Identification of *tlc* and *gltA* mRNAs and Determination of In Situ RNA Half-Life in *Rickettsia prowazekii*

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RNAs of *Rickettsia prowazekii*, an obligate intracytoplasmic bacterium, have been identified and analyzed by an RNase protection assay. Total RNA, a mixture of host cell RNA and rickettsial RNA, was isolated from rickettsia-infected mouse L929 cells by the hot-phenol method. After hybridization with specific antisense RNA probes and digestion with RNase, the protected products were analyzed by electrophoresis and autoradiography. The results show that there is only one mRNA species for the ATP/ADP translocase gene (*tlc*) but two mRNA species for the citrate synthase gene (*gltA*). RNA half-lives were determined by measuring the RNA remaining after addition of rifampin. The half-lives of *tlc* mRNA, *gltA* mRNA I, and *gltA* mRNA II in *R. prowazekii* are 8.4 ± 0.6 , 12.3 ± 1.3 , and 20.5 ± 1.8 min, respectively. However, the half-lives of *tlc* mRNA and *gltA* mRNA I in recombinant *Escherichia coli* strains are 2.9 ± 0.1 and 1.4 ± 0.1 min, respectively. The 16S rRNA in *R. prowazekii* was also examined and shown to be stable.

The steady-state level of a functional mRNA is determined by the balance between the rates of synthesis and decay. Hence, mRNA turnover plays an important role in the control of protein synthesis. The mRNA half-life in *Escherichia coli* is about 3 min (3). However, nothing is known about the stability of mRNA in *Rickettsia prowazekii* in vivo. *R. prowazekii*, the causative agent of epidemic typhus, is an obligate intracellular bacterium with a typical gram-negative bacterial morphology (7, 10). *R. prowazekii* grows directly in the cytoplasm of its eucaryotic host cell with a generation time of 8 to 12 h (6, 11, 12). The analysis of mRNA stability in an intracellular parasite is hampered by the difficulty of rapidly obtaining the parasite free of host cell contamination. In fact, *R. prowazekii* has no functional mRNA after a 4-h purification (9). One approach for circumventing this difficulty would be to analyze rickettsial mRNAs in total RNA rapidly isolated from the rickettsia-infected host cell. In this study, we used an RNase protection assay to analyze the half-life of the RNA transcribed from the *R. prowazekii* citrate synthase gene (*gltA*) (13), ATP/ADP translocase gene (*tlc*) (8), and 16S rRNA gene (*rrs*) (2), which encode a cytoplasmic protein, a membrane protein, and an rRNA, respectively.

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Detection of the rickettsia-specific RNA. RNase protection assays were conducted according to standard methods (4) with total RNA (host cell RNA and rickettsial RNA). Briefly, mouse L929 cells were infected with purified, yolk sac-grown *R. prowazekii* Madrid E. At 36 h postinfection, the rickettsia-infected cell monolayers were lysed on the plates by 1% sodium dodecyl sulfate in 10 mM sodium acetate, pH 4.5. Total RNA was isolated by using the hot-phenol method described by Emory and Belasco (1). For controls, RNA was also isolated from mock-infected L929 cells. Antisense RNA probes labeled with ^{32}P for the RNase

protection assay were generated by in vitro runoff transcription with either T3 RNA polymerase or T7 RNA polymerase (Promega Corp., Madison, Wis.) according to the manufacturer's instructions (5). The DNA template for making the *gltA* probe was constructed by subcloning the 284-bp *Hind*III-*Cla*I fragment of the rickettsial *gltA* gene (13), which contains 104 bp of the open reading frame with a 180 bp upstream sequence, into pBluescriptII(KS+). The DNA template for making the *tlc* probe was constructed by subcloning the 361-bp *Xba*I-*Cla*I fragment of the rickettsial *tlc* gene (8), which contains 181 bp of the open reading frame with an 80-bp upstream sequence, into pBluescriptII(KS+). The DNA template for making the *rrs* probe was constructed by inserting a 525-bp coding sequence of the rickettsial *rrs* gene (2) into pBluescriptI(SK-). Total RNA (100 μg) was hybridized to each probe in the presence of 80% formamide. Since *gltA* and *tlc* contain 25% G plus C bases and *rrs* contains 50% G plus C, the hybridization temperature was 30°C for *gltA* and *tlc* and 40°C for *rrs*. After overnight hybridization, RNase T1 (1,000 U) was added to each sample. After digestion with RNase, the samples were precipitated with ethanol, electrophoresed in 6% polyacrylamide-urea gels, and analyzed by autoradiography according to standard methods (4). The size of the protected band was estimated with DNA size markers.

As shown in Fig. 1, RNase protection assays could clearly detect rickettsia-specific signals in the rickettsia-infected samples but not in the mock-infected samples. Since the probes for *tlc* and *gltA* contain some open reading frame, some upstream region, and some vector sequence and the transcription start sites are not known for *tlc* and *gltA*, the expected size of the protected fragments was predicted from the known length of the open reading frame and the position of -35 and -10 *E. coli*-like promoter. However, since the *rrs* probe contains only coding and vector sequences, the protected fragment should be the same length as the length of the coding sequence. Only one major band with the expected size of about 200 nucleotides (200 nt) was detected when *tlc* probe was used, and one major band with the expected size of 525 nt was detected when *rrs* probe was used. However, when the *gltA* probe was used, there were

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FIG. 1. Analysis of rickettsial RNAs by an RNase protection assay. The autoradiograms for the *tlc* and *gltA* samples were exposed overnight, and that for the *rrs* sample was exposed for 10 min. The RNA samples isolated from mock-infected cells (lanes M), the RNA samples isolated from rickettsia-infected cells (lanes R), and the undigested probes (lanes P) are indicated. Bands that represent *tlc* mRNA, *gltA* mRNA I, *gltA* mRNA II, and 16S rRNA are indicated as *tlc*, *cs1*, *cs2*, and 16s, respectively. The lengths of protected probe fragments for *tlc*, *cs1*, *cs2*, and 16s are about 200, 120, 265, and 525 nt, respectively.

two major bands detected, one with the expected size of 120 nt and another 265 nt in length. Since the *gltA* probe has a 180-bp upstream fragment in addition to the 104-bp open reading frame, these two bands probably represent two mRNA species (mRNA I, with a size of 120 nt, and mRNA II, with a size of 265 nt) which were transcribed from two different start sites of transcription. These data strongly suggested that the rickettsial *gltA* gene is controlled by two promoters *in vivo*. Additional studies to determine whether *gltA* is an monocistronic gene controlled by two promoters or a part of an operon controlled by both the operon promoter and its own promoter will be necessary.

Determination of the chemical half-life of RNA. To measure the chemical half-life of rickettsial RNA, RNA samples were isolated from the rickettsia-infected cells at various times after addition of rifampin to inhibit transcriptional initiation. Since rickettsiae grow within the cytoplasm of their host cells, rifampin added to the cell culture medium needs to pass through two cell membranes to get into the cytoplasm of rickettsiae and, in addition, will be diluted by the cytoplasm of host cells. Therefore, a high concentration of rifampin (0.4 mg/ml) was used, and the first RNA sample was not isolated until 5 min after addition of rifampin. Equal amounts (100 μ g) of total RNA were used for each assay. For comparison, RNA encoded by rickettsial genes was also isolated from *E. coli* MOB386 (8) and MOB264 (13), which contain pMW386 (pUC19 with a rickettsial *tlc* insert) and pMW264 (pBR322 with a rickettsial *gltA* insert), respectively. Quantitative analysis of RNA was performed by densitometric analysis of autoradiograms, and the rates of decay were calculated by least-squares determination of the slope of a logarithmic plot of band density against time. Errors were estimated from the standard deviation of the slope for *E. coli* samples, but the half-lives of rickettsial RNAs were presented as averages $\pm$ standard deviations calculated from three independent experiments, since the

FIG. 2. Decay of rickettsial gene-encoded mRNAs in *R. prowazekii* and *E. coli*. The upper panels show RNase protection assays of RNA stability. The lower panels show logarithmic plots of RNA concentration as a function of time. The labels are the same as those of Fig. 1.

number and physiological state of the rickettsiae could vary from dish to dish of rickettsia-infected cells.

As shown in Fig. 2, the half-lives of *tlc* mRNA, *gltA* mRNA I, and *gltA* mRNA II were 8.4 ± 0.6 , 12.3 ± 1.3 , and 20.5 ± 1.8 min, respectively. The data also showed that rickettsial 16S rRNA was stable *in vivo*. It is remarkable that these half-lives, determined for three specific mRNAs in rickettsiae growing logarithmically within the host cell's cytoplasm, are similar to the half-life determined previously for the degradation of total labeled RNA synthesized *in vitro* by nongrowing, purified *R. prowazekii* (9). In the earlier study this rapid (relative to that in the 4-h purification) degradation of RNA was intended to explain the observation that proteins could not be synthesized *in vitro* by such purified *R. prowazekii* unless the *de novo* synthesis of RNA also occurred, but the half-life obtained may have estimated, perhaps fortuitously, the true half-life of a typical mRNA. The half-lives of *tlc* mRNA and *gltA* mRNA I measured in recombinant *E. coli* strains were 2.9 ± 0.1 and 1.4 ± 0.1 min, respectively. The lack of *gltA* mRNA II in *E. coli* MOB264 was due to the fact that the insert of rickettsial *gltA* in pMW264 contains only an 80-bp upstream region. The difference in the half-lives in these two organisms probably reflects the difference in mRNA degradation systems or the different secondary structures formed in the plasmid- and genome-encoded mRNAs in the two organisms.

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