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Identification of functional promoters in the *msp2* expression loci of *Anaplasma marginale* and *Anaplasma phagocytophilum*

Anthony F. Barbet[a,*], Joseph T. Agnes[c], Annie L. Moreland[a], Anna M. Lundgren[a], A. Rick Alleman[b], Susan M. Noh[c], Kelly A. Brayton[c], Ulrike G. Munderloh[d], Guy H. Palmer[c]

[a]*Department of Pathobiology, College of Veterinary Medicine, University of Florida, Box 110880, Gainesville, FL 32611-0880, USA*
[b]*Department of Physiological Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611-0880, USA*
[c]*Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA*
[d]*Department of Entomology, University of Minnesota, St. Paul, MN 55108, USA*

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Abstract

Organisms in the family *Anaplasmataceae* are important tick-borne pathogens of livestock worldwide and cause recently emergent infections in humans. Despite their medical importance, very little is known about how these organisms regulate gene expression in the mammalian host, the tick vector, or during transition between the host and vector. However, it is clear that gene regulation, in addition to recombinatorial mechanisms, is essential for these small genome pathogens to adapt to distinctly different environments. In this study, we identify and establish the function of three promoter elements in the locus encoding major outer membrane protein expression sites in both *Anaplasma marginale* and *Anaplasma phagocytophilum*. Gene expression from this locus involves both classical and atypical polycistronic transcripts. The identified promoter elements have a structure similar to that defined in *Escherichia coli* and are functional in driving protein expression in a prokaryotic cell-free transcription and translation system and in recombinant *E. coli*. The two strongest promoters identified in vitro and with recombinant *E. coli* were also shown to be functional in *A. marginale* infected cells, as determined by quantification of downstream transcripts. The promoters in both *A. marginale* and *A. phagocytophilum* have similar structure and activity, supporting the conclusion that the two loci are syntenic with conservation of function. In addition, they share structural elements within the promoters that appear to be likely sites for regulation. These data enhance our understanding of how expression of these variable outer membrane proteins may be controlled in the key stages of tick-borne transmission and infection.

Keywords: Outer membrane proteins; Variable antigens; Gene regulation

1. Introduction

Anaplasma marginale is a tick-transmitted rickettsial pathogen that infects and causes acute disease in cattle. Cattle that survive the acute stage remain persistently infected for life and serve as reservoirs for ticks to acquire and subsequently transmit *A. marginale* (Palmer et al., 2000). Similarly, *Anaplasma phagocytophilum* is a closely related tick-transmitted pathogen that infects and causes disease in horses and sheep, and, more recently, has emerged as a prevalent human pathogen (Dumler and Bakken, 1998). Persistent infection in reservoir mammalian

Abbreviations: bp, base pairs of DNA; FAM, 6-carboxyfluorescein; *gfp*, GFP, green fluorescent protein; kDa, kilodaltons; MOPS, 3-(*N*-morpholino) propanesulfonic acid, sodium salt; MSP2, *msp2* major surface protein 2; *omp*, OMP, outer membrane protein; *opag*, operon-associated gene; orf, open reading frame; PCR, polymerase chain reaction; RT-PCR, amplification from RNA using reverse transcriptase and the polymerase chain reaction; SDS, sodium dodecyl sulfate; TAMRA, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine; *tr*, TR, transcription regulator; *tr*, *omp1*, *opag3*, *opag2*, *opag1*, *msp2*, genes located in the MSP2 expression locus of *A. marginale*; *tr1*, *omp-1x*, *p44ESup1*, *msp2/p44*, genes located in the MSP2/p44 expression locus of *A. phagocytophilum*.

* Corresponding author. Tel.: +1 352 392 4700x5819; fax: +1 352 392 9704.
E-mail address: barbeta@mail.vetmed.ufl.edu (A.F. Barbet).

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hosts is required for ongoing transmission, as ticks do not maintain the organisms between generations. The ability to persist in immunocompetent reservoir hosts is due, at least in part, to the expression of an immunodominant, but antigenically variable, outer membrane protein designated major surface protein 2 (MSP2) in *A. marginale* and MSP2/p44 in *A. phagocytophilum* (Caspersen et al., 2002; Eid et al., 1996; French et al., 1999; Wang et al., 2004). MSP2 variants are generated by a unidirectional gene conversion mechanism in which *msp2* pseudogenes are recombined into a single expression site (Barbet et al., 2003; Brayton et al., 2001; Lin et al., 2003). The pseudogenes encode unique hypervariable regions, which include the primary surface domains, and are flanked by truncated 5′ and 3′ regions that are identical to the single expression site *msp2*. Consequently, the expressed variants have conserved N- and C-terminal domains with a central hypervariable domain—which encodes immunodominant T and B cell epitopes (French et al., 1999; Brown et al., 2003). *A. marginale* differs from *A. phagocytophilum* in that *A. marginale* has < 10 *msp*2 pseudogenes while *A. phagocytophilum* has ~ 100; however *A. marginale* extensively uses a related gene conversion event in which segments of the pseudogenes are recombined in a mosaic fashion (Brayton et al., 2002). The resulting combinatorial diversity is capable of generating over 6000 unique *A. marginale* variants.

Both *A. marginale msp2* and *A. phagocytophilum msp2/p44* are transcribed as part of polycistronic mRNAs. *A. marginale msp2* is encoded at the end of a four gene locus that has the structure of a classic prokaryotic operon with adjacent genes separated by ≤35 bp (Barbet et al., 2000). All four genes, *opag3*, *opag 2*, *opag1*, and *msp2*, are predicted to encode surface proteins and all but OpAG1 have been demonstrated to be surface expressed (Löhr et al., 2002). Upstream of this four gene cluster are genes encoding a related outer membrane protein, designated OMP1, and a putative transcriptional regulator, designated TR. Interestingly, *omp1* transcripts can be linked to the *opag3–msp2* transcripts, although the distance between *omp1* and *opag3* (242 bp) is greater than is common for adjacent genes in prokaryotic operons (Löhr et al., 2004). The *A. phagocytophilum msp2/p44* expression locus is syntenic and is composed of a *tr1*, *omp1x*, *p44ESup1*, and *msp2/p44*-orthologues of, respectively, *A. marginale tr*, *omp1*, *opag3*, and *msp2* (Lin et al., 2003; Löhr et al., 2004). Transcriptional analysis has linked expression of these four *A. phagocytophilum* genes, although none of the genes in this locus (here referred to as the *msp2* expression locus based on its synteny) has spacing typical of an operon, with all intergenic spaces exceeding 146 bp.

Despite the importance of the genes in *msp2* loci in generating antigenic variation and our understanding of how variants are generated, there is little, if any, information regarding the promoters responsible for expression of these genes. The presence of a classic operon structure in *A. marginale* as well as atypical polycistronic transcripts in both *A. marginale* and *A. phagocytophilum* suggests that there may be multiple promoters that drive expression of *msp2*. As both *msp2* and *msp2/p44* are expressed in the mammalian reservoir hosts (>37 °C) and in ticks, both on and off the host (26–34 °C), there may be different promoters used in the different life stages. Thus, knowledge of the promoter structure is necessary to understand how genes are regulated in the transition between infection in the mammalian host versus the tick vector. In this paper, we present the identification of promoters in the *msp2* loci of *A. marginale* and *A. phagocytophilum*, comparison of their relative strength under different temperatures, and quantification of *A. marginale* mRNA expressed in both mammalian and tick cells.

2. Materials and methods

2.1. Organisms, infection, and cell cultures

The Florida and St. Maries strains of *A. marginale* were maintained as cryopreserved stabilates of infected bovine erythrocytes in dimethyl sulfoxide-buffered saline prior to being used to individually inoculate seronegative calves, as previously described in detail (Futse et al., 2003; Meeus et al., 2003). During acute rickettsemia (levels of $>10^9$ bacteria per ml of blood), infected erythrocytes were collected (Ambrosio et al., 1986) and genomic DNA was isolated using the Qiagen QIAamp DNA mini kit (Qiagen Inc., Valencia, California). The *A. marginale* St. Maries strain was established in the *Ixodes scapularis* embryonic cell line IDE8 using infected erythrocytes as the inoculum and was maintained in L15-B media as originally described by Munderloh et al. (1996). Uninfected IDE8 cells were handled identically with isolation of RNA as a negative control. For analysis of the effect of temperature on transcript levels, cells cultivated at 34 °C (with >80% infected cells) were re-suspended in new media and divided into three new flasks. The flasks were then incubated at 26 °C, 34 °C, or 37 °C. After a 24 h incubation, the cells were collected by centrifugation, and stored in RNALater (Ambion, Austin, Texas) at −20 °C. Uninfected cells were handled identically. The NY-18 strain of *A. phagocytophilum* was maintained in the promyelocytic leukemia cell line HL-60, and genomic DNA was isolated using the Nucleospin nucleic acid purification kit (Clontech, Palo Alto, California) (Barbet et al., 2003).

2.2. Primer extension analysis

Primer extension analysis was conducted essentially as described previously (Barbet et al., 2000). Briefly, total RNA was isolated from whole blood obtained during acute rickettsemia with the Florida strain of *A. marginale* by extraction with 6 M urea–3 M LiCl. For *A. phagocytophilum*, RNA was isolated from infected HL-60 cells that had