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Unusual chromatin structure associated with monoparalogous transcription of the *Babesia bovis ves* multigene family

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ABSTRACT

Rapid antigenic variation in *Babesia bovis* involves the variant erythrocyte surface antigen-1 (VESA1), a heterodimeric protein with subunits encoded by two branches of the *ves* multigene family. The *ves*1α and *ves*1β gene pair encoding VESA1a and 1b, respectively, are transcribed in a monoparalogous manner from a single locus of active *ves* transcription (LAT), just one of many quasi-palindromic *ves* loci. To determine whether this organization plays a role in transcriptional regulation, chromatin structure was first assessed. Limited treatment of isolated nuclei with micrococcal nuclease to assay nucleosomal patterning revealed a periodicity of 156–159 bp in both bulk chromatin and specific gene coding regions. This pattern also was maintained in the intergenic regions (IGr) of non-transcribed *ves* genes. In contrast, the LAT IGr adopts a unique pattern, yielding an apparent cluster of five closely-spaced hypersensitive sites flanked by regions of reduced nucleosomal occupancy. *ves* loci fall into three patterns of overall sensitivity to micrococcal nuclease or DNase I digestion, with only the LAT being consistently very sensitive. Non-transcribed *ves* genes are inconsistent in their sensitivity to the two enzymatic probes. Non-linear DNA structure in chromatin was investigated to determine whether unique structure arising as a result of the quasi-palindromic nature of the LAT may effect transcriptional control. The in vitro capacity of *ves* IGr sequences to adopt stable higher-order DNA structure is demonstrated here, but the presence of such structure in vivo was not supported. Based upon these results a working model is proposed for the chromatin structural remodeling responsible for the sequential expression of *ves* multigene family members from divergently-organized loci.

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1. Introduction

Many microbes persist within immune hosts through the common mechanism of antigenic variation (Deitsch et al., 1997), a rapid alteration in the structure and antigenicity of specific key components. It has been proposed that microbial components undergoing antigenic variation sensu stricto normally serve some functional role (Borst, 2003). The protozoal hemoparasite, *Babesia bovis*, provides a classic example of this (Allred et al., 1994). Persistent infections are established in immune cattle through the use of at least two mechanisms: cytoadhesion of infected erythrocytes (IE) containing mature parasites, and rapid antigenic variation of the cytoadhesion ligands (Allred et al., 1994; O'Connor et al., 1997, 1999a; O'Connor and Allred, 2000). In the case of *B. bovis*, rapid antigenic variation has been proposed to enable the ligands responsible for cytoadhesion to avoid detection by adaptive host immune responses (Allred and Al-Khedery, 2004). These functions involve the variant erythrocyte surface antigen-1 (VESA1), a heterodimeric protein comprised of size-polymorphic subunits 1a and 1b (Allred et al., 1994; O'Connor and Allred, 2000). VESA1a and 1b are encoded by *ves*1α and *ves*1β genes, respectively, from closely related but distinct branches of the *ves* multigene family, of which there are well over 100 members in total (Allred et al., 2000; Al-Khedery and Allred, 2006; Brayton et al., 2007; Xiao et al., 2010).

Significant to the establishment of persistent infections through antigenic variation is the ability of a microbe to control which and how many members of variant multigene families are expressed. In most systems that have been studied a single member of the multigene family is significantly expressed, generally in a mutually exclusive manner. We are here introducing the term "monoparalogous transcription" to describe the situation wherein transcription occurs from a single paralog of a multigene family in a haploid organism or life cycle stage, without regard for syntenic position. Regardless of ploidy, this situation prevails in order to

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prevent exposure of the organism's entire repertoire of variant antigens to the host immune system (Borst, 2003). Known mechanisms that have evolved to achieve this task can be quite diverse among different microbes. At one end of the spectrum is epigenetic transcriptional silencing of all but one member of a gene family, without DNA recombination. An example of this mechanism is the regulation of *var* genes encoding the PfEMP1 cytoadhesion ligand of *Plasmodium falciparum* (Duraisingh et al., 2005; Voss et al., 2006; Frank and Deitsch, 2006; Chookajorn et al., 2007; Dzikowski et al., 2007). At the opposite extreme is the highly promiscuous transcription of the Variant Surface Protein (*VSP*) gene family of *Giardia lamblia*. The VSP transcript population is winnowed down to one major form by post-transcriptional silencing prior to translation, through an RNA interference (RNAi)-like mechanism (Prucca et al., 2008). Kinetoplastid parasites have even evolved pathways for the biosynthesis of a novel nucleotide, β-D-glucosyl-hydroxymethyluracil (base "J"), which is associated with the maintenance of silencing of non-transcribed variant surface glycoprotein (*VSG*) gene expression sites (Gommers-Ampt et al., 1993; Borst and Van Leeuwen, 1997).

Of the more than 100 known members of the *ves* multigene family, a majority are organized as divergently-oriented pairs of genes. The gene pairs usually include one each of *ves*1α and *ves*1β, although examples of divergently paired *ves*1α genes are also found (Al-Khedery and Allred, 2006; Brayton et al., 2007). The intergenic regions (IGrs) lying between the paired *ves* genes are short, typically 400–450 bp in length. Moreover, the IGrs are quasi-palindromic, with paired, GC-rich inverted repeats in each half. The inverted repeats are highly conserved in sequence and are shared among IGrs from nearly all similarly organized *ves* genes (Al-Khedery and Allred, 2006). Due to the conservation of sequence and organization, IGrs from non-transcribed *ves* loci appear to be functionally equivalent to that derived from the locus of active *ves* gene transcription (LAT), and a preliminary characterization of *ves* promoters has confirmed this possibility (Wang et al., 2012). Despite such apparent functional equivalence, in vivo *ves* transcription appears to occur from a single locus among clonal parasites (Zupanska et al., 2009). Therefore, there are significant questions concerning the regulation of transcription among the various members of the *ves* multigene family of *B. bovis*, and how monoparalogous transcription is achieved and maintained.

An unusual trait of the LAT and the *ves* multigene family is that two distinct apoprotein subunits of the VESA1 holoprotein must be transcribed from the same divergently organized locus (Allred et al., 2000; Al-Khedery and Allred, 2006; Xiao et al., 2010). The presence of paired inverted repeats and divergent organization lend the IGr of the LAT and similarly organized *ves* loci a quasi-palindromic character. Based upon mapping of the 5′-untranslated region (5′-UTR) of both *ves*1α and *ves*1β genes, transcription begins within this region, indicating that bidirectional promoter activity also resides there, an assertion supported by transfection studies (Wang et al., 2012). Palindromic sequences have been demonstrated in a number of organisms to provide promoter functions, sometimes even requiring extrusion as a cruciform or hairpin to facilitate transcription (Kim et al., 1998; Hughes et al., 2009). Thus, the question arises how two divergent genes with heavily overlapping 5′-UTRs are equivalently transcribed to provide stoichiometrically similar amounts of the subunits for assembly of functional holoprotein. Detection of the individual subunits by immunofluorescence suggests that expression of each subunit is sequential within individual cells rather than simultaneous (Xiao et al., 2010), a feat that could be achieved by – among various possibilities – a sequential remodeling of IGr chromatin. To determine whether the quasi-palindromic nature of the IGr might play some role in the regulation of transcription, or in the achievement of sequential bidirectional transcription, we first asked whether transcription within the *ves* multigene family is associated with higher-order chromatin and/or DNA structure. Evidence is presented herein that *ves* IGr sequences are restructured uniquely with regard to nucleosomal occupancy and distribution, and are able to adopt stable higher-order structure in vitro, but the possibility that higher-order DNA structure exists in vivo at the LAT was unsupported. We hypothesize instead that sequential alternative nucleosomal remodeling explains the sequential activation and silencing of individual *ves* genes within the LAT, an integral aspect of the monoparalogous gene expression crucial to successful antigenic variation in this parasite.

2. Materials and methods

2.1. Parasites

The *B. bovis* C9.1 clonal parasite line, the derivation of which has been described previously (Allred et al., 1994; Al-Khedery and Allred, 2006) was used in this study. This line transcribes from a single locus both the *ves*1α and *ves*1β genes that are expressed as protein (Allred et al., 2000; Al-Khedery and Allred, 2006; Xiao et al., 2010). Transcription occurs in a monoparalogous fashion (Zupanska et al., 2009), by a mechanism that excludes transcription from other similarly organized *ves* loci. To ensure continued transcription from the LAT, parasites were periodically re-selected for immunoreactivity with the C9.1 line-specific monoclonal antibody, 4D9.1G1 (O'Connor et al., 1997, 1999b), to maintain population uniformity. Parasites were maintained under microaerophilous stationary phase culture conditions (Levy and Ristic, 1980), as described elsewhere (Allred et al., 1993). Prior to isolation of genomic DNA (gDNA) parasitemias were enhanced to ~10% by a "dilution enrichment" procedure (O'Connor et al., 1997). Bovine leukocytes were removed from parasite-infected cultures by passage through columns of sterile Whatman CF-11 cellulose (G.E. Healthcare; Piscataway, NJ, USA) (Ambrosio et al., 1986).

2.2. Preparation of B. bovis nuclei

Nuclei were isolated from *B. bovis* IEs, essentially as described previously (Lanzer et al., 1992), except that samples were maintained on ice throughout processing. Briefly, IEs were disrupted with 0.05% saponin to release hemoglobin. Parasites were collected by centrifugation at 10,000*g* for 10 min at 4 °C, then washed and resuspended in a hypotonic solution (20 mM piperazine-1,4-bis(2-ethanesulfonic acid), sodium salt (PIPES), pH 7.5, 15 mM NaCl, 60 mM KCl, 14 mM 2-mercaptoethanol, 0.5 mM EGTA, EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.125 mM phenylmethylsulfonate). After addition of 10% (v/v) Nonidet P-40 to a final concentration of 0.06% (v/v), the parasite suspension was disrupted with a pre-chilled Dounce homogenizer equipped with a "B" pestle. Nuclei were pelleted by centrifugation at 7,000*g* for 10 min at 4 °C, and used immediately. DAPI (Invitrogen, Carlsbad, CA, USA) was added to samples to a final concentration of 0.3 μM to enable examination of the condition of the isolated material by fluorescence microscopy.

2.3. Enzymatic treatments of chromatin

Micrococcal nuclease (MNase) digestion of chromatin was conducted as previously described (Greaves and Borst, 1987), with modification. The nuclear pellet was washed and resuspended in pre-chilled MNase reaction buffer (10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tris–HCl, pH 7.4). Aliquots were then incubated with increasing concentrations of MNase (0–6 U ml^{-1}; Fermentas; Glen Burnie, MD, USA) at 37 °C for 3 min. Reactions

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