Sequencing of the PGRP-SA allele

DNA from single homozygous flies was extracted using a 'single fly preparation protocol'[8]. The PGRP-SA open reading frame from *seml* and *y w drs-GFP dpt-LacZ* flies was obtained by PCR using Platinum high-fidelity polymerase (Life Technology) and the following primers: 5′-GATAAATCCGGCAGATAGCCCA-3′ and 5′-ACTTTACTAAACTGATAT GCTC-3′. Single PCR reactions were analysed on 2% agarose gel, mixed together and purified with QIAquick PCR purification kit (Qiagen). DNA was sequenced by Eurogentec (Serraing).

Cloning and transformation of *UAS-dPGRP-SA*

The wild-type *PGRP-SA* cDNA was obtained by PCR using *y w drs-GFP dpt-LacZ* cDNA as a template. *Eco*RI and *Xba*I sites were introduced 5′ and 3′ respectively of the *PGRP* cDNA using the following primers: 5′-GGAATTCCATGCAGCCGGTTCGATTC-3′ and 5′-GCTCTAGAGCTTAGGGATTTGAGAGCCA-3′ and the Platinum high-fidelity polymerase. This fragment was then subcloned into *pUAST*. After sequencing, the construct was injected into w^{1118} embryos.

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1. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. & Ezekowitz, R. A. Phylogenetic perspectives in innate immunity. *Science* **284,** 1313–1318 (1999).
2. Khush, R. S., Leulier, F. & Lemaitre, B. *Drosophila* immunity: two paths to NF-κB. *Trends Immunol.* **22,** 260–264 (2001).
3. Lemaitre, B., Reichhart, J. M. & Hoffmann, J. A. *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl Acad. Sci. USA* **94,** 14614–14619 (1997).
4. Lemaitre, B. *et al.* A recessive mutation, *immune deficiency* (*imd*), defines two distinct control pathways in the *Drosophila* host defense. *Proc. Natl Acad. Sci. USA* **92,** 9465–9469 (1995).
5. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86,** 973–983 (1996).
6. Rutschmann, S. *et al.* The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. *Immunity* **12,** 569–580 (2000).
7. Leulier, F., Rodriguez, A., Khush, R. S., Abrams, J. M. & Lemaitre, B. The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection. *EMBO Rep.* **1,** 353–358 (2000).
8. Rutschmann, S. *et al.* Role of *Drosophila* IKKγ in a Toll-independent antibacterial immune response. *Nature Immunol.* **1,** 342–347 (2000).
9. Lu, Y., Wu, L. P. & Anderson, K. V. The antibacterial arm of the *Drosophila* innate immune response requires an IκB kinase. *Genes Dev.* **15,** 104–110 (2001).
10. Vidal, S. *et al.* Mutations in the *Drosophila dTAK1* gene reveal a conserved function for MAPKKKs in the control of rel/NF-κB-dependent innate immune responses. *Genes Dev.* **15,** 1900–1912 (2001).
11. Meng, X., Khanuja, B. S. & Ip, Y. T. Toll receptor-mediated *Drosophila* immune response requires Dif, an NF-κB factor. *Genes Dev.* **13,** 792–797 (1999).
12. Hedengren, M. *et al.* Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol. Cell* **4,** 827–837 (1999).
13. Levashina, E. A. *et al.* Constitutive activation of Toll-mediated antifungal defense in serpin-deficient *Drosophila*. *Science* **285,** 1917–1919 (1999).
14. Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunol.* **2,** 675–680 (2001).
15. Celine, L. F. *Semmelweis. L'imaginaire* (Gallimard, Paris, 1999).
16. Belvin, M. P. & Anderson, K. V. A conserved signaling pathway: the *Drosophila* Toll-dorsal pathway. *Annu. Rev. Cell Dev. Biol.* **12,** 393–416 (1996).
17. Kang, D., Liu, G., Lundström, A., Gelius, E. & Steiner, H. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc. Natl Acad. Sci. USA* **95,** 10078–10082 (1998).
18. Werner, T. *et al.* A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* **97,** 13772–13777 (2000).
19. Liu, C., Gelius, E., Liu, G., Steiner, H. & Dziarski, R. Mammalian peptidoglycan recognition protein binds peptidoglycan with high affinity, is expressed in neutrophils, and inhibits bacterial growth. *J. Biol. Chem.* **275,** 24490–24499 (2000).
20. Janeway, C. A. Jr Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54,** 1–13 (1989).
21. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* **282,** 2085–2088 (1998).
22. Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S. & Beutler, B. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. *Proc. Natl Acad. Sci. USA* **97,** 2163–2167 (2000).
23. Takeuchi, O. *et al.* Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* **11,** 443–451 (1999).
24. Hemmi, H. *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* **408,** 740–745 (2000).
25. Hayashi, F. *et al.* The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410,** 1099–1103 (2001).
26. Ozinsky, A. *et al.* The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl Acad. Sci. USA* **97,** 13766–13771 (2000).
27. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A. Jr A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* **388,** 394–397 (1997).
28. Tauszig, S., Jouanguy, E., Hoffmann, J. A. & Imler, J. L. Toll-related receptors and the control of antimicrobial peptide expression in *Drosophila*. *Proc. Natl Acad. Sci. USA* **97,** 10520–10525 (2000).
29. Franc, N. C. & White, K. Innate recognition systems in insect immunity and development: new approaches in *Drosophila*. *Microbes Infect.* **2,** 243–250 (2000).
30. Turner, M. W. & Hamwas, R. M. Mannose-binding lectin: structure, function, genetics and diseases associations. *Rev. Immunogenet.* **2,** 305–322 (2000).

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A pol I transcriptional body associated with VSG mono-allelic expression in *Trypanosoma brucei*

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In the mammalian host, African trypanosomes generate consecutive waves of parasitaemia by changing their antigenic coat. Because this coat consists of a single type of variant surface glycoprotein (VSG), the question arises of how a trypanosome accomplishes the transcription of only one of a multi-allelic family of *VSG* expression site loci to display a single VSG type on the surface at any one time[1]. No major differences have been detected between the single active expression site and the cohort of inactive expression sites[2]. Here we identify an extranucleolar body containing RNA polymerase I (pol I) that is transcriptionally active and present only in the bloodstream form of the parasite. Visualization of the active expression site locus by tagging with green fluorescent protein[3] shows that it is specifically located at this unique pol I transcriptional factory. The presence of this transcriptional body in postmitotic nuclei and its stability in the nucleus after DNA digestion provide evidence for a coherent structure. We propose that the recruitment of a single expression site and the concomitant exclusion of inactive loci from a discrete transcriptional body define the mechanism responsible for *VSG* mono-allelic expression.

African trypanosomes are responsible for sleeping sickness in humans, an epidemic disease currently affecting up to half a million people. The parasite *Trypanosoma brucei* alternates between a mammalian host and the tsetse vector during its life cycle. The bloodstream form can undergo antigenic variation by switching VSG surface coats[1], thus avoiding the host immune response and ensuring a persistent infection. To achieve the expression of a single type of VSG on the surface, only 1 out of 20 possible telomeric *VSG* expression sites (ESs) is expressed at a given time[2]. A set of ES-associated genes[4], which confers selective advantages in particular hosts[4,5], are transcribed polycistronically together with the *VSG* from a promoter located 40–60 kilobases (kb) upstream[6,7]. Hence, *VSG* expression site regulation is a genuine example of a mono-allelic expression from a multi-allelic gene family in an early branched eukaryote[8].

In addition to DNA recombination involving individual *VSG* genes[9], antigenic variation can occur by a coupled transcriptional activation/inactivation of ESs, named *in situ* switching. Once active, the choice of this ES is maintained over many generations. Previous

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work has shown that ES *in situ* switching can occur epigenetically[2]. In the procyclic form (the tsetse mid-gut stage of the parasite), no VSG is expressed and an invariant glycoprotein, procyclin, covers the parasite surface[10]. Notably, RNA polymerase I (pol I) transcribes not only the ribosomal DNA but also the genes of these two main surface proteins, *VSG* and *procyclin*, which are characteristic of the different developmental stages[11–14].

Although much molecular biology has been done to identify differences between active and inactive ESs in the bloodstream form, no principal differences have been found[2]. Because ES transcription is accomplished by a pol I activity that is normally associated with rDNA expression in the nucleolus[15], we investigated whether *VSG* ES regulation is mediated by a nuclear compartmentalization process.

We developed antibodies reactive to the large subunit of *T. brucei* pol I. Both rabbit polyclonal (affinity-purified) and mouse monoclonal antibodies recognized a polypeptide in protein extracts from the two developmental stages that is similar to the predicted relative molecular mass (M_r) of the pol I large subunit (196,000) (Fig. 1a). Immunofluorescence analysis using these antibodies in procyclic cells showed that pol I localized to the nucleolus, as expected for a eukaryote[15] (Fig. 1b). But immunofluorescence analysis of the bloodstream form identified a pol-I-containing body in addition to the nucleolus (in 59% and 62% of G1 cells (n = 500) using mono- and polyclonal antibodies, respectively) (Fig. 1c). Throughout this work, we assume that this body will not be seen in cells where it lies above, below or very close to the nucleolus. A detailed reconstruction of an individual nucleus using the Deltavision system shows clearly the discrete three-dimensional nature of the pol I body in the nucleoplasm (see Supplementary Information).

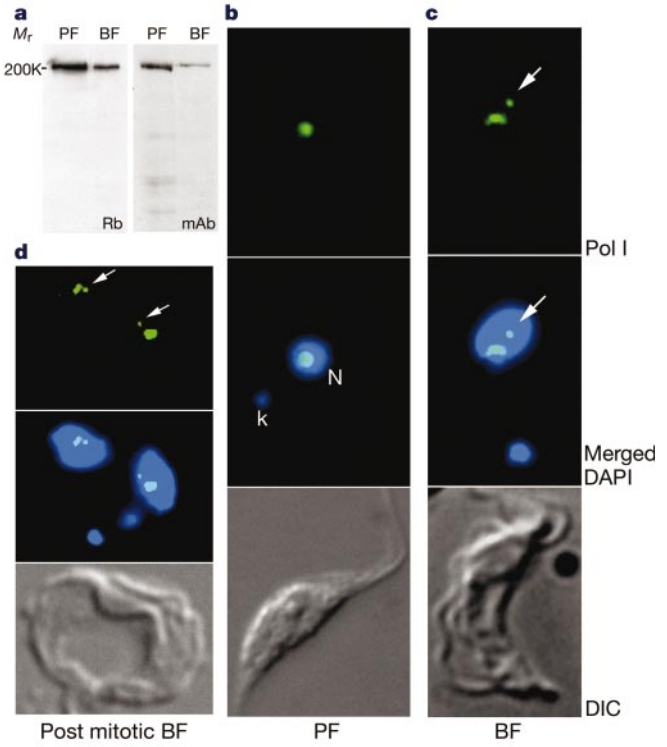

Figure 1 Identification of an extranucleolar body containing pol I in bloodstream-form nuclei of *T. brucei*. **a**, Western blot analysis using rabbit polyclonal (affinity-purified) (Rb) and mouse monoclonal 16B1a (mAb) antibodies reactive to the pol I large subunit. Total protein extracts from procyclic (PF) and bloodstream forms (BF) (5×10^6 cells) were used. **b**, Immunofluorescence analysis of the procyclic form, a tsetse stage of the parasite, using the pol I monoclonal antibody (green; top), merged with DNA stained by DAPI (blue; middle), and differential interference contrast (DIC) image of the cell (bottom). Nuclear DNA (N) and mitochondrial DNA (k) are labelled in a G1 cell. **c**, Immunofluorescence analysis of the wild-type bloodstream form using the pol I monoclonal antibody (green), merged with DNA stained by DAPI (blue), and the DIC image of the cell. Arrows indicate the extranucleolar pol-I-containing body. **d**, Immunofluorescence analysis of a bloodstream-form cell in postmitotic cells where both nuclei are visible and a pol-I-containing body in each nucleus is present (arrows).

To investigate whether this body represented an additional site of ribosomal RNA synthesis in the nucleus of the bloodstream form, we performed RNA fluorescence *in situ* hybridization (FISH) using the rDNA locus as a probe (Fig. 2a). RNA FISH experiments showed only one area of rRNA synthesis in both procyclic and bloodstream-form nuclei that corresponded to the nucleolus. We also analysed the localization of the well-characterized nucleolar marker fibrillarin, which is involved in one of the pre-splicing reactions of 18S rRNA[16]. Fibrillarin was localized to the nucleolus in both procyclic and bloodstream forms, but it did not localize to the pol I extranucleolar body of bloodstream-form nuclei (Fig. 2b, c). Thus, this extranucleolar pol-I-containing body represents a specialized nuclear compartment with a function different to that of the nucleolus.

To determine whether this pol I extranucleolar body was transcriptionally active, we labelled nascent RNA with 5-bromo-UTP (Br-UTP) in permeabilized cell nuclei[17]. Many transcriptional foci were detected in a bloodstream-form trypanosome nucleus (Fig. 3a); however, when α-amanitin was included in the buffer to inhibit transcription by pol II and pol III, the foci were reduced to a maximum of two (Fig. 3b). Double-labelling experiments of nascent Br-UTP RNA and pol I confirmed that both of the α-amanitin-resistant transcriptional foci colocalized with pol-I-containing structures (Fig. 3c). These data indicate that in addition to the expected transcription activity of the nucleolus, this unique pol I body is transcriptionally active. Run-on assays in *T. brucei*

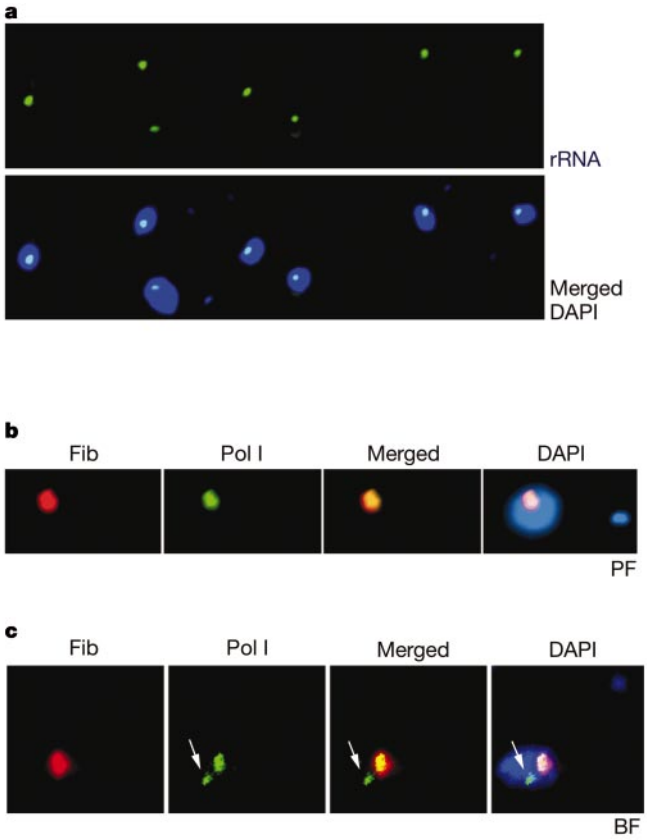

Figure 2 The pol I extranucleolar body has a different function from that of the nucleolus. **a**, Fluorescence *in situ* hybridization using a rDNA as a probe (green; top), and merged with nuclear DNA stained with DAPI, showing a unique signal in each nucleus (bottom). **b**, Double-labelling experiment using an anti-fibrillarin monoclonal antibody (P2G3) and the rabbit polyclonal anti-pol I on a procyclic-form (PF) cell. **c**, Double-labelling experiment as in **b**, but using bloodstream-form (BF) cells, which shows that the pol I extranucleolar body does not contain fibrillarin (arrows).

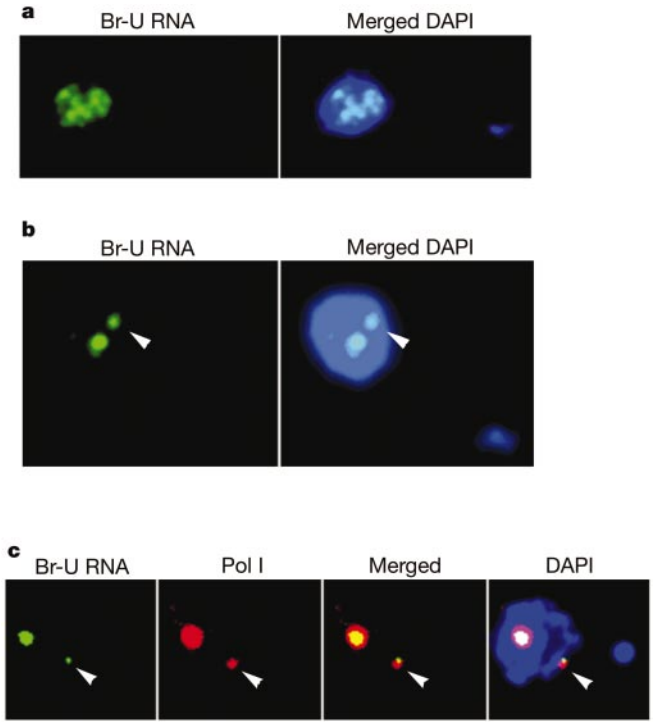

Figure 3 Br-UTP labelling of nascent RNA in permeabilized bloodstream-form nuclei indicates that the extranucleolar pol I structure is transcriptionally active. **a**, Total transcription, as revealed by Br-UTP (Br-U) incorporation foci (green) using a monoclonal antibody reactive to BrdUTP. **b**, Two transcriptional foci detected in the presence of α-amanitin (100 μg ml^{-1}). **c**, Colocalization of the α-amanitin-resistant nascent RNA Br-UTP-labelled foci (green) with the nucleolar and extranucleolar body (arrowhead) containing pol I (red).

have indicated that the rDNA and the *VSG* ESs are transcribed by an α-amanitin-resistant polymerase[11,12,18] that shares biochemical characteristics with pol I (refs 13, 14). We provide here direct evidence for pol-I-mediated α-amanitin-resistant transcription *in vivo*.

DNA FISH using a probe common to all ESs showed their dispersed distribution in the nucleus of the bloodstream form (Fig. 4a), similar to previous results[19]. However, we wanted to determine whether the active *VSG* ES sequences were associated with the pol I transcriptional body. We therefore established another way to visualize the position of DNA sequences in the nucleus that has been successful in other organisms[3]. The active 221ES site was tagged with the *lac* operator sequences, thus allowing specific recognition and visualization through binding of the *lac* repressor (LacI) fused to green fluorescent protein (GFP)[3]. We generated a construct to target the region upstream of the active ES promoter with 256 tandem copies of the operator for the *Escherichia coli* lactose operon. The GFP–LacI fusion protein was expressed in a tetracycline-inducible system[20]. On tetracycline induction of the transgenic trypanosomes, we detected a single dot in G1 nuclei using anti-GFP antibodies (Fig. 4b).

We examined whether the tagged ES was associated with the pol I transcriptional body. First, double-labelling experiments using anti-GFP antibodies and Br-UTP RNA labelling showed colocalization of the α-amanitin-resistant transcripts and the tagged active ES (Fig. 4c). Second, the GFP-tagged active ES was associated with the pol-I-containing extranucleolar body (Fig. 4d). As a control, we analysed the nuclear position of an inactive ES by inserting the *lac* operator cassette upstream of a silent ES (121ES); this construct showed a lack of association with the pol I transcriptional body (Fig. 4e). The restricted localization of α-amanitin-resistant nascent transcripts, pol I and the active ES (but not an inactive ES) is suggestive of a higher order nuclear architecture.

We examined whether our observations were more than the mere

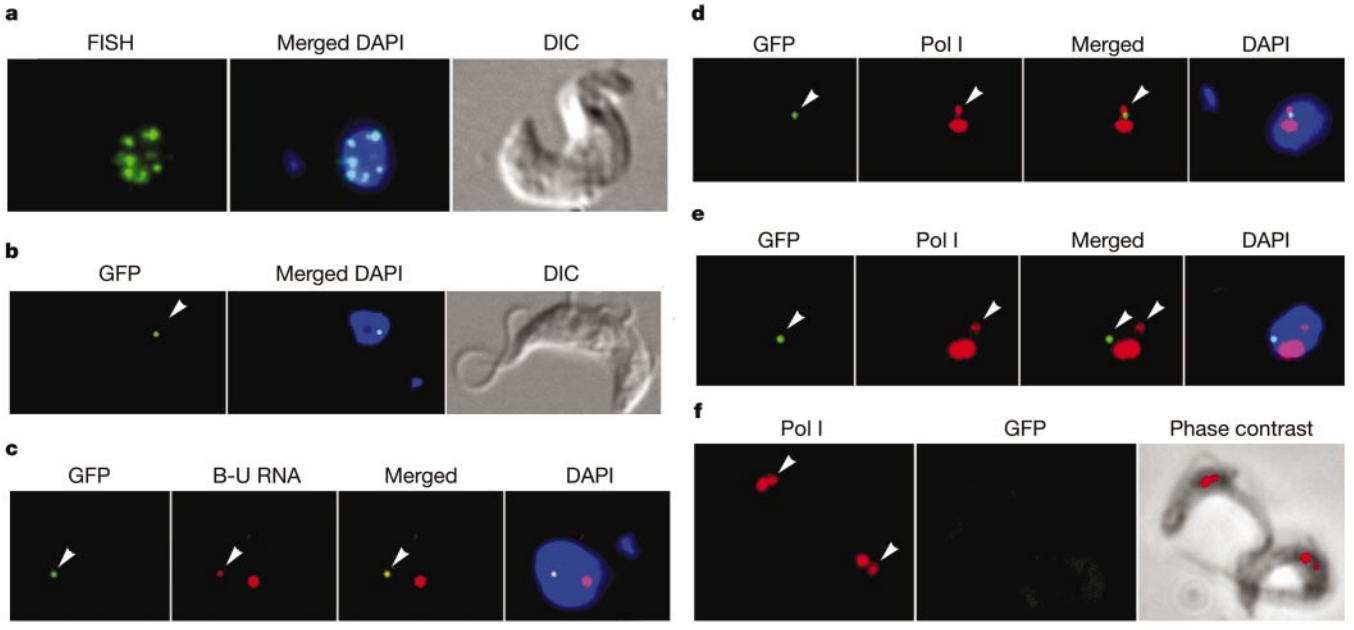

Figure 4 GFP–LacI tagging of the active expression site (ES) sequences in bloodstream-form nuclei reveals the association of this locus with α-amanitin-resistant transcription and with the extranucleolar pol I body. **a**, Distribution of ESs in the nucleus of a bloodstream form detected by DNA FISH using a probe containing the 50-bp repeat sequences that lie upstream of all ESs. **b**, GFP–LacI bound to the ES sequences (green) in a G1 cell detected using an anti-GFP polyclonal antibody. **c**, The small α-amanitin-resistant transcriptional foci (Br-U RNA; red) colocalized with the GFP-tagged active ES sequences (GFP; green). **d**, The extranucleolar pol I body is associated with the GFP-tagged active ES sequences, detected by double-labelling using monoclonal anti-pol-I (red) and polyclonal anti-GFP (green) antibodies (arrowheads), in the 221ES Lac-operator-tagged cell line. **e**, The extranucleolar pol I body is not associated with the GFP-tagged inactive ES sequences in the 121ES Lac-operator-tagged cell line (arrowheads). **f**, The extranucleolar pol I body is present in the absence of DNA (arrowheads). In contrast, the GFP–LacI signal associated with the active ES was not present after DNase I treatment. Complete digestion of DNA was confirmed by lack of DAPI staining (see Methods).

consequence of resident pol I on the active ES DNA. We compared the stability of the pol I body after DNase I digestion of the nuclei with that of the GFP–LacI bound to the 250 operator repeats upstream of the active ES. This GFP–LacI immunofluorescence dot was completely removed by DNase I treatment, as compared with untreated cells (200 counted; see Methods). However, the pol I body was still detected as a pol-I-positive nucleolar structure (Fig. 4f). This result indicates that there is a higher order architecture, and we propose that this extranucleolar structure should be termed the 'ES body' (ESB).

The ESB was solely associated with the expression of the *VSG* ES in the bloodstream form, a finding given more significance by its absence in the procyclic-tsetse form in which *procyclin* genes in addition to rDNA are also transcribed by pol I. The ESB is only present in the bloodstream form, in which the mutually exclusive expression of a single ES is crucial to display only one VSG type on the surface at any one time. This presence indicates that the ESB structure may have a specific role in the specialized regulation of *VSG* ESs. In contrast to the GFP–LacI dot, the ESB pol I molecules at the active ES remained after DNase I treatment of the nuclei (Fig. 4f). The presence of the ESB only in the bloodstream form and even in the absence of DNA strongly supports the existence of a coherent architectural body.

The identification of the ESB explains the unexpected localization of nuclear ES transcripts outside the nucleolus[19]. The detection of adjacently located nuclear transcripts from two different ESs in a switching process[21] can also be explained by the existence of a single structure (the ESB) with which each ES alternatively interacts. The transcription and splicing of messenger RNAs are coupled processes. It seems likely that the ESB will contain the RNA processing machinery, providing an explanation for efficient processing of transcripts from the active ES[22]. We have also detected the ESB in postmitotic nuclei at a point when the DNA has segregated into two nuclei but cytokinesis is not yet complete (Fig. 1d). These data suggest that the ESB is replicated along with the active ES, and that each daughter inherits an ESB with an attached active ES. This process would provide a mechanism for the maintenance and inheritance of the active transcriptional state.

During antigenic switching, the currently accepted ES cross-talking hypothesis[2] predicts mutual exclusion, with activation of a new ES dependent on inactivation of the currently expressed site, resulting in a coupled process[23]. Experiments that attempt to force the expression of two ESs, each targeted with a selectable marker, consistently show that two ESs cannot be fully active at any one time[21]. There is little evidence for chromatin-silencing mechanisms that can repress all inactive ES telomeres except one[2]; instead, inactive ESs are transcriptionally accessible[23]. Given previous data and our current results, we propose a model in which ESB-dependent ES recruitment leads to the activation of a single ES, and inactive ESs are excluded from this structure owing to the presence of the active one. Currently we do not know the mechanism that ensures that only one ES occupies the ESB; some conserved ES sequences may function as a *cis*-acting unique attachment site, similar to the way in which locus control regions[24] may function. Our model implies further that *in situ* switching is mediated by an infrequent process whereby the attachment of the active ES with the ESB becomes unstable, thus allowing occupancy by an inactive ES that displaces the active one, resulting in a coupled activation/inactivation.

It has been suggested that multiple transcriptional complexes can be organized into macro-structures, termed transcriptional factories[25]. But the identification of individual factories is extremely difficult owing to the complexity of pol II and III transcription in eukaryotic nuclei. The ESB that we have identified represents direct evidence for the existence of a highly defined individual transcription factory. Our model, which links such factories to the expression of particular genes, may help to explain other mono-allelic expression[26], including *var* genes in malaria antigenic variation[8] and developmental activation of highly expressed genes in other differentiated eukaryotic cells. □

Methods

Trypanosomes and immunofluorescence

Trypanosoma brucei bloodstream-form (antigenic type 1.2, MITat 1.2, clone 221a) and procyclic-form strain 427 was used in this work[1]. The bloodstream trypanosome transgenic cell line expressing the T7 RNA polymerase and the tetracycline repressor (single marker cell line)[20] was used as an initial cell line for the GFP tagging of the ES. We carried out immunofluorescence experiments on cells fixed in methanol at −20 °C as described[27]. Anti-RNA pol I monoclonal (16B1a) and rabbit polyclonal (affinity-purified) antibodies were developed against a His-fusion polypeptide from the large subunit of RNA pol I (residues 1,365–1,521). Polyclonal antisera were further purified over a CNBr–Sepharose (Pierce) fusion protein column according to the manufacturer's instructions. Immunofluorescence using these antibodies was carried out in 300 mM NaCl and 10% blocking reagent (Roche) in PBS/0.3% Tween 20. As secondary antibodies, we used anti-mouse and anti-rabbit conjugated with Alexa 488 or 596 (Molecular Probes). Images were captured by a Leica DMBRE microscope (×100 lens) with narrow band filters (Leica) and a CCD camera (Princeton) using IPLab software, and pseudo-coloured and merged in Adobe Photoshop.

We performed DNA FISH using a bacterial artificial clone (BAC) clone (40D16) of ~65 kb as a probe, which extended from the 50-base-pair (bp) repeats to ESAG3 of 221ES, and was labelled with digoxigenin (Roche), under described conditions[28]. RNA FISH was performed using a probe containing the region between 18S and 28S of the rDNA (2.1-kb *Hin*dIII–*Eco*RI), as described[28], except that before fixation the cells were permeabilized in 0.5% Triton X-100, 300 mM sucrose, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM PIPES, 2 mM EGTA (pH 6.8) for 5 min at 4 °C.

GFP–LacI repressor tagging of the ES

We generated two tagging constructs to mark the active 221ES and the inactive 121ES. For targeting sequences, we used the upstream regions of both promoters: for the 121 ES, a 578-bp *Spe*I–*Hin*dIII fragment[29]; and for the 221ES, a 594-bp *Swa*I–*Hin*dIII fragment[7]. These targeting sequences were cloned upstream of the 256 *lac* operator repeats (10.1-kb *Hin*dIII–*Sma*I fragment from pAFS59; ref. 3), which were cloned upstream of a 379-bp *Hpa*I–*Psh*AI restriction fragment containing the ES promoter from the DES[29] driving a bleomycin selectable marker, with an unregulated untranslated region[30]. These two constructs were inserted by a single crossover upstream of the ES promoter, resulting in tandem repeat ES promoters that mimic the naturally occurring ES promoter duplication described in 30% of ESs[29].

In vitro culture of trypanosomes and DNA transfection procedures have been described[29,30]. Clonal cell lines were selected and subsequently grown in the presence of 40 μg ml^{-1} phleomycin (Sigma) for the active 221ES targeting experiment, and 1 μg ml^{-1} for the inactive 121ES targeting, as described[29,30]. Southern analysis confirmed the proper insertion of the cassette (data not shown). To express the GFP–LacI fusion in a tetracycline-dependent manner in the Lac-operator-tagged cell line, we used pLew100 (ref. 20) containing the streptomycin acetyl transferase (SAT) gene instead of bleomycin as a selectable marker, and the EGFP (Clonetech) fusion with the LacI (containing SV40nls) from pAFS144 (ref. 3) instead of luciferase. GFP–LacI was detected after induction using an anti-GFP antibody. The levels of GFP–LacI expression varied between cells even in the absence of chromosomal *lac* operator repeats, those cells with lower levels (~10%) were most useful for colocalization studies.

Transcription in permeabilized cells and DNase I treatment

Nascent RNA Br-UTP labelling was essentially done as described[17]. Trypanosomes were washed in medium without fetal calf serum and then in transcriptional buffer (50 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 10 μg ml^{-1} Leupeptin, 80 U ml^{-1} RNA inhibitor (Roche), 0.5 mM EGTA). Afterwards, Saponin (Calbiotech) was added to a final concentration of 50 μg ml^{-1} (10^7 cells ml^{-1}), and samples were mixed by inversion and incubated for 3 min at room temperature. Cells were washed and resuspended at the same density in transcription buffer, and α-amanitin (Sigma) was added to 100 μg ml^{-1}. We next added a mixture of ribonucleoside triphosphates (Roche; final concentration 2 mM ATP, 1 mM CTP, 1 mM GTP) and 0.5 mM Br-UTP (Sigma) to the cell suspension in transcription buffer. Samples were incubated at 33 °C in a water bath for 15 min, and the reaction was stopped by fixing the cells with methanol at −20 °C. Br-UTP-labelled RNA was detected using a monoclonal anti-BrdUTP (Biocell). Nineteen per cent of the nuclei showed an additional extranucleolar BrUTP focus. The double-labelling experiments showed either closely associated (partially overlapping) or co-localized, but not separate individual signals, except when the inactive 121ES was tagged (Fig. 4e). The DNase I experiment was carried out using the transgenic cell line with the *lac* operator in the 221ES (same as in Fig. 4d), and expression of the GFP–Lac repressor was achieved by inducing cells for 16 h with 1 μg ml^{-1} tetracycline. The DNase I (GibcoBRL) treatment (1,270 U ml^{-1} for 10 min at room temperature in 10 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM DTT) was performed on cells fixed with methanol as above. After the cells were washed three times for 15 min in PBS/0.5% Tween 20, the simultaneous immunofluorescence detection of pol I and GFP was carried out as above. No DNA was detected in the nuclei after this treatment, as revealed by the lack of staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). In the control sample (induced cells without DNase I treatment), about 20% of nuclei showed the GFP dot.

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1. Cross, G. A. M. Identification, purification and properties of variant-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology* **71,** 393–417 (1975).
2. Borst, P. & Ulbert, S. Control of VSG gene expression sites. *Mol. Biochem. Parasitol.* **114,** 17–27 (2001).
3. Robinett, C. C. *et al.* *In vivo* localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. *J. Cell Biol.* **135,** 1685–700 (1996).
4. Pays, E., Lips, S., Nolan, D., Vanhamme, L. & Perez-Morga, D. The VSG expression sites of *Trypanosoma brucei*: multipurpose tools for the adaptation of the parasite to mammalian hosts. *Mol. Biochem. Parasitol.* **114,** 1–16 (2001).
5. Bitter, W., Gerrits, H., Kieft, R. & Borst, P. The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*. *Nature* **391,** 499–502 (1998).
6. Pays, E. *et al.* The genes and transcripts of an antigen expression site from *T. brucei*. *Cell* **57,** 835–845 (1989).
7. Zomerdijk, J. C. B. M. *et al.* The promoter for a variant surface glycoprotein gene expression site in *Trypanosoma brucei*. *EMBO J.* **9,** 2791–2801 (1990).
8. Borst, P. & Chaves, I. Mono-allelic expression of genes in simple eukaryotes. *Trends Genet.* **15,** 95–96 (1999).
9. Robinson, N. P., Burman, N., Melville, S. E. & Barry, J. D. Predominance of duplicative VSG gene conversion in antigenic variation in African trypanosomes. *Mol. Cell. Biol.* **19,** 5839–5846 (1999).
10. Roditi, I. *et al.* Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of *Trypanosoma brucei*. *J. Cell Biol.* **108,** 737–746 (1989).
11. Kooter, J. M. & Borst, P. Alpha-amanitin-insensitive transcription of variant surface glycoprotein genes provides further evidence for discontinuous transcription in trypanosomes. *Nucleic Acids Res.* **12,** 9457–9472 (1984).
12. Rudenko, G., Bishop, D., Gottesdiener, K. & Van der Ploeg, L. H. T. Alpha-amanitin resistant transcription of protein coding genes in insect and bloodstream form *Trypanosoma brucei*. *EMBO J.* **8,** 4259–4263 (1989).
13. Rudenko, G., Lee, M. G.-S. & Van der Ploeg, L. H. T. The PARP and VSG genes of *Trypanosoma brucei* do not resemble RNA polymerase II transcription units in sensitivity to Sarkosyl in nuclear run-on assays. *Nucleic Acids Res.* **20,** 303–306 (1992).
14. Laufer, G., Schaaf, G., Bollgonn, S. & Günzl, A. *In vitro* analysis of alpha-amanitin-resistant transcription from the rRNA, procyclic acidic repetitive protein, and variant surface glycoprotein gene promoters in *Trypanosoma brucei*. *Mol. Cell. Biol.* **19,** 5466–5473 (1999).
15. Scheer, U. & Hock, R. Structure and function of the nucleolus. *Curr. Opin. Cell Biol.* **11,** 385–390 (1999).
16. Hartshorne, T. & Agabian, N. RNA B is the major nucleolar trimethylguanosine-capped small nuclear RNA associated with fibrillarin and pre-rRNAs in *Trypanosoma brucei*. *Mol. Cell. Biol.* **13,** 144–154 (1993).
17. Pombo, A. *et al.* Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III. *EMBO J.* **18,** 2241–2253 (1999).
18. Zomerdijk, J. C. B. M., Kieft, R. & Borst, P. Efficient production of functional mRNA mediated by RNA polymerase I in *Trypanosoma brucei*. *Nature* **353,** 772–775 (1991).
19. Chaves, I. *et al.* Subnuclear localization of the active variant surface glycoprotein gene expression site in *Trypanosoma brucei*. *Proc. Natl Acad. Sci. USA* **95,** 12328–12333 (1998).
20. Wirtz, E., Leal, S., Ochatt, C. & Cross, G. A. M. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **99,** 89–101 (1999).
21. Chaves, I., Rudenko, G., Dirks-Mulder, A., Cross, M. & Borst, P. Control of variant surface glycoprotein gene-expression sites in *Trypanosoma brucei*. *EMBO J.* **18,** 4846–4855 (1999).
22. Vanhamme, L. *et al.* Differential RNA elongation controls the variant surface glycoprotein gene expression sites of *Trypanosoma brucei*. *Mol. Microbiol.* **36,** 328–340 (2000).
23. Navarro, M., Cross, G. A. & Wirtz, E. *Trypanosoma brucei* variant surface glycoprotein regulation involves coupled activation/inactivation and chromatin remodeling of expression sites. *EMBO J.* **18,** 2265–2272 (1999).
24. Grosveld, F. Activation by locus control regions? *Curr. Opin. Genet. Dev.* **9,** 152–157 (1999).
25. Cook, P. R. The organization of replication and transcription. *Science* **284,** 1790–1795 (1999).
26. Ohlsson, R., Tycko, B. & Sapienza, C. Monoallelic expression: 'there can only be one'. *Trends Genet.* **14,** 435–438 (1998).
27. Matthews, K. R. & Gull, K. Evidence for an interplay between cell cycle progression and the initiation of differentiation between life cycle forms of African trypanosomes. *J. Cell Biol.* **125,** 1147–1156 (1994).
28. Ersfeld, K. & Gull, K. Partitioning of large and minichromosomes in *Trypanosoma brucei*. *Science* **276,** 611–614 (1997).
29. Navarro, M. & Cross, G. A. DNA rearrangements associated with multiple consecutive directed antigenic switches in *Trypanosoma brucei*. *Mol. Cell. Biol.* **16,** 3615–3625 (1996).
30. Navarro, M. & Cross, G. A. *In situ* analysis of a variant surface glycoprotein expression-site promoter region in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **94,** 53–66 (1998).

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Thymus medulla consisting of epithelial islets each derived from a single progenitor

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The thymus is organized into medullary and cortical zones that support distinct stages of T-cell development. The formation of medulla and cortex compartments is thought to occur through invagination of an endodermal epithelial sheet into an ectodermal one at the third pharyngeal pouch and cleft, respectively[1–5]. Epithelial stem/progenitor cells have been proposed to be involved in thymus development[6,7], but evidence for their existence has been elusive. We have constructed chimaeric mice by injecting embryonic stem (ES) cells into blastocysts using ES cells and blastocysts differing in their major histocompatibility complex (MHC) type. Here we show that the MHC class-II-positive medullary epithelium in these chimaeras is composed of cell clusters, most of which derive from either embryonic stem cell or blastocyst, but not mixed, origin. Thus, the medulla comprises individual epithelial 'islets' each arising from a single progenitor. One thymic lobe has about 300 medullary areas that originate from as few as 900 progenitors. Islet formation can be recapitulated after implantation of 'reaggregated fetal thymic organs'[8] into mice, which shows that medullary 'stem' cells retain their potential until at least day 16.5 in fetal development. Thus, medulla–cortex compartmentalization is established by formation of medullary islets from single progenitors.

To search for signs of thymic epithelial progenitor cell activity in thymus epithelium, we generated chimaeric mice by injecting ES cells (either CBA (MHC class II, I-A^k)) or (BALB/c (I-A^d)) into MHC-mismatched blastocysts (C57BL/6 (I-A^b)) (Fig. 1a). Because MHC class II molecules are highly expressed on medullary epithelium[9], we used the intense expression of class II molecules, together with cytokeratin which is expressed by thymic epithelium[10], to trace the origin of the medullary epithelium to either the ES cell or the blastocyst. A total of 103 CBA–C57BL/6 and 91 BALB/c–C57BL/6 chimaeras were generated. In all chimaeras, the contribution of ES cell versus blastocyst-derived tissue was analysed in skin (an ectodermal tissue), muscle (a mesodermal tissue) and liver (an endodermal tissue) to include tissues from all three germ layers (Fig. 1a). The relative contribution of ES cells and blastocysts to adult tissues was determined for each chimaera by amplifying a microsatellite marker yielding polymerase chain reaction (PCR) fragments specific for ES or blastocyst genomic DNA. Of 103 CBA–C57BL/6 chimaeras, 48 showed ES cell contribution to all three analysed tissues, 12 to two out of three tissues, 6 to only one of three tissues, and 37 showed no ES cell contribution. We used this analysis to select chimaeras with roughly equal contributions of ES cell and blastocyst ('balanced chimaeras') to increase the probability that both ES- and blastocyst-derived tissues would contribute to the thymus epithelium in ontogeny.

Thymi of mice with balanced chimaerism were analysed further by three-colour-histology to compare the expression of I-A^k, I-A^b and cytokeratin as a pan-thymic epithelial marker[10]. The location of medullary areas in thymic sections was identified by monoclonal