A/B and C (6-1R11 and



**Figure 2.** Nucleotide sequence of the SSrRNA coding regions of all three units. Nucleotide sequences were determined from both strands of units A, B and C using the synthetic primers made from the conserved regions in eukaryotic SSrRNAs (34). In addition, primers SP5 (5'GGGCGTGTGCGTTTGGC3') and SP3 (5'AACGCCCCAGCGAAAACGCCGG3') were synthesized from the sequences 5' and 3' to the coding region, respectively, for the completion of sequencing of both strands. Units A and B had identical sequences in the regions examined. The sequence for unit C is presented below the sequence for units A/B only where it differs. Dots represent sequence identity between units A/B and C. Numbers 1–1693 and under lines indicate the coding sequence for SSrRNA. Numbers with '-' sign and '+' sign indicate sequence positions 5' and 3' to coding sequences, respectively.



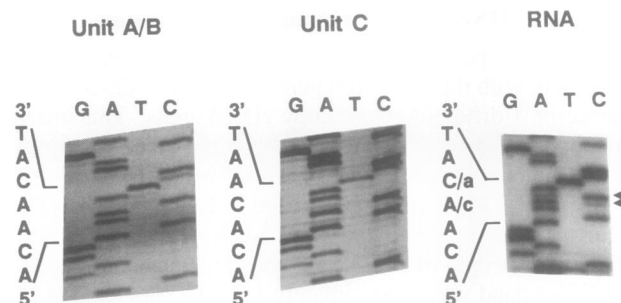
**Figure 3.** Ratio of unit-specific rRNAs in cultured parasites measured with unit-specific oligonucleotide probes. Slot blots of RNA denatured with formaldehyde were prepared using a manifold apparatus (BRL) and nylon membrane (14). Varying concentrations of *B. bigemina* total RNA isolated from the cultured parasites were applied to two membranes (slots 1 to 4 of each received 3 ng, 6 ng, 12 ng and 24 ng, respectively). Filters were hybridized at 60°C one each with 6-1R11 (specific for units A/B) and 6-1R14 (specific for unit C) as described in (30). Radioactivity incorporated in various slots was counted in AMBIS machine and cpm versus RNA concentration were plotted. (Under these conditions the corresponding cloned DNA controls hybridized with similar quantities of probe.)

6-1R14, respectively) to Southern blots of genomic DNA. Conditions for specific hybridization of these oligonucleotides which differ from one another by only two nucleotides at positions 10 and 11, were established first with the cloned DNA from units A and C under conditions essentially as described in (30). Specific hybridization was observed at 55°C and 60°C. Therefore, 60°C was selected to verify the existence of the two different coding sequences in genomic DNA. Oligonucleotides specific for units A/B and C selectively hybridized to 10.65/10.80 kb and 13.35 kb EcoR I restriction fragments, respectively, which corresponded to the size of the EcoR I fragments carrying their respective rDNA units (data not shown).

#### Analysis of RNA from parasites in erythrocyte culture

Since the synthetic oligonucleotides differentiated units A/B and C in genomic DNA, we analyzed the RNA prepared from the cultured parasites for the presence of these transcripts. The results shown in Figure 3 indicated the presence of both transcripts in cultured parasites. The ratio of transcripts A/B to C in the RNA was 4:1 as calculated from blots hybridized at 60°C. The DNA-RNA hybrids are more stable (about 5°C) than DNA-DNA hybrids (14), thus the stringency of hybridization was increased to 65°C. The ratio remained unchanged (data not shown). Since this method cannot distinguish unit A from B, it is not known whether only one or both units were transcribed. Both SSrRNA transcripts were also detected in the intraerythrocytic parasite using RNA-PCR (primers BbgUS8-1R and BbgUS1-1) coupled with diagnostic Fok I digestion of the products (data not shown).

Total RNA was sequenced in the region of the sequence differences using BbgUS8-1R as a sequencing primer, and the sequence was compared with that of cloned DNAs for units A/B and C (Figure 4). The RNA sequence ladder at bases 1259 and 1260 looked predominantly like the sequence of units A/B. However, minor bands were also visible that correspond to unit C (see arrows in Figure 4). These results, further supported the



**Figure 4.** Comparison of RNA sequence ladder to cloned DNA sequence ladder of units A and C. Sequence in the coding region 1269–1250 is shown. RNA sequence was generated using  $^{32}\text{P}$ -end-labeled BbgUS8-1R as primer. DNA sequence was generated by  $\alpha$ - $^{35}\text{S}$ -dATP incorporation. The two arrows indicate the paired transversions observed in sequencing of the cloned genes.

relative abundance of units A and/or B in the RNA analyzed. The RNA prepared from *in vitro* culture of another isolate of *B. bigemina*, PR, was also sequenced (data not shown). The results showed a sequence ladder identical to that shown for the RNA of JG29, indicating the conservation of the two different SSrRNA units among strains.

#### DISCUSSION

Malaria parasites are thus far unique in possessing rDNA units which are few in total number, structurally distinct and developmentally regulated (5–10). We have examined the rDNA of *B. bigemina*, a closely related arthropod-borne, Apicomplexan, hemoparasite, to ascertain whether this observation could be extended to another genus. The results obtained show that there are three structurally distinct rDNA units in *B. bigemina*, which is low relative to other eukaryotes but similar to the number detected in *Plasmodium* spp. (2–4). Obvious similarity of *B. bigemina* rDNA to that of *Plasmodium* spp. ends with the number of units, however, since significant differences were detected in most other attributes including the sizes of the mature rRNAs, the extent of sequence divergence among rDNA units within a species, and the extent of differential control of expression.

The major rRNA molecules of *B. bigemina* are smaller than those of most eukaryotic organisms (31), including the malaria parasite, where the small and large rRNA coding sequences are 0.4 kb (8,9) and 1.1 kb (18) smaller, respectively. It is of interest that the regions of the SSrRNA molecule which are reduced in *B. bigemina* rDNA relative to *Plasmodium* spp. are those which contribute most to the structural differences in *Plasmodium* spp. This results in the situation where, though structurally distinct, the *B. bigemina* rDNA units are much more similar to one another than is found in *Plasmodium* spp.

Sequence analysis of the rDNA units reveals that the SSrRNA produced by *B. bigemina* rDNA units A, B, and C are identical save 2 adjacent transversions in unit C. These differences have been confirmed in both the genome and the SSrRNA of a cloned parasite line, and the presence of the same paired mutation in the Puerto Rico isolate suggests that the difference is a fixed rather than a random mutation. The potential effect of such a small mutation in variable region V (29), which corresponds to the loop in *Escherichia coli* at 1120–1150 (28), on the functioning of the ribosome is subject to speculation since single base mutations

in other regions of the SSrRNA have significant effects on the functioning of the ribosome (32,33). Although not known to grossly affect ribosome function, mutations in this region may have subtle effects (e.g. efficiency of translation or stability of certain classes of mRNAs). Structurally different ribosomes in *Plasmodium* are due to mutations that occur principally in the variable regions constituting a 3.5%–17% variation in overall SSrRNA sequence (8,9). This raises significant questions about why such a situation exists. These divergent SSrRNA transcripts may form the core of functionally distinct ribosomes essential for the proper development of the organism or they may be the product of genes that for other reasons are independently evolving yet form essentially equivalent ribosomes. These hypotheses remain to be tested, but our study suggests that *B. bigemina* is able to handle the obligate two host life-cycle without developing major structural differences in its SSrRNAs.

Although transcripts of the three rDNA units of *B. bigemina* do not accumulate equally in the erythrocytic stages (the ratio of (A+B):C is approximately 4:1), the transcripts of each unit are much more equally represented than the 20:1 ratio of the A to C units of either *P. berghei* (8) or *P. falciparum* (9). In *Plasmodium* spp. the A:C ratio reverses during the transitional stages between the vertebrate and arthropod hosts. Studies are underway to determine whether the (A+B):C ratio in *B. bigemina* is altered in the tick stages of its life cycle. Further, future analysis of the large rRNA will make it possible to fully assess the accumulation of each of the three rDNA units.

## ACKNOWLEDGEMENTS

The authors wish to thank Ms. Ileana Crespo-Tizol, Ms. Raina DiVenere, and Mr. Stephen Kania for their able technical assistance. This work was supported by USAID Grant DAN-4178-A-00-7056-0.

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