Analysis of Convergent Gene Transcripts in the Obligate Intracellular Bacterium *Rickettsia prowazekii*

Andrew Woodard, David O. Wood*

Department of Microbiology and Immunology, University of South Alabama College of Medicine, Mobile, Alabama, United States of America

**Abstract**

Termination of transcription is an important component of bacterial gene expression. However, little is known concerning this process in the obligate intracellular pathogen and model for reductive evolution, *Rickettsia prowazekii*. To assess transcriptional termination in this bacterium, transcripts of convergent gene pairs, some containing predicted intrinsic terminators, were analyzed. These analyses revealed that, rather than terminating at a specific site within the intervening region between the convergent genes, most of the transcripts demonstrated either a lack of termination within this region, which generated antisense RNA, or a putative non-site-specific termination that occurred throughout the intervening sequence. Transcripts terminating at predicted intrinsic terminators, as well as at a putative Rho-dependant terminator, were also examined and found to vary based on the rickettsial host environment. These results suggest that transcriptional termination, or lack thereof, plays a role in rickettsial gene regulation.

**Introduction**

The obligate intracellular bacterium, *Rickettsia prowazekii*, is the causative agent of epidemic typhus, a louse-borne disease usually associated with non-hygienic conditions arising in crowded human populations during war, famine, and as a result of extreme poverty. Due to its potential as an instrument of bioterrorism, *R. prowazekii* is classified as a category B Select Agent. The unique life style of this pathogen involves growth in the widely different environments of an arthropod louse vector and a human host. In addition, a zoonotic reservoir, the flying squirrel, has been identified in the United States [1,2]. *R. prowazekii* pathogenicity results from the intracytoplasmic growth of the rickettsiae, leading to cell lysis and the subsequent infection of additional host cells. In *R. prowazekii*, this lifestyle is dependent on a relatively small genome that contains a high proportion of pseudogenes and non-coding sequences [3].

Rickettsial gene expression studies have focused primarily on transcription initiation. *R. prowazekii* has been shown to utilize regulated promoters and to organize genes into operons [4,5,6,7,8,9], and rickettsial RNA polymerase was shown to exhibit properties that distinguish it from the *Escherichia coli* polymerase, such as the requirement for a supercoiled template for promoter binding, [10,11]. However, an area of gene regulation that has not been evaluated in rickettsiae is transcriptional termination. In fact, very few functional terminators have been experimentally confirmed outside of model organisms such as *E. coli* and *Bacillus subtilis* [12]. The conservation of genes associated with the termination process (*nusA*, *nusB*, *nusG*, *nusH*, and *rho*) suggests that transcription termination has a role in rickettsial gene regulation [3]. Preliminary studies in our laboratory indicated that rickettsial termination of the *groA* gene transcript exhibited unusual properties. Transcripts of this gene were shown to extend through an intervening region and into the coding sequence of the converging *put* gene [13].

To gain insight into this aspect of rickettsial gene regulation, we elected to focus on areas of the rickettsial genome where gene organization suggests the most probable location for termination events; namely, the intervening regions between convergent genes [12]. Analysis of these regions revealed functional intrinsic terminators and a putative Rho-dependent terminator. Also identified were convergent genes that exhibit a lack of termination or a non-site-specific termination and the generation of rickettsial antisense RNAs resulting from the extension of transcripts into convergent gene sequences. Most importantly, we discovered that *R. prowazekii* may regulate termination efficiency of specific terminators depending on the rickettsial host.

**Materials and Methods**

**Bacterial strains, host cell lines, and culture conditions**

*R. prowazekii* strain Madrid E was cultured and purified from the yolk sacs of embryonated hen eggs, as described previously [14]. Purified rickettsiae were suspended in a sucrose-phosphate-glutamate-magnesium buffer solution (0.218 M sucrose, 3.76 mM KH₂PO₄, 7.1 mM K₂HPO₄, 4.9 mM potassium glutamate, and 10 mM MgCl₂), and stored frozen at −80°C. Murine fibroblast L929 cells (American Type Culture Collection, Manassas, VA, ATCC Number CCL-1) were cultured at 34°C with 5% CO₂ in modified Eagle’s medium (Mediatech, Inc., Herndon, VA), supplemented with 10% heat-inactivated newborn calf serum (HyClone Laboratories, Logan, UT), and 2 mM glutamine...
free DNase I (Qiagen), eluted in 100 µl of RNase-free water, and treated with 1 U of RQ1 DNase (Promega Corp., Madison, WI) at 37 °C, and immediately treated with 1 µl of the radiolabeled probe (5.0 × 10^3 cpm/µl) and 10 µl of Hybridization Buffer III. Hybridization samples were brought to 94 °C for 4 minutes and then incubated in a dry heat block overnight at 42 °C. RNase digestion buffer was added to the sample, and the sample incubated for 30 minutes at 37 °C. Inactivation Solution III was then added and samples were placed at −20 °C for a minimum of 15 minutes. The samples were centrifuged at 16,000 × g for 15 minutes, and the resulting pellet suspended in 10 µl of Gel Loading Buffer II. Experimental, and control samples as well as 5.0 × 10^3 cpm of the labeled RNA Century-Plus Marker (Applied Biosystems) were incubated at 94 °C for 3 minutes, analyzed by electrophoresis using a 5% acrylamide/8 M urea gel, and detected using a phosphorimager. Controls were included for each RPA analysis to confirm the validity of the assay (data not shown). Controls for each RPA analysis included: a positive control for the hybridization consisting of the labeled probe and 300 pg of its unlabeled complement. A negative control of labeled probe only, at the amount used in the experimental assay, was included to ensure that all unprotected probe would be digested. DNA contamination would be reflected by full length protection of every probe under all conditions. Therefore, the absence of fully protected probe in all assays for each RNA preparation confirmed the absence of DNA contamination. All RPA results shown are representative of a minimum of three independent RNA preparations.

**Results**

Transcriptional termination and convergent gene pairs

To analyze rickettsial transcriptional termination, we focused on the most likely location for termination, the intervening sequences between convergent genes [12]. Of the 104 convergent gene pairs annotated in the *R. prowazekii* genome [3], we selected 12 genes (Table 1), representing 6 well-separated gene pairs that, with two exceptions, met the following selection criteria. First, each gene is transcribed at detectable levels. This was evaluated using microarray data [4] or by direct measurements using RPA. In addition, in the current study intragenic positive control probes were included to confirm gene transcription (Table 1). Secondly, the gene products were detected by proteomic analysis, with the exception of RP826 and RP777. The latter gene was listed as a pseudogene in Madrid E and therefore not annotated or screened in proteomic analyses [15,16] (unpublished results). Two of these gene pairs (RP703-RP704 and RP826-RP827) were also included due to the prediction by TransTermHP of a strong, bidirectional, intrinsic terminator within the intervening regions [12].

Transcript detection was accomplished using ribonuclease protection assays (RPA). RPA analysis uses single-strand, labeled RNA probes that are antisense to target mRNAs. If mRNA specific to the probe is present, it will hybridize to the probe and protect it from digestion with nucleases that specifically digest single strands. The protected probe can then be analyzed by gel electrophoresis. The extent of protection allows for the estimation of termination sites and reveals a complete picture of protected transcripts within the selected region. Conversely, if the transcript does not stop and reads through the intervening region, the probe will appear as fully protected. If there are non-site specific termination events through the intervening region, multiple bands
of differing sizes will be visualized. In Table 2, the sizes of the intergenic regions, the probe size, and the amount the probe overlaps the coding regions of the genes are presented for the probes used in this study.

We assayed rickettsial RNA extracted from rickettsiae grown in hen egg yolk sacs and in L929 mouse fibroblast cells. Previous studies had indicated that mRNA assayed at 34°C from rickettsiae grown in L929 cells had a half-life of approximately 15 minutes [6], a property that would preclude the isolation of mRNA from this source. However, we found that rickettsial mRNA is present in the egg yolk sac rickettsial RNA preparation and can be detected at levels comparable to mRNA isolated from rickettsiae grown in L929 cells. The recovery of mRNA from yolk sac rickettsiae is most likely due to performing all manipulations at 4°C during rickettsial purification. This permitted us to assay rickettsial RNA from different rickettsial host backgrounds.

RPA analysis of transcripts specific for three of the gene pairs is presented in Figure 1. The results reveal that the lack of specific termination sites is not an uncommon occurrence in *R. prowazekii*. For example, the RP145-RP146 convergent gene pair is transcribed as evidenced by the fully protected intragenic probes that are targeted to sites within the two genes (Fig. 1B, Lanes 1 and 3). However, RP145 transcripts do not exhibit a specific termination site within the intervening region (Fig. 1B, Lane 2). Since there is a 106 base pair overlap of the probe with RP145 (Table 2), this indicates the absence of a transcriptional stop immediately following the RP145 stop codon (a 106 base protected fragment would have been visible by gel electrophoresis).

Rather, the presence of a diffuse banding pattern suggests non-site-specific termination throughout the intervening region. In contrast to the RP145 transcript, the probe targeted to the intervening region downstream of RP145 was fully protected (Fig. 1B, Lane 4) demonstrating that the RP146 transcript extends into the RP145 coding region generating antisense RNA to RP145 transcripts (the probe extends 116 bp into the coding region). The RP195-RP196 gene pair exhibited a similar termination profile: RP195 transcripts exhibit no defined termination site (Fig. 1C, Lane 2) and the probe specific for RP496 was fully protected (Fig. 1C, Lane 4). Once again this demonstrates that RP496 transcripts are extending into the RP195 coding region generating antisense RNA (the probe extends 281 bp into the coding region). The protection profile observed with the RP777-RP778 gene pair demonstrated that both gene transcripts exhibited a diffuse pattern indicative of non-site-specific termination throughout the intervening region (Fig. 1D, Lanes 2 and 4). Thus, these three convergent gene pairs of *R. prowazekii* do not terminate transcripts at specific sites within the intervening region, as might be expected based on gene orientation. In fact, read-through into the opposing gene, generating antisense RNA, is observed for two gene transcripts (Fig. 1, B, Lane 4 and C, Lane 4). These results were obtained whether the RNA was extracted from rickettsiae propagated in hen egg yolk sacs or in L929 tissue culture cells.

Transcriptional termination at predicted terminators

To further elucidate rickettsial transcription termination, we selected two rickettsial gene pairs (RP703-RP704 and RP826-RP827) predicted by bioinformatic analysis to exhibit strong intrinsic terminators. Probes were designed to span the predicted intrinsic terminator sites of these gene pairs (Table 1). Analysis of these intrinsic terminator regions revealed a variable pattern of termination based on the source of the RNA. The RP703-RP704 gene pair exhibits a long intervening region of 1910 bp and a predicted strong, bi-directional terminator.

### Table 1. Targeted genes and intragenic probes.

| Gene   | Annotation* | Gene Size (bp) | Probe Size | Bases from Start |
|--------|-------------|----------------|------------|------------------|
| RP067  | parC        | 2217           | 619        | 883              |
| RP068  | Unk         | 1323           | 627        | 628              |
| RP145  | aspS        | 1818           | 375        | 1052             |
| RP146  | Unk         | 1839           | 441        | 816              |
| RP495  | gltA        | 831            | 346        | 450              |
| RP496  | rbn         | 858            | 367        | 364              |
| RP703  | ccmF        | 2013           | -          | -                |
| RP704  | scoS        | 4932           | -          | -                |
| RP777  | metK        | 1143           | 316        | 105              |
| RP778  | dnaE        | 3549           | 399        | 2592             |
| RP826  | Unk         | 327            | -          | -                |
| RP827  | Unk         | 747            | -          | -                |

*Annotations from [3] and ERGO™ (Integrated Genomics, Chicago, IL).

### Table 2. Gene pairs and intergenic probes.

| Gene Pair         | Annotation* | Intergenic Region (bp) | Probe Size | Overlap Gene A | Overlap Gene B |
|-------------------|-------------|-------------------------|------------|----------------|----------------|
| RP067-RP068       | parC – Unk  | 341                     | 593        | 122            | 130            |
| RP145-RP146       | aspS – Unk  | 569                     | 791        | 106            | 116            |
| RP495-RP496       | gltA – rbn | 137                     | 512        | 94             | 281            |
| RP703-RP704 (1)   | ccmF – scoS | 1910                    | 577        | 370            | -              |
| RP703-RP704 (2)   | ccmF – scoS | 1910                    | 833        | -              | -              |
| RP703-RP704 (3)   | ccmF – scoS | 1910                    | 563        | -              | -              |
| RP703-RP704 (4)   | ccmF – scoS | 1910                    | 665        | -              | 293            |
| RP777-RP778       | metK – dnaE | 366                     | 682        | 160            | 156            |
| RP826-RP827       | Unk – Unk   | 253                     | 466        | 127            | 86             |

*Annotations from [3] and ERGO™ (Integrated genomics, Chicago, IL).

Unk = Unknown.

doi:10.1371/journal.pone.0016537.t001

doi:10.1371/journal.pone.0016537.t002
(5’-AAAAAAA GCCCATTTT TTC AAAGTGGGC TTTTTTT-3’) located 32 bases from the annotated end of RP704. Interestingly, the RP703 transcripts did not reach this termination site but exhibited a diffuse pattern of termination reminiscent of the transcripts presented in Figure 1 (data not shown). Thus, we were unable to evaluate the efficiency of RP703 transcript termination at the predicted site. However, functional evaluation of this site was possible for the RP704 transcript. The probe for this region was designed to extend 293 bases into RP704 and yield a protected product of 329 bases if the transcript terminated at the predicted site. A representative RPA analysis is presented in Figure 2B. When using RNA isolated from rickettsiae propagated in hen egg yolk sacs, a probe spanning the predicted intrinsic terminator was fully protected with little evidence of a stop at the intrinsic terminator (Fig. 2B, Lane 1). In contrast, the majority of the transcripts terminated at the predicted site when RNA isolated from rickettsiae grown in L929 cells was analyzed (Fig. 2B, Lane 2).

The large intervening region between RP703 and RP704 provided an excellent opportunity to examine the progression of transcription termination of transcripts that read through the predicted terminator as in rickettsiae harvested from hen egg yolk sacs. We used overlapping probes (minimum 42 base overlap) spanning the entire intervening region of 1910 bp and extending 370 bases into the RP703 gene. Probes 1 and 2 were essentially fully protected (Fig. 2D, Lanes 1 and 2) indicating complete read-through. Less full-length probe was detected for probe 3 (Fig. 2D, Lane 3), and only a negligible amount of full-length probe 4 (Fig. 2D, Lane 4) was detected. This is another example of the absence of a distinct termination site and an incremental, non-site-specific termination.

Differential termination at an intrinsic terminator, based on RNA source, was not unique to the RP703-704 gene pair. RP826-RP827 was an additional convergent gene pair with a predicted strong, bidirectional, intrinsic terminator (5’-AAAAA GGCTTTTA TTAA TAAAGACC CTTTT) that exhibited a similar termination profile (Fig. 3). Interestingly RPA analysis of RP826 transcripts revealed no difference between RNA obtained from hen egg yolk sac grown rickettsiae and L929 cell grown rickettsiae (Fig. 3B, Lanes 1). The majority of transcripts correspond to the predicted size for termination at the intrinsic terminator (347 bases). In contrast, there was a dramatic difference in the ratio of terminated versus read-through transcripts for RP827 depending on the rickettsial host (Fig. 3B, Lanes 2). Once again the majority of transcripts detected in RNA isolated from hen egg yolk sac grown rickettsiae completely protected the full-length probe indicating termination at the predicted terminator site. The opposite result was observed for RNA isolated from rickettsiae grown in L929 cells where the majority of the RP827 transcripts protected a 187 base portion of the probe indicating termination at the predicted terminator site. Thus, R. prowazekii exhibits intrinsic...
terminators that appear to function efficiently in rickettsiae grown in L929 tissue culture cells but are predominantly bypassed in rickettsiae growing in hen egg yolk sacs.

Identification of a putative Rho-dependent termination site

The intervening region between RP067 and RP068 does not contain a predicted intrinsic terminator. However, we detected a specific termination site by RPA for the RP068 message when using RNA isolated from rickettsiae grown in L929 cells (Fig. 4B, Lane 4). Similar to the results of gene pairs described in Figure 3 above, RP067 transcripts display a banding pattern indicating non-site-specific termination extending through the intervening region (Fig. 4B, Lane 2). Once again, when we analyzed transcripts from these genes using RNA isolated from rickettsiae grown in hen egg yolk sacs, a very different pattern was observed for the RP068 transcripts (Fig. 4C, Lane 4). No evidence of termination was observed. The entire probe was protected indicating read-through of the transcripts into the RP067 coding region. The lack of a predicted intrinsic terminator and the differentially regulated partial termination at this site suggests that the detected terminator is a Rho-dependent terminator. While we suspect that this is a Rho-dependent terminator, sequences associated with such terminators are not as easily identifiable as those of intrinsic terminators [17], and we were unable to confirm this identification.

A summary of the transcriptional results for all the genes examined in this study is presented in Table 3.

Discussion

Until now termination of transcription in *R. prowazekii* has received little attention. Analysis of the *R. prowazekii* genome reveals a highly reductive genome containing many pseudogenes and a high proportion of non-coding regions. However, the retention of genes involved in the termination process (*nusA*, *nusB*, *nusG*, *mfd*, and *rho*) suggests that transcription termination has a role in rickettsial gene regulation. By targeting multiple areas hypothesized to be regions of termination, this study provides the first comprehensive analysis of transcription termination in this pathogen.

Examination of the *R. prowazekii* genome identified the presence of stem loop structures preceding oligo (T) sequences typical of bacterial termination sites. As demonstrated by our results with two predicted terminators found in the intervening regions of the RP703-RP704 and RP826-RP827 gene pairs, such classical terminators do function in *R. prowazekii*. However, one character-
istic of rickettsial transcriptional termination evident from these studies is the absence of site-specific termination within many intervening regions. Of the 12 gene transcripts analyzed, all but one (RP826) exhibited either a putative non-site-specific termination or complete read-through of the intervening region that extended into the opposing gene coding sequence. This suggests that specific termination may not be prevalent in this relatively slow-growing, obligate, intracellular bacterium. The presence of protein products from the converging genes demonstrates that the absence of specific termination and the subsequent extension of transcript into the convergent gene do not impede nascent mRNA synthesis to a point affecting the survival of the rickettsiae. The exceptions may be those intrinsic terminators whose efficiencies appear to be regulated.

The efficiency of an intrinsic terminator in *E. coli* can be modulated as various factors (e.g. NusA) interact and influence the transcription complex [17]. We have identified similar alterations of terminator efficiency in *R. prowazekii*. RNAs isolated from rickettsiae grown in different host cell environments exhibited regulated transcription termination. Interestingly, rickettsial growth in the hen egg yolk sac generated RNAs that, in all but one case (RP826), failed to terminate at an intrinsic termination site, possibly reflecting a more suitable rickettsial growth environment. Although increased transcription might be assumed to require more specific termination to prevent a negative impact on convergent genes, the need to prevent wasteful transcription may assume priority if rickettsiae are not in an optimum

---

**Figure 3.** RPA analysis identifying transcripts of the RP826-RP827 intervening region. (A) Schematic indicating the relative position of the RPA probes that span the intergenic region. Probe 1 is designed to hybridize to RP826 transcripts while probe 2 hybridizes to RP827 transcripts. (B) RPA analysis of rickettsial RNA isolated from rickettsiae grown in egg yolk sacs or in L929 cells. Lane numbers correspond to probe numbers in the schematic. M, sized markers with sizes (in bases) indicated. The marker lane on the right was digitally moved horizontally using Microsoft Office PowerPoint to maintain consistent lane organization. Arrows identify the band resulting from termination of RP837 transcripts at the predicted terminator. doi:10.1371/journal.pone.0016537.g003

**Figure 4.** RPA analysis characterizing transcripts of the RP067-RP068 gene pair region. (A) Schematic indicating the relative positions of the RPA probes targeted to the convergent gene pair. The probes include two internal probes to confirm gene transcription and two intervening probes of opposite orientation to detect intergenic mRNA. Probes 1 and 2 hybridize to RP067 transcripts while probes 3 and 4 hybridize to RP068 transcripts. (B) RPA analysis of RNA isolated from rickettsiae harvested from L929 cells. Lanes were digitally moved horizontally using Microsoft Office PowerPoint to maintain consistent lane organization. (C) RPA analysis of RNA isolated from rickettsiae harvested from egg yolk sacs. Lane numbers correspond to probe numbers in the schematic. Numbers at the bottom of each lane in B and C correspond to the size of the probe in bases. M, sized markers with sizes (in bases) indicated. The arrow indicates a band resulting from specific termination of RP068 transcripts. doi:10.1371/journal.pone.0016537.g004
Table 3. Summary of Gene Termination Events.

| Gene   | Annotation* | Egg  | L929 |
|--------|-------------|------|------|
| RP0067 | parC        | NSTa | NST  |
| RP068  | Unk         | R    | R/St |
| RP145  | aspS        | NST  | NST  |
| RP146  | Unk         | R    | R    |
| RP495  | glmA        | NST  | NST  |
| RP496  | rbo         | R    | R    |
| RP703  | ccmF        | NST  | NST  |
| RP704  | sacS        | R    | ST   |
| RP777  | metK        | NST  | NST  |
| RP778  | dnaE        | NST  | NST  |
| RP826  | Unk         | ST   | ST   |
| RP827  | Unk         | R    | ST   |

*Annotations from (1) and ERGOTM (Integrated Genomics, Chicago, IL).

**R = Read through into opposing gene, ST = Specific Termination, NST = Non-Specific Termination.

Unk = Unknown.

doi:10.1371/journal.pone.0016537.t003

The presence of rickettsial antisense RNAs generated by the lack of termination between convergent genes was an intriguing finding. The existence of antisense RNA was evident from the complete protection of probes that extended, in some cases, hundreds of bases into convergent genes. While the presence of antisense RNA in bacteria is uncommon, most such RNAs are associated with specific small regulatory RNAs or with intergenic promotion rather than termination [19,20]. Recently, examination of the Helicobacter pylori transcriptome revealed the widespread occurrence of antisense transcripts leading to the speculation that some of these may be due to imperfect termination [21]. These results are similar to an earlier study in E. coli that detected extensive antisense transcription throughout the genome [22]. Using a whole genome tiling microarray analysis, antisense transcripts have also been identified in the obligate intracellular bacterium, Anaplasma phagocytophila [23]. Interestingly, in contrast to the E. coli and H. pylori results and our identification of several examples of antisense transcripts generated by read-through into convergent genes, the A. phagocytophila whole genome study identified only one gene, p44, associated with this phenomenon [23]. Bacterial antisense RNAs have been shown to regulate gene expression [20]. The common occurrence of transcriptional read-through into convergent genes and the regulation of terminator function suggests a role in rickettsial intracellular survival and growth.

Supporting Information

Table S1 Primer sequences used to generate RPA probes. (DOC)

Acknowledgments

We thank Lonnie Driskell and Andria Hines for excellent technical assistance and manuscript review. We also thank Amanda Lackey and Aimee Tucker for manuscript review and helpful discussions and Jon Audia and Herbert Winkler for providing a portion of the hen egg yolk sac rickettsial preparations used in this study.

Author Contributions

Conceived and designed the experiments: DOW. Performed the experiments: AW. Analyzed the data: DOW AW. Contributed reagents/materials/analysis tools: DOW. Wrote the paper: DOW AW.
21. Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeib S, et al. (2010) The primary transcriptome of the major human pathogen Helicobacter pylori. Nature 464: 250–255.
22. Selinger DW, Cheung KJ, Mei R, Johanson EM, Richmond CS, et al. (2000) RNA expression analysis using a 30 base pair resolution Escherichia coli genome array. Nature Biotechnol 18: 1262–1268.
23. Nelson CM, Herron MJ, Felsheim RF, Schloeder BR, Grindle SM, et al. (2008) Whole genome transcription profiling of Anaplasma phagocytophilum in human and tick host cells by tiling array analysis. BMC Genomics 9: 364.