6-15-2009

A Unique Group I Intron in Coxiella Burnetii is a Natural Splice Mutant

Rahul Raghavan

Linda D. Hicks

Michael F. Minnick
University of Montana - Missoula, mike.minnick@mso.umt.edu

JOURNAL OF BACTERIOLOGY, June 2009, p. 4044–4046 Vol. 191, No. 12
0021-9193/09/$08.00+0 doi:10.1128/JB.00359-09

A Unique Group I Intron in *Coxiella burnetii* Is a Natural Splice Mutant[▽]

Rahul Raghavan, Linda D. Hicks, and Michael F. Minnick*

Division of Biological Sciences, The University of Montana, Missoula, Montana 59812

Received 13 March 2009/Accepted 10 April 2009

Cbu.L1917, a group I intron present in the 23S rRNA gene of *Coxiella burnetii*, possesses a unique 3′-terminal adenine in place of a conserved guanine. Here, we show that, unlike all other group I introns, Cbu.L1917 utilizes a different cofactor for each splicing step and has a decreased self-splicing rate in vitro.

Coxiella burnetii is an obligate intracellular gammaproteobacterium that causes Q fever in humans (3). In addition, *C. burnetii* infects domestic ruminants and has been isolated from a wide variety of wild vertebrates and arthropods (9). Inside the host cell, *C. burnetii* replicates in a parasitophorous vacuole that has features of a mature phagolysosome (20). Genome sequences of four *C. burnetii* isolates have been published recently and show diversity in their pseudogene content and other pathoadaptive features (1, 18). Previously, we described two highly conserved self-splicing group I introns in the 23S rRNA gene of ten *C. burnetii* isolates belonging to eight genotypes (14, 15). Here, we present the unique self-splicing mechanism of one of the introns (Cbu.L1917), which is altered from and slower than the canonical group I intron-splicing process described to date.

Group I introns are ribozymes that catalyze a two-step transesterification reaction that results in a free intron and spliced exons (21). All group I introns share conserved features such as a secondary structure that consists of about 10 paired (P) elements and their terminal nucleotide, which until the discovery of Cbu.L1917 was always a guanine (15). The conserved terminal guanine (ΩG) plays an important role in group I intron self-splicing. In the first splicing step, the 3′-OH group of an exogenous guanosine bound to the G-binding site (GBS) in P7 carries out a nucleophilic attack on the 5′ splice site to release the 5′ end of the intron. The guanosine is now covalently attached to the free 5′ end of the intron and is removed from the GBS, allowing ΩG to occupy the site and mark the 3′ splice site. The second splicing step is chemically equivalent to the reverse of the first step, where the free 3′-OH group of the 5′ exon attacks the 3′ splice site, releasing the intron and leaving the exons spliced together (19). When we first characterized Cbu.L1917 as a self-splicing intron by functional analyses, we were unclear about its classification as a group I intron due to its unique 3′-terminal adenine (ΩA). Since ΩA marks the site for Cbu.L1917's second splicing step, we wanted to determine the cofactor used in its first splicing step.

Genomic DNA was isolated from *C. burnetii* Nine Mile phase II (RSA 439; clone 4) using a High Pure PCR template preparation kit (Roche Diagnostics, Basel, Switzerland), and the region coding for Cbu.L1917 and its flanking exons were amplified using specific primers (L1917_flank, Table 1) by PCR, as previously described (14). The amplicons were purified (QIAquick nucleotide removal kit; Qiagen, Valencia, CA) and used as a template for in vitro transcription utilizing the T7 promoter sequence (underlined in L1917_flank, Table 1) and a MEGAscript high-yield transcription kit (Ambion, Austin, TX). The resulting RNA was electrophoresed in a 5% (wt/vol) acrylamide-8 M urea gel, and the precursor RNA (unspliced intron with flanking exons) was excised from the gel; eluted overnight at 37°C into a buffer containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate; and subsequently purified with an RNeasy minikit (Qiagen). In vitro intron splicing was performed for 30 min at 37°C using 1 μg of precursor RNA in a buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM $MgCl_2$, and 0.8 mM ribonucleoside triphosphate mix (0.2 mM each of rATP, rGTP, rCTP, and rUTP). Unincorporated nucleotides were removed using a NucAway kit (Ambion), and the spliced intron RNA (which has the nucleotide cofactor used in the first splicing step covalently attached to its 5′ end) was used as a template for cDNA synthesis with a primer that nestles within the intron sequence (L1917_internal, Table 1). Template RNA was removed by RNase treatment, and the cDNA was tailed using terminal deoxynucleotidyltransferase (5′ rapid amplification of cDNA ends system; Invitrogen, Carlsbad, CA) and 2 mM dATP. The tailed cDNA was purified (QIAquick nucleotide removal kit; Qiagen) and PCR amplified using a primer that hybridizes to the poly(A) tail and a primer complementary to a sequence within the intron (L1917_tail, Table 1). The amplicons were cloned into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen). Twenty random clones were sequenced with a BigDye Terminator cycle sequencing ready reaction kit and an automated DNA sequencer (ABI3130x1; ABI, Foster City, CA). Sequencing results showed that all 20 clones had guanine incorporated at the 5′ end of the intron RNA, revealing that the cofactor used by Cbu.L1917 in its first splicing step is GTP.

To confirm the above observations, we performed in vitro splicing in the presence of either GTP or ATP (Fig. 1). Unspliced precursor RNA (Cbu.L1917 with flanking exons) was synthesized and purified as described above. In vitro splicing was carried out with 2 μg of precursor RNA and the same buffer and reaction conditions as those described above, except that 0.2 mM of either rATP or rGTP was provided along with relevant controls (without essential $MgCl_2$ or nucleotide cofactors). After the splicing reactions (37°C, 30 min), equal

* Corresponding author. Mailing address: Division of Biological Sciences, The University of Montana, Missoula, MT 59812. Phone: (406) 243-5792. Fax: (406) 243-4184. E-mail: mike.minnick@mso.umt.edu.

▽ Published ahead of print on 17 April 2009.

TABLE 1. PCR primers used in the study

Designation	Sequence	
	5′ primer	3′ primer
L1917_flank[a]	TAATACGACTCACTATAGGGAGGTGGCTGCGACTGTTTAC	GGAATTTCGCTACCTTAGGACCG
L1917_internal	TATTGACGTTATGTTAATCATG	
L1917_tail	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT	CGCTATAGAGATCGGACTC
L1917_mut[b]		GGAATTTCGCTACCTTAGGACCGTT**C**ATTG

[a] T7 promoter sequence is underlined.
[b] Mutation site is in boldface.

volumes of gel loading buffer (Ambion) were added to the RNA and heated at 95°C for 5 min to disrupt secondary structures. The samples were then electrophoresed in a 5% (wt/vol) acrylamide-8 M urea gel (150 V, 60 min). The gel was stained with ethidium bromide and visualized using a Gel Doc system (Bio-Rad, Hercules, CA). As shown in Fig. 1, the intron splices only in the presence of GTP but not ATP, confirming that GTP is the cofactor in the first splicing step of Cbu.L1917.

Results from the above experiments demonstrate that, unlike all other group I introns studied to date, Cbu.L1917 uses a different cofactor for each splicing step (guanine for the first step and its terminal adenine for the second step). Earlier studies showed that if the 3′-terminal guanine was mutated to adenine, the efficiency of group I intron splicing was markedly reduced (2, 10, 13). To investigate how the natural guanine-to-adenine mutation observed in Cbu.L1917 affects its splicing efficiency, we compared the rates of intron splicing between wild-type Cbu.L1917$_{(\Omega A)}$ and Cbu.L1917$_{(\Omega A \rightarrow G)}$.

During the first step of group I intron splicing, the nucleotide cofactor (guanosine, GMP, or GTP) is covalently linked to the 5′ end of the intron (19). By using ^{35}S-GTP in the splicing reaction and quantifying its incorporation into spliced introns at various time points, the rate of intron splicing can be calculated. To perform a comparative analysis, we first mutated the terminal adenine to guanine using a PCR-based strategy. A primer that replaces the terminal adenine with guanine (L1917_mut, Table 1) and a primer with T7 promoter sequence (L1917_flank, 5′ primer, Table 1) were used in the PCR. The amplicon with the T7 promoter region and the ΩA-to-G mutation was used as the template for in vitro transcription using a MEGAscript kit (Ambion) to produce the unspliced precursor RNA. The RNA was purified using an acrylamide gel as described above, and 500 ng was used per reaction. Splicing reactions were carried out as described above but with 25 μCi of γ-^{35}S-GTP (Perkin-Elmer, Waltham, MA). The reactions were started by the addition of 50 mM $MgCl_2$, and the samples were incubated at 37°C for either 2 min or 10 min. The reactions were stopped by chilling the mixtures on ice and by adding 10 mM EDTA. Unincorporated nucleotides were removed using an RNeasy minikit (Qiagen). The RNA was mixed with scintillation cocktail (Aquasol-2; Perkin-Elmer) and counted using a liquid scintillation system (Beckman Coulter, Fullerton, CA). The amounts of ^{35}S-GTP incorporated into spliced introns are presented as counts per minute (Fig. 2). The rate of splicing for each intron was determined from the slope (m) of the plots (8). As shown in Fig. 2, the slope of wild-type Cbu.L1917$_{(\Omega A)}$ ($m = 10.90 \pm 1.32$) is significantly lower ($P = 0.015$, paired t test) than that of Cbu.L1917$_{(\Omega A \rightarrow G)}$ ($m = 15.09 \pm 2.20$). This observation is consistent with earlier reports showing that when ΩG of a group I intron was mutated to adenine, it resulted in a significant loss in splicing efficiency (2, 10, 13).

The GBS binds an exogenous guanine during the first splicing step and then the terminal guanine during the second step

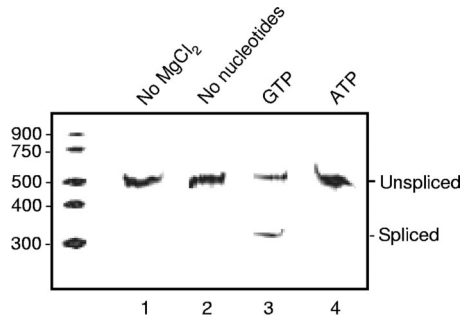

FIG. 1. Cbu.L1917 splices in the presence of GTP but not ATP. Two micrograms of precursor unspliced intron RNA was spliced in the presence of either 0.2 mM rGTP (lane 3) or 0.2 mM rATP (lane 4). Splicing reaction mixtures without $MgCl_2$ (lane 1) or without nucleotides (lane 2) were included as controls. RNA size standards are shown to the left in bases.

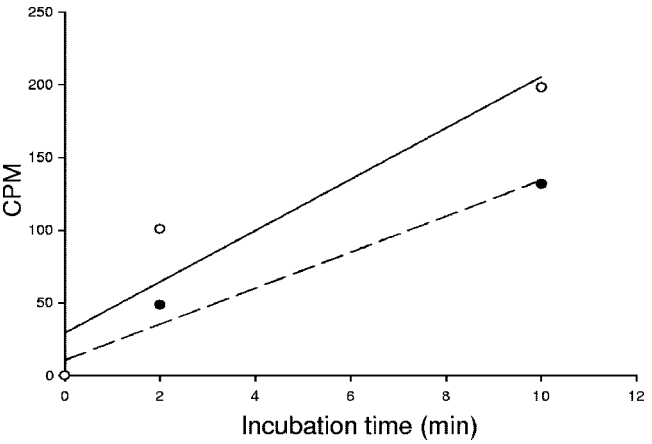

FIG. 2. Comparison of intron splicing rates. Cbu.L1917 with terminal adenine (ΩA) or terminal guanine (ΩG) was spliced in the presence of γ-^{35}S-GTP. CPM recorded after 2- and 10-min incubations were plotted, and the slopes were determined. One graph representative of three independent experiments is shown. The slope for Cbu.L1917$_{(\Omega A)}$ (filled circles, dashed line) is significantly lower than that of Cbu.L1917$_{(\Omega A \rightarrow G)}$ (open circles, solid line) ($P = 0.015$, paired t test).

to facilitate group I intron self-splicing. The GBS has evolved to accommodate guanine efficiently to the exclusion of other nucleotides (10, 13). The guanine cofactor interacts with a G · C base pair in the GBS to form a stable base triple (4, 19). However, in the case of Cbu.L1917, the GBS is forced to accommodate an adenine in place of guanine during the second splicing step. The resulting less-compatible binding of ΩA to GBS is likely the reason for the decreased splice rate exhibited by Cbu.L1917. In fact, earlier studies have shown that when the G · C pair in GBS is mutated to A · U such that it can efficiently accommodate ΩA during intron splicing, the rate of intron splicing is restored (2, 10). It is likely that some *C. burnetii* proteins help Cbu.L1917 splice more efficiently in vivo, thereby negating the deleterious effects of the ΩG-to-A mutation (7). Another possibility is that an intron with decreased splicing rate was fixed in *C. burnetii* due to genetic drift.

The genomes of host-associated bacteria tend to be A+T rich. Even though the mechanisms underlying this GC-to-AT shift are not well understood, it is thought to be due to a variety of factors like mutational bias, loss of DNA repair genes, or metabolic cost (11, 16). The *Coxiella* chromosome and Cbu.L1917 both have low G+C ratios (42.5% and 36.8%, respectively). The terminal guanine might have mutated to an adenine as part of the GC-to-AT conversion that occurred during the evolution of *C. burnetii* from a free-living bacterium to an obligate intracellular pathogen (14, 18).

Obligate intracellular bacteria are susceptible to genetic drift due to constant availability of nutrients, low effective population size, and bottlenecks during transmission, resulting in stochastic loss of some beneficial genes and accumulation of some slightly deleterious mutations (Muller's ratchet) (11, 12). This process has resulted in the accumulation of slightly deleterious mutations in such vital genes as *groEL* and 16S rRNA in the obligate endosymbiotic bacterium *Buchnera aphidicola* (5, 6) and gene decay and pseudogene formation in the facultative intracellular pathogen *Francisella tularensis* (17). Similarly, it is possible that the ΩG-to-ΩA mutation in Cbu.L1917 that occurred during the evolution of *C. burnetii* was fixed in the population in spite of the slightly deleterious loss in splicing rate.

We thank Patty McIntire (UM Murdock Sequencing Facility) for sequence analyses.

This work was supported by the NIH Rocky Mountain Regional Center of Excellence for Biodefense and Emerging Infectious Disease grant U54 AI065357-040023 to M.F.M.

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