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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 *ves1α* and *ves1β* 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. Nonlinear 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.

Keywords

Antigenic variation; *Babesia bovis*; Chromatin remodeling; Chromatin structure; Nucleosome remodeling; Promoter structure; Transcription

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

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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 *ves1α* and *ves1β* 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 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 *ves1α* and *ves1β*, although examples of divergently paired *ves1α* 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 *ves1α* and *ves1β* 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 *ves1α* and *ves1β* 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 MgCl₂, 0.1 mM CaCl₂, 10 mM Tris·HCl, pH 7.4). Aliquots were then incubated with increasing concentrations of MNase (0–6 units ml^{−1}; Fermentas; Glen Burnie, MD, USA) at 37°C for 3 min. Reactions were stopped by addition of “stopping buffer”, yielding final concentrations of 0.2% (w/v) SDS, 10 mM EDTA, 5 mM EGTA. DNase I cleavage sensitivity was assessed by resuspending nuclear pellets in pre-chilled DNase I reaction buffer (10 mM Tris·HCl, 2.5 mM MgCl₂, 10 mM CaCl₂, pH 7.5) at 25°C. Aliquots were incubated with increasing concentrations of DNase I (0–3 units ml^{−1}; Fermentas) at 37°C for 10 min. Reactions were stopped by addition of 10x stopping buffer (0.1% SDS, 50 mM Tris, 150 mM NaCl, 25 mM EDTA, pH 8.0) (Chen and Yang, 2001). gDNAs from enzymatic treatments were isolated by a classic SDS-proteinase K procedure, with phenol and chloroform extractions, and precipitation with isopropanol (Maniatis et al., 1982). In some experiments purified nuclease-treated gDNAs were further digested with restriction endonucleases (indicated in the text for individual experiments), resolved by size on 1.2% (w/v) agarose gels in Tris-Acetate-EDTA (TAE) buffer, and transferred onto Hybond N⁺ nylon membrane (GE Healthcare, Piscataway, NJ, USA) for hybridization analysis, using 10x standard saline citrate (10x SSC: 1.5 M NaCl, 150 mM sodium citrate, pH 7.0) by neutral capillary transfer.

2.4. Southern blot analyses

Oligonucleotides were end-labeled for probe use, using γ-[³²P]ATP and T4 polynucleotide kinase, as previously described (Reddy et al., 1991). End-labeling of 1 Kb Plus Ladder DNA for detection of the linear fragment controls was performed by a kinase exchange reaction, using T4 polynucleotide kinase, following manufacturer’s instructions (Invitrogen). Cosmid 1E10 IGr sequences were labeled for probe use by random primer extension, using α-[³²P]dCTP and a DECAprime II Random Priming DNA Labeling Kit (Ambion; Grand Island, NY, USA). Pre-hybridization, hybridization and washes were conducted as described previously (Al-Khedery et al., 1999). Finally, membranes were exposed to Hyperfilm MP (GE Healthcare) at −80°C, using an α,β-emitter phosphor intensifying screen.

2.5. Relative sensitivities of individual loci to enzymatic digestion

Relative sensitivities of individual loci to MNase or DNase I were determined by quantifying the loss of full-length restriction fragments with progressively higher enzyme concentration. Band peak areas on autoradiographs (see Supplementary Fig. S1) were integrated, using ImageJ (open-source software; National Institutes of Health; rsbweb.nih.gov/ij/index.html), after subtraction of background signal. The corrected peak areas were subtracted from the 0 U ml^{−1} values for that same sample, to give Δarea for each enzyme treatment. The Δarea values were divided by the size of the restriction fragment being followed (in Kb). Size-corrected Δarea values were then divided by the corrected peak area value of the 0 U ml^{−1} control and plotted as the proportion of signal lost. DNase I-

treated sample values were also corrected for % AT content by dividing the corrected Δ area values by (%A + T)(size in Kb) of each restriction fragment sequence.

2.6. Mapping of nucleosomal positioning

Nucleosomal positioning was mapped by a low-resolution combination of MNase partial digestion, agarose gel fractionation, and pseudo-end-labeled hybridization, essentially as described (Carey and Smale, 2000). Briefly, DNA was purified from MNase partially-digested chromatin as described earlier and digested with *EcoRI* or *KpnI*. Fragments were resolved on agarose gels and transferred to nylon membranes for Southern blot probing. The probes used were end-labeled oligonucleotides designed to hybridize at or near the restriction site (one nucleosomal unit distance or less). In this way it is possible to approximate the distance of a cleavage hypersensitive site from the anchoring restriction site. The essential principle behind this approach is illustrated in Fig. 7. for the LAT and LH α 2 probe.

2.7. Establishment of structural controls

A control containing a segment of perfect palindromic sequence was constructed from coding sequences of the *B. bovis* C9.1 line p9.6.2. *ves1 α* cDNA (GenBank accession no. [AF173158](#)). Primers PIRF-*PinAI* (forward) plus PIRR-*BamHI* (reverse) were used to amplify nucleotides 1982–2195. The product was digested with *PinAI* (New England Biolabs, Beverly, MA, USA) and ligated to create a perfect palindrome. The ligation product was digested with *BamHI* and inserted into a pBluescript II KS(+) vector (Invitrogen) to generate plasmid “pL3”. This proved a difficult sequence to clone and sequencing revealed that pL3 had a 20 bp deletion which disrupted the palindromic nature of flanking sequences but left a central 46 bp perfect palindrome. A 532 bp fragment of pL3 containing this palindrome, B0.5, was obtained by digestion with *BssHII* and isolation of the fragment from agarose gels.

2.8. Strategies for detection of in vivo higher-order DNA structure

Two strategies were used to preserve in vivo higher-order DNA structure within chromatin. The first method involved purification of nuclear DNA from partially digested chromatin under buffer conditions previously demonstrated to preserve structure and prevent junction migration in meiotic yeast chromatin (Allers and Lichten, 2000). Briefly, the nuclear pellet was lysed with extraction buffer comprised of 20 mM hexamine cobalt chloride ($\text{Co}^{3+}(\text{NH}_3)_6\text{Cl}^{-3}$), 2 M NaCl, 100 mM Tris·HCl, 25 mM EDTA, 2% (w/v) polyvinyl pyrrolidone 40, 3% (w/v) cetyltrimethylammonium bromide (CTAB), pH 7.5. Following addition of 2-mercaptoethanol to 0.16% (v/v), proteinase K to 500 $\mu\text{g ml}^{-1}$ and RNase A to 20 $\mu\text{g ml}^{-1}$ the lysate was incubated at 37°C for 1 h. The DNA was extracted with chloroform: isoamyl alcohol (24:1, v/v) by vigorous vortexing, prior to phase separation by centrifugation at 16,000 *g* for 3 min at 4°C. The aqueous phase was collected and mixed with 3 vol. of dilution buffer (1% (w/v) CTAB, 50 mM Tris·HCl, 10 mM EDTA, 4 mM $\text{Co}^{3+}(\text{NH}_3)_6\text{Cl}^{-3}$, pH 7.5). Spontaneously precipitated gDNA was transferred to a fresh tube, with wash and precipitation steps carried out as previously described (Allers and Lichten, 2000) to replace $\text{Co}^{3+}(\text{NH}_3)_6\text{Cl}^{-3}$ with Mg^{2+} . Finally, the purified DNA was dissolved in TMSpe buffer (10 mM Tris·HCl, 2 mM MgCl_2 , 50 mM spermidine, pH 7.5).

The second approach to stabilizing structure involved cross-linking of chromatin DNA with 4'-aminomethyl-4,5',8-trimethylpsoralen·HCl (psoralen), essentially as previously described (Bell and Byers, 1983). Parasites, released from IEs with saponin as described above, were suspended in pre-chilled cell suspension buffer (0.7 M sorbitol, 50 mM Tris·HCl, 50 mM EDTA, pH 8.0) and incubated at 4°C for at least 15 min in the dark prior to cross-linking. Cross-linking was initiated by the slow addition of the parasite suspension to a 35 mm Petri

dish (BD Falcon, San Jose, CA, USA) containing 9 vol. of cross-linking solution (0.12 mg ml⁻¹ 4'-aminomethyl-4,5',8-trimethylpsoralen HCl, 50 mM Tris-HCl, 50 mM EDTA, pH 8.0). The mixture was irradiated for 15 min at 365 nm (Blak-Ray, Model C50), at 4°C. Parasites were lysed by addition of an equal volume of 10% (w/v) SDS. The DNA was isolated by phenol:chloroform extraction and isopropanol precipitation as previously described (Maniatis et al., 1982). Isolated gDNAs were digested and resolved by two-dimensional (2-D) gel electrophoresis as described elsewhere (Bell and Byers, 1983). Following gel separation, cross-links were reversed by short-wavelength irradiation (120 mJ each side; Stratagene UV Stratalinker) (Sanz et al., 2007), and the fragments transferred onto Nylon membrane under alkaline conditions for hybridization.

2.9. Electron microscopy

Copper grids (200 mesh) were coated with Formvar (Schneider et al., 2007) and stabilized with a thin carbon coating. DNAs were spread onto the grids essentially as previously described (Kleinschmidt, 1968). The grids were stained with 1% (w/v) uranyl acetate solution, then washed with 90% ethanol and air-dried. Low-angle rotary shadowing with a platinum/palladium electrode was then performed to enhance structural details. Carbon coating and rotary shadowing were performed by the University of Florida Interdisciplinary Center for Biotechnology Research Electron Microscopy Laboratory, USA.

2.10. 2-D gel analysis of DNA structure

A neutral/neutral 2-D gel electrophoresis system was used to separate structured from linear form gDNAs (Bell and Byers, 1983). As a control for migration of linear DNAs under these conditions, an arc of linear DNA fragments was formed by the use of 200 ng of "1Kb Plus DNA Ladder" (Invitrogen). Unless indicated otherwise, gel conditions were as follows: the first dimension was performed in 0.6% (w/v) agarose in Tris-borate-EDTA-Mg²⁺ buffer (TBE-Mg²⁺; 0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA) (Maniatis et al., 1982) supplemented with 3 mM MgCl₂. Separation was performed at 1 V cm⁻¹ for 62 h at 4°C. The sample lane was excised and loaded onto a second gel comprised of 1.2% (w/v) agarose in TBE-Mg²⁺ buffer supplemented with 0.5 µg ml⁻¹ of ethidium bromide. Separation was at 3 V cm⁻¹ for 22 h at 4°C. To guarantee maximal stabilization of possible DNA higher-order structures, a Mg²⁺-supplemented 5x sample buffer (25 mM Tris-HCl, 0.25 mM EDTA, 25 mM MgCl₂, 0.25% (w/v) bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400, pH 7.5; recipe kindly provided by M. Lichten, National Institutes of Health, USA) was employed. For Southern analyses, the gels were exposed to ultraviolet light for approximately 3 min to fragment DNAs. To facilitate DNA transfer Mg²⁺ was removed by washing twice in 10 mM EDTA, pH 8.0 and twice with 5 mM EDTA, pH 8.0, followed by deionized water. The DNA fragments were denatured in 1.5 M NaCl, 0.5 N NaOH and transferred onto Hybond XL nylon membranes (GE Healthcare) under alkaline conditions. The membranes were washed with 2x SSC and the DNA fragments cross-linked to the membrane with UV light (Stratalinker 2400 UV Crosslinker; Stratagene, Inc.; La Jolla, CA, USA) for Southern hybridization. Individual spots were identified by sequential probing of blots with locus-specific oligonucleotide probes, followed by probing with end-labeled 1 Kb DNA ladder standards, with stripping of blots between probings (Al-Khedery and Allred, 2006).

2.11. Oligonucleotides used in this study

Oligonucleotides were used in a variety of roles in this study and included the following: AZT02: AACAACTGGGCCAAGGGTC (β-tubulin coding sequences only); BAK6: GATTATCCAGTACCGGTAGATTC (IGr of six divergent *ves* loci, including that of the LAT); BAK52: CAATCTATGTGTCTATGCCAACG (LAT *ves1α* intron 1 and a second, uncharacterized fragment); BAK68: GTAGGCGCTGAGAACCACCAC (phagemid 10-1

ves locus only); GAPDHE1R: TGAACAGAATCGTACTTCAGCAAGT (glyceraldehyde-3-phosphate dehydrogenase coding sequences only); LH1: CCATACAAACTCTCCTTTCTGAC (phagemid 15-1 *ves* locus); LH2: CCAGCACTATTACAGTTACAAGTA (phagemid 15-1 *ves* locus only); LH α 1: ACCTAGTAGTGGAGTAGTACTATCT (LAT *ves*1 α coding sequences and a second, uncharacterized fragment); LH α 2: ACTGATACACAAGTCACAGACCA (LAT *ves*1 α coding sequences only); PIRF-*Pin*AI: ACGTACCGGT/CTGACAGCACTAAAGAACCC (LAT *ves*1 α coding sequences; adds *Pin*AI at 5' end); PIRR-*Bam*HI: ACGTGGATCC/GAGACTACCCATTACCATACC (LAT *ves*1 α coding sequences; adds *Bam*HI at 5' end).

2.12. Accession numbers

GenBank accession numbers of the *ves* and other loci under study herein are: LAT, [DQ267461](#); cosmid 1E10, [AY279553](#); cosmid S621, [AY279554](#); phagemid 10-1, [AY279556](#); and phagemid 15-1, [AY279557](#) (all described in Allred and Al-Khedery, 2006). Other loci are β -tubulin, gi|[182636838:1061341-1063168](#); and GAPDH, gi|[182636837:c617497-616255](#) (Brayton et al., 2007).

3. Results

3.1. Nucleosomal patterning of *B. bovis* chromatin

Currently, our knowledge regarding chromatin organization in Apicomplexan parasites is quite limited. One characteristic that stands out is the lack of any identifiable ortholog to the nearly ubiquitous histone, H1, a characteristic that holds true in *B. bovis* (Brayton et al., 2007). To understand gene regulation there is a need to first understand chromatin structure. Therefore, *B. bovis* chromatin nucleosomal organization was first examined, using MNase to determine the nucleosomal spacing of “bulk chromatin”. At lower concentrations MNase primarily cuts DNA between nucleosomes within chromatin linker regions, identifying nucleosomal spacing (Telford and Stewart, 1989; Zaret, 2005). As shown in Fig. 1A, partial digestion of bulk nuclear chromatin yielded a ladder of ethidium bromide-stained bands exhibiting a mean periodicity of 156.3 bp, similar to the ~150 bp periodicity observed for chromatin nucleosomal spacing in *P. falciparum* (Cary et al., 1994; Lanzer et al., 1994b; Horrocks et al., 2002). To query whether the spacing of bulk chromatin also held for *ves* loci, MNase-treated chromatin was Southern blot probed with oligonucleotide BAK6 (Fig. 1B). This probe hybridizes to a sequence found within the center of the IGRs of six different *ves* loci (approximately at the location of the “X” in Fig. 7), including that of the LAT (Al-Khedery and Allred, 2006), and therefore provides averaged information on nucleosomal spacing in the *ves* family, but no specific information on nucleosome locations in individual loci. These loci yielded a mean nucleosomal periodicity of 161 bp. The difference from the ethidium bromide-stained gel is likely due to inaccuracies in determining band sizes on agarose gels.

Southern blots of similarly processed *B. bovis* chromatin were hybridized with oligonucleotide probes to specifically detect nucleosomal organization based on gene identity and activity. As representatives of consistently expressed housekeeping genes, β -tubulin and GAPDH (AZT02 and GAPDHE1R probes; not shown) were chosen. Among the *ves* family, unique probes to the non-transcribed *ves* loci, phagemid 10-1 (BAK68) and phagemid 15-1 (LH2), were generated. End-labeled probes were applied to Southern blots made from *B. bovis* chromatin partially digested with MNase, then digested with either *Kpn*I or *Eco*RI. Restriction enzyme and probe combinations were chosen to allow not only locus-specific probings but whenever possible also to provide pseudo-end-labeled mapping of nucleosomal band peaks relative to an “anchoring” restriction site (Carey and Smale,

2000). Whenever possible, the same blots were stripped and reprobed, to facilitate direct comparisons of probing data. Applying this approach, a plot of band sizes for each locus again yielded evidence of uniform nucleosomal spacing with a mean periodicity for the various loci of 156.9 ± 4.0 bp (Fig. 1C). Significantly, this included the IGr of the non-transcribed phagemids, 15-1 and 10-1 *ves* loci, each of which spans slightly more than three nucleosomes (Fig. 2, vertical black bars). Thus, the non-transcribed *ves* loci had the same apparent nucleosomal spacing and organization as the actively transcribed genes, β -tubulin and GAPDH, and each resembled ethidium bromide-stained bulk chromatin. On the other hand, the consistent nucleosomal spacing seen in non-transcribed *ves* genes was not found in the nucleosomal organization of the LAT (Fig. 2). When hybridized with the LAT-specific oligonucleotide probes, LH α 1 and LH α 2, a very reproducible pattern of five closely spaced, sharp, nuclease-sensitive bands were observed within the IGr, instead of the three uniformly spaced, “fuzzy” nucleosomal bands seen in non-transcribed *ves* gene IGr. This region was flanked by broad zones with inconsistent nucleosomal positioning, beyond which regularly spaced nucleosomes could again be detected within coding sequences of the *ves1* α and *ves1* β genes (Fig. 2). This pattern of nuclease accessibility is associated with the transcriptional activity of the LAT. Previously, the LAT was shown to be the only transcriptionally active *ves* locus in the *B. bovis* C9.1 line genome (Allred et al., 2000; Al-Khedery and Allred, 2006; Zupanska et al., 2009; Xiao et al., 2010). Although there are numerous *ves* loci with similar divergent organization (Al-Khedery and Allred, 2006; Brayton et al., 2007), and IGr from silent *ves* loci are capable of driving transcription in transient transfection assays (Wang et al., 2012), to date we have been unable to detect in situ transcriptional switching from the LAT to other *ves* loci, even when attempts were made to select for such switching (unpublished data). Regardless, to ensure continued expression of VESA1 derived 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.

3.2. Nuclease-sensitivity of genomic loci

Given the clear difference in organization of the transcriptionally active LAT from that of silent loci, the relative accessibility of these loci to nucleases was quantified. As a measure of accessibility, the rates of loss of full-length restriction fragments cut from genomic DNAs following partial cleavage with MNase were determined. To facilitate making this determination directly without between-probings variability, the BAK6 oligonucleotide probe was used to detect six previously characterized *ves* loci (Al-Khedery and Allred, 2006), including the LAT. The MNase partially digested chromatin was purified and further digested with *Kpn*I or *Eco*RI to yield discrete, full-length bands. BAK6 hybridizes with the LAT, which is actively transcribed, and five additional *ves* loci that are not transcribed. On Southern blots of *Kpn*I-cut DNA it is possible to discriminate four of the bands clearly for quantification (cosmid S621, 4.8 Kb; phagemid 15-1, 4.6 Kb; LAT, 2.9 Kb; and phagemid 10-1, 1.7 Kb). The rate of loss of each full-size restriction fragment relative to the MNase-undigested (0 U) control sample was first corrected for background, then normalized based on the size of the band to correct for size-dependent susceptibility of each target (see Supplementary Fig. S1). When analyzed in this manner, the LAT was found to be similar to cosmid 6-2-1 but more sensitive than other non-transcribed *ves* loci (Fig. 3A). The silent phagemid 15-1 locus was intermediate in sensitivity and similar to the actively transcribed β -tubulin gene. Interestingly, the phagemid 10-1 *ves* locus was reproducibly much less sensitive to MNase digestion than any of the other sequences followed. In similar experiments performed with the BAK52 probe, which detects only the LAT *ves1* α gene and one silent *ves* locus (presumably the source of BAK52 sequences within the LAT), the LAT was routinely dramatically more sensitive (not shown). Thus, the *ves* loci observed can be grouped into three overall levels of MNase accessibility and sensitivity to digestion. A

similar pattern of three major chromatin accessibility states has previously been proposed for *P. falciparum* chromatin as an explanation for *var* gene intrinsic switch rates (Horrocks et al., 2004).

DNase I was similarly employed as a second enzymatic probe of *ves* chromatin structure. Whereas MNase primarily digests inter-nucleosomal linker DNA from chromatin (Telford and Stewart, 1989; Zaret, 2005), DNase I cleaves the DNA essentially anywhere along its length through recognition of the minor groove, but preferentially wherever DNA is not protected by protein. However, cleavage is not truly random, as DNase I displays a moderate preference for AT-rich DNA (Chen and Yang, 2001). Application of DNase I as an enzymatic probe, but with mathematical correction for both fragment length and %AT of each probed locus, gave results that were qualitatively similar to those obtained with MNase (Fig. 3B). Again, the LAT was the most sensitive of all loci observed, the phagemid 10-1 locus the least sensitive, and the remaining *ves* loci and GAPDH all had a very similar intermediate sensitivity, including the cosmid 6-2-1 locus. Interestingly, DNase I-sensitivity of the β -tubulin gene was more similar to that of the LAT than it was to the remaining loci, suggesting a differential effect of localized structure on the abilities of MNase and DNase I to access some actively expressed genes.

3.3. Ability of *ves* loci to adopt higher-order structure

The quasi-palindromic nature of the IGr found in many *ves* loci, the hypersensitivity of the LAT to nuclease digestion, and the unusual pattern of MNase digestion products mapping to the IGr of the LAT, led us to hypothesize that local adoption of higher-order DNA structure could explain those traits of this locus and might play a role in gene regulation. The approach taken was to use a 2-D gel electrophoresis method capable of distinguishing non-linear from linear DNA (Bell and Byers, 1983) to analyze gDNA that had been isolated either under conditions which stabilize higher-order structure (Allers and Lichten, 2000), or after cross-linking with psoralen (Bell and Byers, 1983) to preserve structure. Prior to application to gDNA isolated from structurally preserved chromatin, a positive control molecule was prepared. The plasmid, pL3, was constructed containing a 46 bp perfect central inverted repeat with a *Pin*AI restriction site (ACCGGT) at the center, flanked by unique sequences (not shown). A 532 bp fragment containing the inverted repeat, called “B0.5”, was isolated from pL3 for use as a control. To ensure the presence of nonlinear structure (in the case of B0.5, a hairpin structure) a standardized method for “thermal remodeling” of the control DNAs was developed. Control DNAs were suspended in TE buffer, heated to 80.1°C for 8 min, then cooled in a series of steps to: 65°C, 37°C, 25°C and finally 4°C, with incubation for 10 min at each step. These conditions were adopted after a series of preliminary experiments in which temperature, [Na⁺], [Mg²⁺], and buffer composition were all examined, resulting in consistent structural remodeling of B0.5 and other sequences without full denaturation or apparent strand separation (Huang, Y-L. 2010. Higher-order structure in the *Babesia bovis* locus of active *ves* transcription. Ph.D. Dissertation, University of Florida, USA). Thermal remodeling of B0.5 DNA resulted in a mixture of linear and cruciform-like forms as imaged by low angle rotary shadowing electron microscopic observation of spread DNAs. Observation of purified *ves* IGr-containing sequences treated in vitro by the same protocol revealed a similar mix of linear and higher-order structured forms (Fig. 4). When analyzed by 2-D gel electrophoresis, the thermally-remodeled DNAs resolved into two or more spots. The B0.5 control resolved as two spots: One form was comprised of double-stranded linear DNA which co-migrated as part of a smooth arc with other linear DNAs (“1 Kb-Plus Ladder” size standards). The second form was observed as an off-axis spot comprised of the cruciform-like structure, migrating more slowly in the first dimension but more rapidly in the second dimension (Fig. 5). Similarly treated *ves* IGr-containing sequences resulted in at least two spots for each.

Again, linear forms of each molecule migrated with linear size standards as a part of a smooth linear-form arc. IGr-derived structured forms, unlike B0.5, migrated slightly faster in the first dimension and significantly faster in the second dimension, resulting in off-axis spots laying slightly ahead and below the linear-form spots, beneath the linear forms arc (Fig. 5). The pattern of off-axis migration was somewhat dependent upon the size of the fragment. Analysis of thermally-remodeled fragments of varying sizes by comparing their migrations relative to similarly-sized linear DNAs revealed the formation of a distinct arc that intersects and crosses that of linear DNA at approximately 3 Kb. Some fragments demonstrated exaggerated migration in both dimensions (e.g., phagemid 10-1 sequences).

Application of the 2-D gel analysis to structurally-preserved gDNA isolated either in the presence of buffers designed to preserve structure (Figs. 5, 6) or following psoralen cross-linking (not shown) revealed that the vast majority of gDNA shows no evidence of adopting higher-order structure in vivo (Fig. 6). Probing of DNAs stabilized either with structure-preserving buffer or with psoralen, using the LHa2 oligonucleotide specific for sequences near the 5' end of the LAT *ves1α* gene failed to consistently reveal off-axis DNA. No such spots were ever observed when blots were probed with oligonucleotides to other *ves* IGr-containing fragments (not shown). Because our ability to detect such off-axis structured forms was not consistent, our hypothesis concerning involvement of higher-order DNA structure in *ves* gene regulation is considered unsupported. The use of a probe of high specific activity, random-labeled cosmid 1E10 IGr, which hybridizes to fragments from many *ves* genes, detected a complete arc of linear DNAs. Additionally, a low-abundance off-axis smear was detected that exhibited crossover of the linear arc, as described in Fig. 5, but without apparent association with specific loci.

4. Discussion

The organization of chromatin in babesial parasites has not previously been described. Despite relying upon a complicated two-host life cycle involving both vertebrate and invertebrate hosts, babesial parasites have very small genomes. Thus, *B. bovis*, which infects cattle during the haploid asexual portion of its life cycle and tick species from the genera *Boophilus* and *Rhipicephalus* as definitive hosts during the sexual portion, has a genome of only about 8.2 Mbp (Brayton et al., 2007). As a result of this small size *B. bovis* is thought to use approximately 70% of its genome to encode proteins (Brayton et al., 2007). The proportion of the genome found to be involved in gene regulation will undoubtedly account for much of the remainder once some understanding is gained of non-coding RNAs in apicomplexans. Against this backdrop we sought to provide some fundamental information regarding *B. bovis* chromatin, beginning with nucleosomal organization. The sensitivity of chromatin to nuclease digestion has long been recognized as a useful metric for distinguishing differing chromatin structures. As with other apicomplexans, no clear ortholog of histone H1 can be identified in the *B. bovis* genome by bioinformatic means, suggesting either that other proteins may be fulfilling this role or a linker function comparable with that of H1 is lacking altogether. Similarly to *P. falciparum* (Cary et al., 1994; Lanzer et al., 1994a; Horrocks et al., 2002), the nucleosomes of *B. bovis* were found to be closely spaced, on average 157 bp apart (measurements with all detection methods in this study yielded a mean of 156.8 ± 3.8 bp). The close nucleosomal spacing calls into question how the chromatin of *B. bovis* and other apicomplexans is packaged into higher-order arrangements for compaction within the nucleus. In the absence of H1, nucleosomal units may have greater opportunity for inter-nucleosomal interactions, perhaps facilitating the rapid spreading of silencing or activation signals within a region of chromatin. Seemingly, the very close spacing of coding sequences leaves little room for insulator elements between genes of average spacing, although such elements have been hypothesized to exist among similarly clustered virulence genes in *P. falciparum* (Crowley et al., 2011).

This situation raises the possibility of the genome being organized into regions that participate in coordinated activation or silencing. The association of H3K9me₃ solely with a few variant multigene families in *P. falciparum* (Lopez-Rubio et al., 2009) would be consistent with such a scenario, with regard to maintenance of widespread silencing of those families. The answer to this question in *Babesia* must await genome-wide mapping of epigenetic regulatory markers. This possibility would raise a further logistical problem for *ves* genes, as most are clustered closely together, but the clusters are mostly small and scattered around the genome, without the usual bias to subtelomeric regions (Brayton et al., 2007). Moreover, the majority of *ves* genes within the clusters are organized functionally for bidirectional expression, having IGRs which are functional in vivo for expression of marker genes when present on episomal DNA (Wang et al., 2012). The monoparalogous nature of *ves* transcription (Zupanska et al., 2009), like that of most variant multigene families, raises the question how gene pairs within a cluster are individually and discriminately marked for transcription or silencing.

One significant ramification of the small genome size of *B. bovis* is that the mean IGR length is only 589 bp (Brayton et al., 2007), or less than four nucleosomal units. Within the divergently organized *ves* loci the distance is only 400–450 bp. Within this region are dual bidirectional promoter activities transcribing both *ves1α* and *ves1β* genes (Wang et al., 2012), with heavily overlapping 5'-UTRs (Allred et al., 2000; Wang et al., 2012). It remains to be determined critically whether the LAT IGR is able to transcribe simultaneously in both directions or must somehow alternate between transcribing *ves1α* and *ves1β* sequences. Live-cell immunofluorescence labeling of *B. bovis* IEs with monospecific antibodies discriminating VESA1a and 1b subunits has revealed considerable variability in the timing of appearance of the two subunits on the IE membrane of individual cells containing young parasites. This occurs even though similar distributions are eventually reached and both are transcribed from the same bidirectional promoter region (Allred et al., 2000; Al-Khedery and Allred, 2006; Xiao et al., 2010). One possible explanation for this result is that transcription may occur first from one gene of the pair, followed by localized remodeling to support transcription of the second gene. This could result in temporally staggered transcription, translation and transport, effecting the observed sequential appearance of the two subunits on the IE surface. Should that scenario hold, the positioning of nucleosomes within the LAT IGR would likely be dynamic. In fact, what was observed within the LAT was the presence of five discrete, closely-spaced bands where three would normally be expected (Fig. 2). This result suggests a unique and relatively stable chromatin structure exists within the IGR of the LAT, associated with in vivo transcription. Similarly, the overall accessibility and sensitivity of the LAT IGR to both MNase and DNase I was exaggerated relative to non-transcribed *ves* genes. This combination of traits led us to question whether the MNase partial digestion products observed within the IGR of the LAT are, in fact, due to changes in nucleosomal occupancy only, or represent MNase hypersensitive sites resulting from DNA which has adopted some higher-order structure.

A 2-D gel electrophoresis approach was chosen to directly assay for non-linear DNA forms through. This methodology should enable determination whether DNA within the LAT IGR adopts a unique higher-order conformation absent from non-transcribed loci. The 2-D gel electrophoresis approach separates DNA forms based on structure in the first dimension and apparent size in the second dimension (Bell and Byers, 1983). The relative influence of each trait varies with size and shape of the DNA fragments, altering fragment migration relative to linear double-stranded (ds)DNA in a manner associated with, but not strictly dependent upon, fragment size (Figs. 5, 6). Thermally remodeled DNAs migrated off the arc formed by linear DNA, whereas that portion of the DNA failing to adopt such structure migrates on the linear DNA arc. The higher-order structural forms created by thermal remodeling in control experiments appear to be intramolecular, as evidenced by the DNA concentration-

independence of their formation, inhibition of their formation by elevation of [NaCl] or [Mg²⁺] in the annealing buffer, and failure to observe bimolecular structures by electron microscopy of spread DNAs (additional data, not shown). When applied to chromatin whose native structure was stabilized in its in vivo chromatin conformation, either through the use of specialized buffers (Allers and Lichten, 2000) or psoralen cross-linking (Bell and Byers, 1983), off-arc spots were observed only when probed with a LAT-specific oligonucleotide probe. However, it was not possible to consistently detect such structure and our hypothesis of higher-order structure being associated with transcription must be considered unsupported. The extensive off-arc smear observed with the use of a long probe of high specific activity (Fig. 6) likely reflects mitotic intermediates present in the asynchronous population.

To explain the above observations and how they may relate to the apparent sequential expression of VESA1 subunits we propose a working model, illustrated in Fig. 7. Inactive *ves* loci contain a regular series of evenly-spaced nucleosomes. When the bidirectional *ves* promoter region (comprised of at least two bidirectional promoter activities (Wang et al., 2012); twin black dual-ended flags) becomes activated, a nucleosome is expelled, perhaps that associated with the center of the IGr (indicated by “X” and “removed” arrow). This allows the lateral, discrete repositioning of a nucleosome on one side or the other of the IGr (shown is one from the *ves1β* 5′ end; arrow). Nucleosomes near the 5′ ends of both genes adopt less regular positioning, probably due to epigenetic modifications associated with activation. The result is an overall hypersensitivity to MNase and DNase I, and flanking regions of poorly ordered nucleosome positioning. The repositioned IGr nucleosome prevents the proximal promoter from firing but leaves the distal promoter “open” for transcription (in this case, of the *ves1β* gene). This would result in long 5′ UTRs beginning on the distal side of the IGr, as described previously (Allred et al., 2000; Wang et al., 2012). A second lateral repositioning of nucleosomes is proposed that inactivates the first gene. The mechanism for this repositioning is unclear but may be stochastic. Repositioning would “open” the distal promoter for the second gene, allowing its transcription (in this case, the *ves1α* gene). Because the MNase digestion data herein was obtained from developmentally asynchronous parasites, all possible combinations of activation and nucleosomal positioning are detected. This results in two new fragments arising from the discretely-repositioned nucleosomes (Fig. 7). When the two new bands (red fragments) are superimposed upon the three-band pattern derived from regularly-positioned nucleosomes, the five-band pattern results. This model is consistent with the lack of higher-order DNA structure and unusual five-band pattern observed by partial MNase digestion of chromatin. Further, the apparent sequential expression of VESA1 apoprotein subunits reported previously (Xiao et al., 2010) from the dual bidirectional promoter activities present within the IGr (Wang et al., 2012) may be explained by this model. Alternative explanations may also hold. For example, the previously observed dual bidirectional promoters may instead merely be segments of a region with broad promoter activity. Further, the five band pattern could reflect developmental remodeling associated with transcription but unassociated with any sequential remodeling. Although conjectural, our model provides a maximally parsimonious explanation for these seemingly disparate results and a framework for future experimental approaches to decipher the monoparalogous expression of *ves* genes, a phenomenon that is key to long-term survival by this parasite.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Babesia bovis* chromatin possesses nucleosomal spacing with a periodicity of approximately 157 bp
- The *B. bovis* locus of active *ves* transcription (LAT) undergoes unusual nucleosomal repositioning when active
- The *LAT* can adopt a higher-order DNA structure in vitro but evidence for such structure in vivo is not supported
- A model is presented for nucleosomal repositioning that may provide for sequential bidirectional expression from the LAT

Fig. 1.

Mean nucleosomal spacing of *Babesia bovis* chromatin. (A) *Babesia bovis* chromatin was subjected to partial digestion with 0, 2, 4 or 6 U ml⁻¹ of micrococcal nuclease (MNase) (indicated for the vertical lanes), followed by *KpnI* restriction endonuclease digestion. Shown is an ethidium bromide-stained gel to reveal behavior of bulk chromatin. Lane S is the DNA size standards with sizes in Kb indicated on the left. Numbers 1–10 on the right indicate the number of nucleosomal units represented by each band. The associated plot of this gel indicates a slope of 156.3 bp per nucleosome. (B) *Babesia bovis* chromatin was partially digested with 0, 1, 2, 4 or 6 U ml⁻¹ of MNase (vertical lanes), digested with *KpnI* and hybridized with radiolabeled BAK6 oligonucleotide. This probe revealed the average nucleosomal banding pattern for intergenic region (IGr) sequences of six divergently oriented members of the *ves* family (slope = 161.0 bp per unit). Note that BAK6 does not

provide pseudo-end-labeling information, as it hybridizes within the middle of the IGr, a significant but variable distance from the *KpnI* sites of the six loci. Numbering on the left side indicates the size in Kb and on the right side is the number of nucleosomal units represented by each band. (C) Linear regression plots of ethidium bromide-stained bulk chromatin (filled circles), BAK6-probed *ves* loci (filled squares), the transcriptionally inactive phagemid 10-1 *ves* locus (open circles), and AZT02-probed β -tubulin sequences (open squares). Each gave plots of essentially indistinguishable slope, with a mean slope for all probes used in this study of 156.8 ± 3.8 bp.

Fig. 2.

Nucleosomal organization of individual intergenic regions (IGr's). Micrococcal nuclease (MNase) partially-digested chromatin was probed with the oligonucleotides BAK68 (which detects the phagemid (ph)10-1 locus), LH2 (ph15-1 locus), and LH α 1 and LH α 2, each of which hybridizes near the 5' end of the locus of active *ves* transcription (LAT) *ves1 α* gene coding sequences, near a *KpnI* site 487 bp downstream of the IGr. In this figure, probes are positioned at or very near to downstream restriction sites to enable pseudo-end-labeled mapping of the nucleosomal positioning relative to the probe and restriction site. The restriction sites used, *EcoRI* or *KpnI*, vary in their distance from the IGr in individual loci. The black bars to the left of the gel lanes represent the region of the gel containing the IGRs of each *ves* locus. Two lanes are shown for each probing, showing 4 and 6 U ml⁻¹ of

MNase-treated samples, where the nucleosomal ladder in this size range was most visible. Note the unusual cluster of five discrete MNase-sensitive bands within the IGr region of the LAT gel lanes, and the flanking zones of poorly positioned nucleosomes. Results from two different experiments, obtained with two different oligonucleotide probes, are shown for the LAT to demonstrate the high reproducibility of the five-band pattern. The probe used for each sample is shown above in brackets and the locus detected is given underneath. Numbering on the left side of ph10-1 lanes indicates the number of nucleosomal units within each corresponding band. Numbering on the right sides of ph15-1 and LAT lanes indicates DNA size standards (in Kb).

Fig. 3.

Nuclease sensitivities of individual loci. The progressive loss of full-length restriction fragments with increasing nuclease digestion is shown. The image intensities of full-length restriction fragments were quantified and plotted as the proportion of signal lost relative to the signal originating from the 0 U sample. (A) Micrococcal nuclease (MNase) digestion; (B) DNase I digestion. Both sample types were normalized for fragment lengths. Additionally, the DNase I samples were normalized for the %AT content of the individual loci to correct for the preferential digestion of AT-rich DNA by DNase I (Chen and Yang, 2001). Analyzed were the locus of active *ves* transcription (LAT), the non-transcribed S621, 15-1, and 10-1 *ves* loci, and β-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes.

Fig. 4.

Electron micrographs of structured DNA control molecules. The B0.5 control sequence and 1.8 Kb fragment derived from the non-transcribed phagemid 15-1 locus were visualized as spread DNAs, by low-angle rotary shadowing electron microscopy. Each of the DNAs was gel-purified and thermally remodeled at 80.1°C (see Section 3.3) to generate non-linear structures prior to spreading. Examples are shown of linear and non-linear, structured forms for each. Phagemid 6-1 DNA containing 1.8 Kb from the locus of active *ves* transcription (LAT) gave results consistent with phagemid 15-1 (data not shown). Size bars represent 200 nm.

Fig. 5.

Two-dimensional gel analysis of control DNAs. The migration of thermally remodeled control DNAs is shown following ethidium bromide staining. The same image is shown with both the linear (L) and non-linear (N) structured form arcs marked by line overlays to facilitate visualization, with the identities of individual spots indicated. B0.5, 0.5 Kb palindromic control; locus of active *ves* transcription (LAT), phagemid 6-1; 10-1, phagemid 10-1; 1-1, phagemid 1-1; 1E10, cosmid 1E10 sequences.

Fig. 6. Two-dimensional gel analysis of locus of active *ves* transcription (LAT) chromatin DNA. *Babesia bovis* chromatin DNA was isolated in its native conformation using structure-protective buffers (Allers and Lichten, 2000), digested with *EcoRI*, gel-resolved in two dimensions (Bell and Byers, 1983), blotted and hybridized with random primer-labeled cosmid 1E10 sequences to reveal numerous *ves* loci. Note the heavy, discrete arc of linear DNA fragments and the light, smeared arc of non-linear forms crossing over that of the linear DNA fragments.

Fig. 7.

Working model of chromatin remodeling at the locus of active *ves* transcription (LAT) during transcriptional activation. The LAT is organized as a divergently-oriented pair of *ves* genes, one each of *ves1α* (thick green arrow) and *ves1β* (thick blue arrow). These genes share a central intergenic region (IGr) of 434 bp, indicated by paired posts with dual flags representing the dual bidirectional promoter activities present there (Wang et al., 2012). Nucleosomal patterning of non-transcribed loci is comprised of evenly-spaced nucleosomes (dark gray circles) approximately 160 bp apart, with three located within the IGr (top). Upon transcriptional activation, chromatin remodeling is proposed to occur, with removal of one nucleosome (shown here as the central nucleosome, "X"), lateral repositioning of another to block one promoter, and inconsistent positioning of flanking nucleosomes (shown in light gray). This allows transcription in one direction. A second remodeling event, with lateral repositioning of nucleosomes, ends transcription from the first gene and allows transcription from the apposing gene. When the chromatin is partially digested with micrococcal nuclease (MNase), then cut with *KpnI* (site K in this example), fragments of the sizes shown at the bottom arise. Hybridization with a LHα2 probe, which hybridizes to *ves1α* sequences 42 bp upstream of the *KpnI* site, results in detection of fragment bands that arise from nucleosomes at both their "normal" (black bars) and repositioned (red bars) sites, giving rise to the five-band structure observed on autoradiographs (Fig. 2).