MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Dec. 2010, p. 552–569 Vol. 74, No. 4
1092-2172/10/$12.00 doi:10.1128/MMBR.00024-10

Cell Biology of the Trypanosome Genome

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INTRODUCTION

Trypanosomes are a group of unicellular protozoan species which include organisms causing devastating diseases around the world. *Trypanosoma brucei* is responsible for human African trypanosomiasis (HAT) (also called sleeping sickness) in humans and the wasting disease nagana in livestock in sub-Saharan Africa. Recent surveys estimate the annual burden of HAT to be 70,000 cases, with more than a million disability-adjusted life years lost (53). Tsetse flies of the genus *Glossina* transmit *T. brucei* to the human bloodstream, where the parasite survives freely, escaping the host immune response by antigenic variation of the surface coat. In contrast, the parasites *Trypanosoma cruzi* and *Leishmania* species penetrate host cells upon infection and replicate intracellularly. *T. cruzi* is responsible for Chagas' disease in Central and South America, while 20 different *Leishmania* species infect humans in 88 countries worldwide, causing different debilitating to fatal diseases (36). The diseases caused by trypanosome parasites have been classified as neglected tropical diseases, as there are no vaccines and the chemotherapeutic treatments available are deficient due to drug resistance and toxicity (www.who.int/mediacenter/factsheets/fs259/en/).

The last decade has witnessed a revolution in our understanding of eukaryotic genomes through the completion of an increasing number of genome sequencing projects for diverse eukaryotes. These have included the trypanosomes *T. brucei*, *T. cruzi*, and *Leishmania major* (15, 47, 84). This primary genetic information, however, is only the first stage on a long path toward understanding how the genome actually functions in the individual living cell (128). For this deeper understanding, we must also unravel how the one-dimensional DNA sequence is arranged in the three-dimensional space of the nucleus, how the myriads of nuclear factors are spatially organized, and how they interact in order to create a functional architecture of the nucleus. This cell biology of the genome will enable us to comprehensively understand the expression and functionality of genetic information *in vivo* from a genome-wide perspective.

In this review, we take trypanosome primary genome sequence information and data from molecular cell biological and biochemical experiments on trypanosomes and integrate them with concepts of nuclear architecture developed from work on other eukaryotic models. In doing so, we focus pre-

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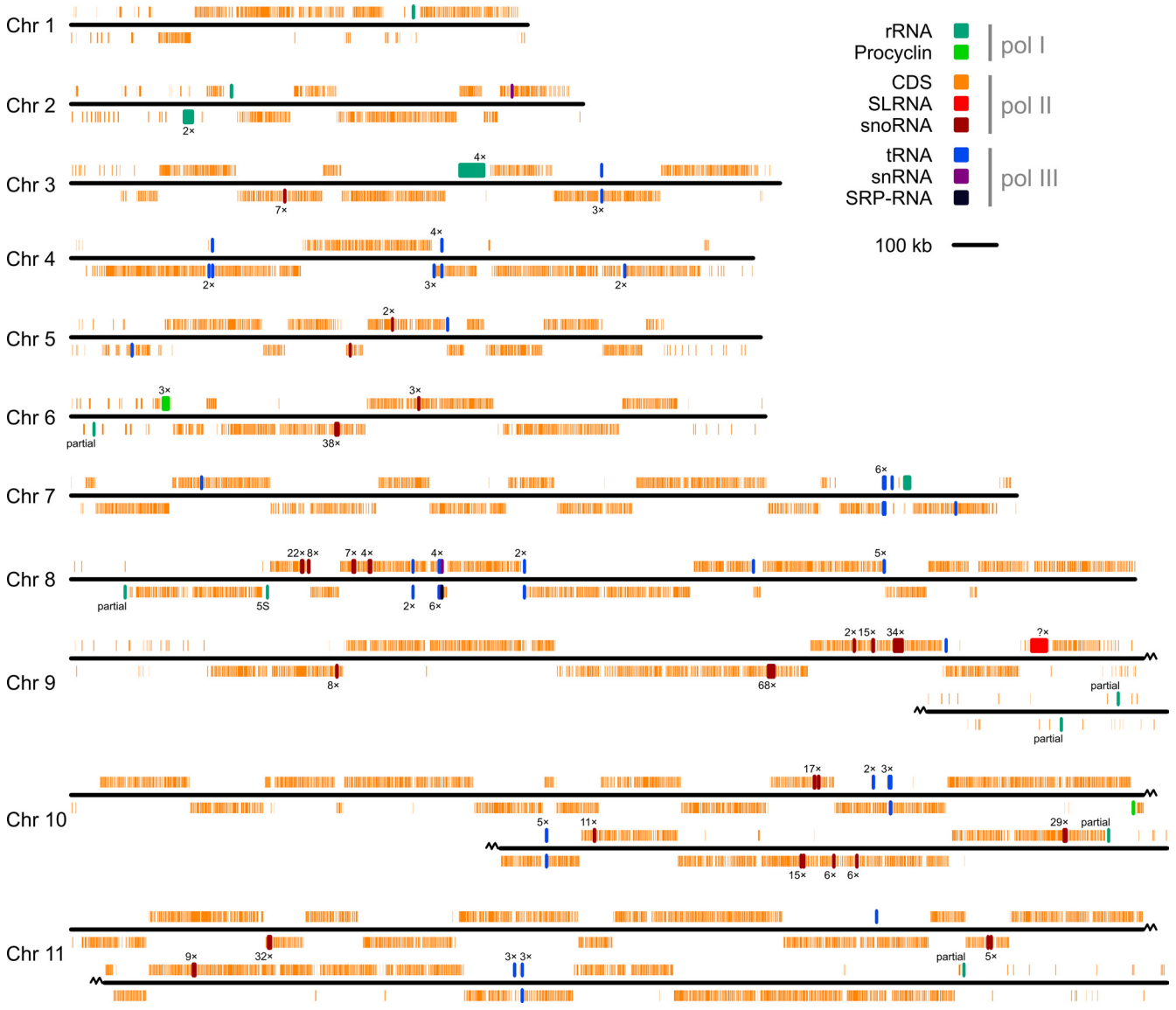

FIG. 1. Map of the *T. brucei* genome showing the organization of genes according to their class and transcribing polymerase. The map is based on the v4 annotation available at www.genedb.org. Colored bars indicate the positions and lengths of different genetic elements relative to the chromosome backbone (black line). Bars above the lines indicate transcription toward the right; bars below the lines indicate transcription toward the left. When a number of a similar elements are present in close proximity in the genome, this is indicated by $n\times$ next to bar (where n is the number of elements). Noncoding RNA genes have been given a minimum bar length to facilitate visualization. Only the largest assembled contig is represented for each chromosome. Chromosomes 9 to 11 have been split across two lines.

dominantly on the genome of *T. brucei*, for which it is now possible to draw on experimental data from both genomic approaches and a broad range of molecular and cellular work of relevance to the topic at hand. However, we also make comparisons to other trypanosomatid parasites, in particular *T. cruzi* and *L. major*, bringing data from these organisms into our discussions and highlighting similarities and differences where they exist. Through these data we argue that the concept of the cell biology of the genome is crucial to our understanding of the parasitology of trypanosomes but also that it can aid our understanding of the evolution of nuclear organization in eukaryotes.

LINEAR ORGANIZATION OF THE TRYPANOSOME GENOMES

Trypanosomes are diploid organisms. The "housekeeping" portion of the *T. brucei* genome is encompassed by 11 pairs of megabase-size chromosomes (MBCs) per genome, as shown in Fig. 1. *L. major* and *T. cruzi* harbor 36 and ~28 chromosome pairs, respectively, and it is likely that this more fragmented karyotype is more similar to the ancestral state (48). The *T. brucei* genome contains in addition ~100 minichromosomes (MCs) (30 to 150 kb) and ~5 intermediate-size chromosomes (ICs) (200 to 900 kb) (77). The MC and IC populations are

probably aneuploid, being inherited in an apparently non-Mendelian fashion (186) and showing a mixed ploidy for genetic markers (2). The genetic information of *T. brucei* totals ~35 Mb (per haploid genome) and is enclosed in a nucleus with a diameter of ~2.5 μm (51, 138). Several aspects of trypanosomal genome organization and nuclear gene expression regulation are unusual compared to those of other eukaryotic model organisms (24, 68, 117, 140). These can be summarized by looking individually at the genomic organization and expression of genes transcribed by the three classes of eukaryotic DNA-dependent RNA polymerases (pol) found in trypanosomes: pol I, pol II, and pol III (Fig. 1).

RNA Polymerase I Transcription Units

African trypanosome species harbor the only known pol I that transcribes not only rRNA genes but also protein-coding genes (69). Importantly, these protein-coding genes include those coding for the variable surface glycoprotein (VSG), which covers the surface of the bloodstream-form cell, and also procyclin, the major surface protein of the tsetse fly midgut stage (69). In this way, pol I transcription in African trypanosomes is intimately linked to both differentiation between life cycle stages and also survival in the mammalian host. As in other eukaryotes, trypanosomal pol I also transcribes the 45S rRNA precursor. The *T. brucei* genome has five clusters of 45S rRNA repeats per haploid genome (two on chromosome 2 and one each on chromosomes 1, 3, and 7) (Fig. 1), while there is just one cluster in the *L. major* genome on chromosome 27 (84). The procyclin genes are found internally on chromosomes 6 and 10 (Fig. 1), associated with other protein-coding genes in polycistronic units of in total three to six genes (103, 147).

The pol I transcription units containing the VSG genes are located in the subtelomeres of the MBCs and ICs. They are classified either as those of the initial life cycle stage transmitted by fly bite (metacyclic expression sites [MESs]) or those used later in parasitemia during proliferation in the host bloodstream (bloodstream expression sites [BESs]). Significantly, the genes coding for metacyclic VSG (MVSG), the surface coat protein of the metacyclic life cycle stage, are the only clear examples among protein-coding genes in *T. brucei* of monocistronic transcription (64, 65) and exclusive control at transcription initiation (62).

BESs are polycistronic units (88) of up to 60 kb in length, each containing a set of ES-associated genes (ESAGs) and the VSG gene itself at the 3′ end (16, 78). Only a single BES is expressed at any one time, and this monoallelic expression is crucial for the parasite's evasion of the host immune response by antigenic variation (20, 146, 148). In the bloodstream, parasites change their surface coat by stochastic switching of the expressed VSG. These cells will escape any specific immune response that the host has mounted against previously expressed VSGs, resulting in a persistent infection characterized by waves of parasitemia. Switching is the consequence of one of two distinct processes. First, the type of VSG expressed can be changed by homologous recombination of the VSG gene in the active BES with other silent VSG gene sequences throughout the *T. brucei* genome. About 2,000 VSG genes are located in arrays in the proximal subtelomeres of the MBCs, and up to 200 might be present at the telomeres of the numerous MCs (9, 15). Most genes in the arrays are pseudogenes, but these still contribute to immune evasion though the formation of productive VSG genes by mosaic formation (115). Second, by *in situ* switching, bloodstream-form cells silence the previous BES and activate one of the other BESs found elsewhere in the genome. Approximately 20 BESs are found in trypanosome genomes, with the number varying depending on the strain in question (78, 194).

pol I-driven transcription of protein-coding genes does not appear to be a normal feature of trypanosomatid organisms except for *T. brucei* and related African trypanosomes. However, pol I complexes from organisms such as *T. cruzi* and *Leishmania* species are competent to transcribe protein-coding genes, either as a result of the duplication of protein-coding genes into an rRNA locus (104) or through genetic modification (21, 54, 123, 192), suggesting that the mechanisms that allow for pol I-based transcription of protein-coding genes in *T. brucei* are not specific to this organism.

RNA Polymerase II Transcription Units

The trypanosome genomes are organized into large directional gene clusters (DGCs) containing protein-coding genes in a head-to-tail orientation (Fig. 1). The extent of this genome organization is very striking. Polycistronic transcription is, of course, also known in other eukaryotes, such as *Caenorhabditis elegans*. However, while the polycistrons of *C. elegans* contain ~15% of its protein-coding genes (19), protein-coding genes in trypanosomes are organized in DGCs on a genome-wide scale, and trypanosomatid DGCs contain tens of genes. In the *T. brucei* genome, there are 388 clusters of two or more unidirectional genes (77). However, many of these are small clusters which are unlikely to be true polycistronic units, and the number of polycistrons is believed to be many fewer (Fig. 1) (discussed further below). The regions that separate two DGCs are called strand switch regions (SSRs), and these can be either convergent or divergent (i.e., formed by DGCs with genes oriented either toward or away from the SSR, respectively). An extreme example of chromosome organization is the *L. major* Friedlin chromosome 1, which harbors only two, opposing DGCs transcribed toward the telomeres (130).

Transcription of the genes in the DGCs is polycistronic (83, 129), although the precise sequences specifying both transcription initiation and also termination for most polycistrons are still uncertain (68, 117). A pol II promoter for the spliced leader (SL) array has been characterized (61). There have also been reports that sequences from actin (13) and HSP70 (99) loci can act as promoters when placed upstream of exogenous reporter genes. However, it was not clear that either putative promoter was a true pol II initiation site in its endogenous position, and in subsequent analyses neither sequence gave significant activity (120). The mapping of a pol II promoter-like activity to a trypanosome SSR when placed into mammalian cells (11) might suggest that the promoters in *T. brucei* are similar to those in other eukaryotes, despite not yet being identifiable bioinformatically. However, interpreting the significance of such heterologous activity for endogenous SSR activity is difficult.

It is challenging to determine the exact extent of the tran-

scription units because of the cotranscriptional nature of the *trans*-splicing events. It seems intuitive that pol II transcription initiates in the SSRs and continues through the complete DGCs until it terminates either in the next SSR or at a specific site between two polycistrons arranged in a head-to-tail manner. In addition, transcription would have to initiate and terminate at units transcribed by different RNA polymerase classes, like tRNAs, located outside the SSRs. It could, however, also be envisioned that transcription starts promiscuously throughout a DGC with a directional bias, perhaps in addition to sites with enhanced promoter activity near the start of DGCs. In support of the former view, experiments on *L. major* Friedlin chromosomes 1 and 3 showed that transcription initiation and termination take place at SSRs (116, 118). However, the interpretation of the SSR as the unique site of transcription initiation remains controversial (68), since marker genes introduced into a copy of chromosome 1 lacking the divergent SSR can still be transcribed at levels sufficient for selection (43). The idea that transcription initiation and termination were occurring at the SSRs was strengthened by the finding that acetylated histones are concentrated at divergent SSRs (putative sites of transcription initiation) but are absent from convergent SSRs (putative sites of transcription termination) in *T. cruzi* (154). Moreover, recent data from genome-wide chromatin immunoprecipitation (ChIP-seq and ChIP-chip) studies of *T. brucei* and *Leishmania* show that certain histone modifications and transcription factors are specifically enriched at genome regions predicted to be associated with either transcription initiation or termination (162, 174, 191). In *T. brucei*, two histone modifications associated with open chromatin (H4K10ac and H3K4me3) and two histone variants (H2AZ and H2BV) are enriched in divergent SSRs and other putative pol II transcription start sites, whereas variants of H3 and H4 are found near the ends of DGCs (162, 191). Interestingly, the putative transcription start sites are also predicted from deep sequencing of mRNA, where they have a signature of increased levels of partially processed mRNA (92), which might be a by-product of proximal transcription initiation. In *Leishmania major*, acetyl-H3 is enriched at all divergent SSRs (as well as other locations that are predicted to be transcription start sites) in a manner that is more pronounced in dividing cells than in stationary-phase cells (174). Some, but not all, of these acetyl-H3 sites are also associated with upstream enrichment of transcription factors TATA-binding protein (TBP) and $SNAP_{50}$ (174).

Combining the data from ChIP-seq (162) and RNA-seq (92) experiments with the genome organization seen in Fig. 1 leads to the prediction that there are ~150 pol II polycistronic transcription units in the housekeeping regions of the *T. brucei* genome (i.e., the ~23 Mb of assembled sequence that excludes the largely pseudogenic subtelomeric regions). This implies that the mean polycistronic gene cluster is 153 kb in size and contains 55 genes. These numbers are very similar to the sizes predicted for *Leishmania major*, wherein there are estimated to be 184 polycistronic gene clusters (174) with a mean length 180 kb and 46 genes.

Processing of the polycistronic transcripts to monocistronic RNAs and capping are accomplished by cotranscriptional *trans*-splicing of a capped, short SL RNA exon to the 5′ untranslated region (5′UTR) and polyadenylation of the 3′UTR of every individual mRNA produced (80, 98, 144, 172). The processes of *trans*-splicing of the downstream mRNA and polyadenylation of the upstream mRNA are coupled (98). However, both are uncoupled from the direct influence of the transcribing polymerase (170), which is crucial for the ability of trypanosome pol I to produce functional mRNA. It is also means, of course, that SL RNA has to be produced in very high quantities to service all mRNA production in the cell. The 135 nucleotide (nt)-long SL RNA is encoded in an array of monocistronic tandem 1.4-kb repeats located on chromosome 9 (Fig. 1). These arrays are also transcribed by pol II (61, 93). In contrast to the case for pol II-transcribed protein-coding genes, a promoter sequence for the SL RNA genes has been identified (67, 74).

The requirement for the addition of the SL to trypanosome mRNA may provide a mechanism for modulation of mRNA levels between transcripts, since differences in the efficiency of *trans*-splicing would be expected to produce different levels of mRNA. Mutation analysis has established the importance of the conserved AG dinucleotide at the splice site as well as an upstream polypyrimindine tract for efficient *trans*-splicing of trypanosome mRNA (80, 119, 160, 164). Additionally, block mutations introduced downstream of the splice site can also affect *trans*-splicing of mRNA in *T. brucei* (105, 164). However, while these experiments have delimited many of the features necessary for efficient splicing, it is not clear if endogenous variation in intergenic regions between trypanosome genes causes differential *trans*-splicing efficiency or whether this is a mechanism used for mRNA level regulation.

Trypanosome pol II also appears to be required for the transcription of snoRNAs (68), which are involved in maturation of rRNAs, snRNAs, and spliced-leader RNA. These snoRNA genes are found in many clusters throughout the *T. brucei* MBCs (Fig. 1) (101, 131) and are transcribed as polycistrons (44). Given their position, embedded in DGCs and codirectional with transcription (Fig. 1), it seems likely that snoRNA gene transcription is concurrent with that of the surrounding protein-coding genes.

Interestingly, only a subset of homologs of the eukaryotic basal transcription initiation factors has so far been identified in trypanosomes (68, 84, 117). While it is uncertain whether some homologs escape their identification due to low sequence similarities or are indeed absent, it is already clear that trypanosomes employ a very divergent pol II transcription initiation complex.

RNA Polymerase III Transcription Units

The third generic eukaryotic polymerase, RNA polymerase III, transcribes a number of small, noncoding RNAs that act in translation (tRNAs and 5S rRNAs) or other cellular processes (7SL RNA). Trypanosome tRNAs are found widely interspersed between the DGCs on the MBCs (Fig. 1) (84). 5S rRNA genes are arranged in a single cluster on chromosome 8 of *T. brucei* (Fig. 1) but spread out into 11 clusters in the *L. major* haploid genome (84). Interestingly, pol III transcription of snRNAs and of the small cytoplasmic 7SL RNA in trypanosomes is dependent on regulatory elements that are located in an adjacent tRNA gene (Fig. 1) (132, 178).

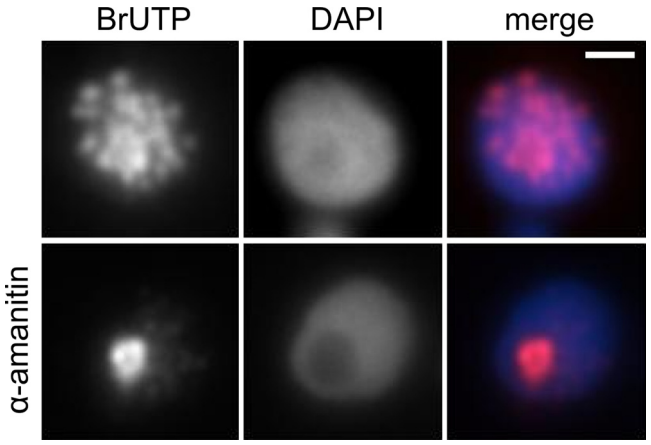

FIG. 2. Compartmentalization of transcription in the trypanosome nucleus. Images show the incorporation of BrUTP into saponin-permeabilized nuclei in the absence (top row) or presence (bottom row) of α-amanitin, which inhibits transcription by pol II and pol III. A stain of the nuclear DNA (DAPI) is also shown. Images courtesy of Eva Gluenz (University of Oxford, United Kingdom).

COMPARTMENTALIZATION OF THE NUCLEUS

A number of general concepts have emerged from studies on the architecture of the eukaryote nucleus, mostly on nuclei of vertebrate model organisms (55, 94, 127, 128, 168). Paramount among these is the finding that nuclear functions such as transcription and replication are not homogeneously distributed throughout the nuclear space but are compartmentalized. Compartmentalization of the nucleoplasm allows for specialization in function of particular regions of the nucleus, separating conflicting processes as well as increasing the effective concentration of specific *trans*-acting factors at their points of action. Labeling of trypanosome cells by incorporation with BrUTP or BrdUTP shows that the concept of compartmentalization is also true for transcription (Fig. 2) (3, 41, 134) and replication (45, 138, 190) in the trypanosome nucleus. With both methods, incorporation of the labeled nucleotide is stronger in specific regions of the nucleus. However, the replication machinery must visit every part of the genome during S phase. Moreover, the structure of the trypanosome genome (Fig. 1) shows that most parts of this also are parts of the DGCs and therefore most likely transcribed. These features have important consequences for the organization of the nucleus.

Models of Nuclear Compartmentalization

As for many other aspects of nuclear and cellular architecture, a crucial question arises as to whether the compartmentalization of transcription is determined by a prebuilt, underlying structure or is based on self-organization as a consequence of transcription itself (125, 128). Deterministic models hypothesize that structural components inside the nucleus assemble into a scaffold that is subsequently utilized by transcriptional processes that are, as a consequence, compartmentalized. This requires transcriptional information to be "hard-wired" into the structure of the nucleus irrespective of active processes. In contrast, self-organizing models suggest that transcriptional processes organize themselves into compartmentalized structures as a result of their own activity and without the need for predefined structures. According to this view, the distribution of transcription that we detect in the nucleus is the consequence of the sum of transcriptional events taking place (32, 125). Evidence from model systems is arguably more supportive overall for self-organization than for deterministic models (128). For example, disturbance of nuclear lamins (structural proteins of the peripheral lamina) has a comparatively small impact on the nuclear architecture, in disagreement with a deterministic model (167, 171). Conversely, interference with almost any active nuclear processes leads to rapid alterations in architecture (95), while ectopic expression of rRNA leads to the generation of micronucleoli, in support of a self-organizing principle underlying the organelle (89).

The organization of transcription and replication in discrete foci in mammalian nuclei, in combination with other evidence, has led to the model of transcription and replication factories (31). In this model, many RNA polymerase complexes are clustered in a transcription factory in which they interactively work on different templates that are pulled through the factory by transcription itself. Following these ideas, it can be envisioned how genes that are widely distributed on the linear chromosomes but transcribed by the same enzymatic complexes clustered in transcription factories. This would, in turn, greatly influence the three-dimensional organization of the chromosomes in the nucleus. In *Saccharomyces cerevisiae*, in addition to the positioning of the pol I-transcribed rRNA genes in the nucleolus, pol III-transcribed tRNA and 5S rRNA genes are also clustered in a transcription-dependent manner (71, 175).

Looking at the linear organization of the trypanosome chromosomes (Fig. 1), it is immediately clear that the transcription units of pol I, pol II, and pol III are distributed across several sites (nearly ubiquitously in the case of pol II). However, the pol I and pol II complexes themselves are highly enriched at specific sites within the nucleus, as exemplified in Fig. 3. Analogous clustering of pol III-transcribed genes would necessarily put significant constraint on the positioning of the MBCs in the nucleus, including the formation of large loops. However, unlike pol I and pol II, pol III is distributed into several discrete clusters in the *T. brucei* nucleus (Fig. 3, pol III), suggestive of a wide spatial distribution of pol III-transcribed genes. Similarly, fluorescent *in situ* hybridization (FISH) to detect the 5S rRNA locus in *T. brucei* showed that the two alleles are not clustered and are also not always associated with the nucleolus (49). Therefore, while there is evidence for the clustering of pol I and pol II transcription units in the nucleus (discussed further below), there is as yet no evidence for a major impact of clustering of pol III transcription units on the nuclear organization of trypanosomes. Whether this is also the case in *L. major*, with its 11 widely distributed 5S rRNA loci, remains to be seen.

The Nucleolus

A nuclear compartment is a macroscopic region within the nucleus that is morphologically and/or functionally distinct from its surrounding (127). The most distinctive nuclear compartment is the nucleolus, the site of ribosome biogenesis

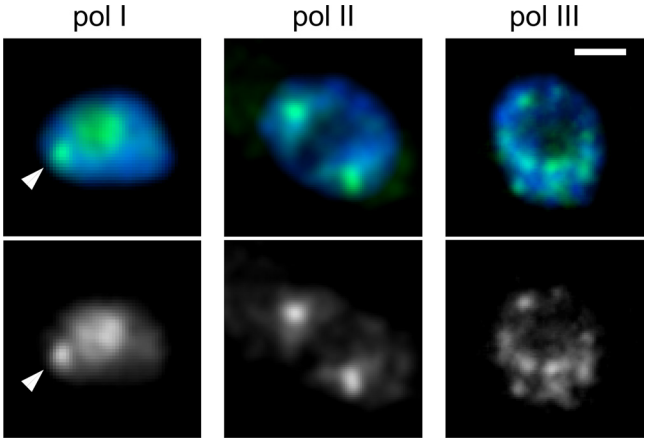

FIG. 3. Distribution of subunits of pol I, pol II and pol III in the *T. brucei* nucleus. (pol I) Native fluorescence of RPA2, the second-largest subunit of pol I, N-terminally tagged in bloodstream-form cells with the TY epitope and yellow fluorescent protein (YFP). Cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS). (pol II) Largest subunit of pol II detected by immunofluorescence with an anti-RPB1 polyclonal antibody (a gift from V. Bellofatto, New Jersey Medical School). Cell were fixed in 4% formaldehyde and 5% acetic acid in PBS. (pol III) Native fluorescence of C-terminally YFP-TY-tagged RPC1, largest subunit of pol III. Cells were fixed in 3% formaldehyde in PBS. In the overlay images, the YFP signal is pseudocolored in green and the DAPI signal in blue. Bar, 1 μm.

(153). The 45S ribosomal repeats cluster in the nucleolus. Pre-rRNA is synthesized and processed in the nucleolus, and the 40S and 60S complexes are assembled there. In animal and plant somatic cells, a tripartite substructure of the nucleolus can be seen by electron microscopy (EM) (152). The fibrillar center most likely stores inactive rRNA genes (153). The surrounding dense fibrillar component is electron dense due to a high concentration of ribonucleoproteins (RNPs). Early processing of rRNA is attributed to this area. The exact location of rRNA transcription is disputed but is considered to be in the fibrillar center, the dense fibrillar component, and/or the interface of these compartments (173). Later processing and ribosome subunit assembly take place in the granular component. It has been proposed, somewhat controversially, that this tripartite structure is not found outside the amniotes and that the vast majority of eukaryotes have a bipartite nucleolus, consisting of only a fibrillar and a granular compartment (153, 173). rRNA transcription is proposed to occur in the fibrillar compartment of these bipartite nucleoli (173).

T. brucei has a single nucleolus of 0.5 to 1 μm in diameter (51). Analysis of the trypanosome interphase nucleus revealed a bipartite organization of the nucleolus into a fibrillar and a granular component (106, 138). The existence of two substructures becomes even more evident when cells are treated with detergent prior to fixation (137). At the light microscope level, rRNA FISH and marker gene integration showed that the ribosomal repeats are tightly clustered within the *T. brucei* nucleolus (26, 134). More specifically, ribosomal genes, the largest subunit of pol I, and transcriptional activity all concentrate at the periphery of the nucleolus (96, 135). In addition, there is evidence that assembly of signal recognition particles, which contain 7SL RNA, also takes place in the trypanosome nucleolus (109) as it does in the mammalian nucleolus (150). Immunofluorescence of nucleolar proteins also indicates that the trypanosome nucleolus is itself further compartmentalized (91). *T. brucei* Nopp140 (TbNopp140) occupies subcompartments within the nucleolus and is not homogeneously distributed throughout this compartment. This becomes even more evident upon RNA interference (RNAi)-mediated knockdown of NoLP, when the Nopp140-containing subcompartments move further apart. NoLP itself localizes to a perinucleolar ring, and a similar pattern was observed with an anti-NOPP44/46 antibody (87, 143). These nucleolar subcompartments and those of ribosomal genes, pol I, and other proteins have not so far been correlated with the ultrastructural compartments identified by electron microscopy.

The nucleolus is a prime candidate for being a largely self-organized structure. Its structural integrity depends on both pol I transcription of rRNA genes and the interactivity of the ribosomal components (153). Knockouts and temperature-sensitive mutants of essential pol I subunits in yeast lead to nucleolar breakdown despite rRNA transcription by pol II (136). In trypanosomes, the nucleolus acquires an abnormal substructure upon RNAi-mediated knockdown of the pol I-specific subunit RPB5z (37), with abnormal nucleoli showing either an unusually heterogeneous density or an electron-dense ring. Interestingly, the decrease in transcriptional activity upon transformation from a proliferative form to a nonproliferative, infectious form of *T. cruzi* is also accompanied by dispersal of the nucleolus (46). Furthermore, depletion of TOR1 kinase in *T. brucei* results in dispersal of the nucleolus and also pol I (8), possibly as a consequence of pol I transcription inhibition. These observations are all consistent with a model of self-organization for the trypanosome nucleolus.

Extranucleolar Bodies in Trypanosomes

The expression site body. Procyclin genes, along with the 45S ribosomal genes, are transcribed by pol I associated with the nucleolus (96) (discussed below). Transcription of the active bloodstream expression site by pol I, however, does not take place in the nucleolar compartment (25, 26). Instead, it occurs in a pol I compartment that is molecularly and functionally distinct from the nucleolus, the expression site body (ESB) (Fig. 3, pol I) (134). The ESB does not contain the nucleolar marker protein fibrillarin, and its integrity is DNase resistant. It harbors only the active BES; inactive BESs are distributed elsewhere in the nucleoplasm (26, 134). When cells are forced to rapidly switch between expression of two BESs due to selection for double drug resistance, the two selected BESs are found close together in the nucleus in what is believed to be a natural intermediate of the *in situ* switching process (25). These observations support the notion of a privileged location in the nucleus that can be occupied by only one BES at any one time (20, 134). This is also consistent with the fact that it was not possible to obtain cells that switch between three BESs when forced by drug selection (179), since intermediates involving three BESs are not a prediction of the model.

The existence of the ESB raises two important and interconnected questions: (i) how the singularity of the ESB is achieved and (ii) whether the ESB is a deterministic structure

or assembles in a self-organized fashion. In other words, is the ESB prebuilt independently and does it then accept only one BES for active transcription, or is the monoallelic choice made by a formation of the ESB on the concomitantly activating BES? A possible scenario is that an enhancer-like DNA element is required for ESB formation (135). The monoallelic expression of odorant receptors in the olfactory sensory neurons of the mouse is proposed to be the consequence of activation by a *cis*-acting DNA element combined with a negative feedback of the odorant receptor's gene product on the other genes (161). One could also envision an essential protein component of the ESB that builds up the ESB in an autoreinforcing process and that is quantitatively limited so that only a single ESB can be built. Both the DNA enhancer model and the limiting-protein model could be part of either a deterministic or a self-organized process. However, in contrast to the olfactory neurons of the mouse, bloodstream-form trypanosomes are actively engaged in the cell cycle and have to inherit the monoallelic choice of VSG gene through mitosis (134). Transcriptional activity also has to be inherited by the two sister BESs during replication. It is difficult to explain this with a deterministic model. How would it be possible to ensure that both sister BESs are incorporated into prebuilt ESB structures? With a model of a self-organized ESB, however, it can more readily be seen how inheritance of transcriptional status at replication would lead to two daughter ESBs following sister chromatid separation at mitosis. In agreement with this model, a partial reduction in the levels of the cohesin subunit TbSCC1 leads to the production of two ESBs in post-S-phase cells, presumably due to premature separation of the chromatids carrying the active ES (97). However, the full inheritance of transcriptional status does not appear to be immediate at replication, since reduction of chromatid cohesion also encourages VSG expression site switching (97). Derepression of BESs by knockdown of the telomeric protein RAP1 also leads to more than one extranucleolar pol I focus (193), suggesting a role for telomeric chromatin in maintenance of the singularity of the ESB. It is not clear, however, whether these additional foci contain BESs, whether they are transcriptionally active, and therefore whether they can be classified as full ESBs.

The ESB is probably essential not only for monoallelic expression but also for full-length BES expression in general. The BES transcription unit is much longer than a rRNA repeat, and its transcription is coupled to pre-mRNA processing. It is therefore likely that the ESB contains RNA processing and elongation factors which are not required for rRNA transcription (148). If the active BES was transcribed by pol I at the same site as the 45S rRNA precursor (the nucleolus), it is possible that the concentration of these factors might not be sufficient for full-length transcription. The ESB may thus be required for molecular crowding of these components with pol I in order to ensure efficient ES expression. Considering that it transcribes just a single template, the concentration of pol I inside the ESB appears to be quite large compared to that in the nucleolus by immunofluorescence against pol I subunits (Fig. 3, pol I). The organization of the polymerase complexes within the ESB is unknown. Still, it is fascinating to picture the pol I enzymes in the ESB clustered with the right spacing in order to ensure an optimal polymerase loading of the active BES.

The ESB is a trypanosome-specific nuclear body with great importance for antigenic variation. Despite efforts through proteomic and bioinformatic approaches, factors specific for the ESB have not yet been found. A detailed molecular characterization of the ESB is a clear target for advancing our understanding of monoallelic expression and protein-coding gene expression by pol I.

Sites of SL RNA transcription and processing. Every mRNA produced by a trypanosome cell must be processed by the addition of the SL RNA miniexon to the 5′ end. *trans*-splicing with SL RNA requires the availability in the cell of large quantities of spliced leader transcripts, all of which are generated by pol II-driven transcription from a single (diploid) genomic tandem-repeat locus. It is therefore of little surprise that the largest subunit of pol II is highly concentrated at the genomic SL RNA loci (Fig. 3, pol II) (41, 180). While in *T. cruzi* epimastigotes and *L. tarentolae* predominantly only one SL RNA focus was observed (41, 79), two separate foci were detected in most G_1 cells in *T. brucei*, consistent with the diploidy of the SL RNA repeat locus (25, 180). A single SL RNA transcription spot might be the consequence of pairing of the two alleles induced by active transcription (32). This would be in agreement with the observations that (i) the pol II focus disappears and SL RNA genes disperse in trypomastigote *T. cruzi*, which has decreased transcriptional activity, and (ii) the pol II focus is lost after treatment with transcription-inhibiting drugs (41). The latter data also suggest that the high concentrations of pol II surrounding the SL RNA loci are at least partly self-organized and a consequence of transcriptional activity itself rather than a predetermined nuclear structure.

SL RNA transcripts concentrate in the nucleus in an area that colocalizes with the snRNP protein SmE and SLA1 RNA (18, 82, 177), an RNA that guides modification of the SL RNA (102). This suggests the existence of a spatially defined SL RNP assembly compartment or SL RNP factory in the nucleoplasm (177). It is presently debated, however, whether the SL RNA maturation and SL RNP assembly also involve a cytoplasmic step (18, 114, 195).

Given the high concentration of pol II at foci in the nucleus, it is possible that other genes, in particular protein-coding genes in DGCs, might also be transcribed by the polymerase in these SL RNA transcription foci. Since the DGCs themselves occupy most of the nucleus, this would require that chromosomes be dragged through a pol II focus in a transcription factory-like manner. The fact that the tubulin gene loci do not colocalize with the SL RNA loci in *T. cruzi* (41) does not entirely rule out this idea, as the pass rate of a specific locus could be low. However, labeling of active transcription in permeabilized nuclei by incorporation of BrUTP shows a much broader distribution of transcriptional foci outside the nucleolus that just those associated with the SL RNA (Fig. 2), although it must be noted that this represents transcriptional activities of both pol II and pol III.

Other nuclear bodies. Cajal bodies are involved in the biogenesis of nuclear RNPs in plant and animal cells (94, 139). Some nucleolar proteins, snoRNPs, and snRNPs colocalize in them. Coilin and the nucleolar protein Nopp140 are charac-

teristic markers of Cajal bodies (139). The failure to detect a coilin homolog in trypanosomes and the exclusive localization of TbNopp140 to the nucleolus strongly suggest that bodies truly homologous to the Cajal bodies in animals are not present in trypanosomes (91). It has therefore been proposed that nuclear assembly of RNPs takes place in analogous bodies such as those found in yeast (82). One candidate for such a compartment was identified cytochemically (106) and molecularly as an SLA1 RNA-containing nuclear area that did not colocalize with SL RNA (82).

Speckles are nuclear bodies containing many pre-mRNA splicing factors, including snRNPs and signal recognition proteins, which form in the interchromatin space of mammalian nuclei (95). There are only two known examples of *cis*-spliced introns in *T. brucei* (84, 113). A further two possible intronic genes were identified by sequence analysis (84), but these do not appear to be spliced in the most recent RNA-seq analyses (92, 163). In contrast, *trans*-splicing occurs for every mature mRNA. Both processes appear to require the spliceosome, and all snRNAs and many spliceosomal proteins are present in the trypanosome genomes (84). Evidence exists for a speckle-like organization of splicing components in the *T. brucei* nucleus: the splicing factor Prp31 has a localization reminiscent of mammalian speckles (100), SmE is also found in a speckle-like distribution in addition to the SL RNP factory, and specific spliceosomal Sm protein 2-1 and U2 snRNA have been found to colocalize in speckle-like structures (177). Also, Lsm proteins, which are involved in RNA processing and turnover, were observed in the nucleoplasm of *T. brucei*, concentrated in an area near the nucleolus (176). Thus, it seems that molecular components involved in splicing do compartmentalize into specific nuclear areas in trypanosomes.

Promyelocytic leukemia protein (PML) bodies are nuclear bodies of particular medical interest, since a translocation resulting in a fusion of their marker protein PML and the retinoic acid receptor-α leads to disruption of the PML bodies in promyelocytic leukemia patients (94, 168). Although they are believed to be linked to transcriptional regulation, the exact functions of PML bodies remain unclear (127). It is therefore difficult to interpret the significance of the fact that both PML bodies and the PML protein have not been described in trypanosomes.

DIFFERENTIAL GENE REGULATION AND POSITIONING IN TRYPANOSOMES

As outlined above, nuclear architecture is not necessarily a fixed feature of the cell, responding to a range of perturbations and treatments. During their life cycles, trypanosomes undergo significant changes to their morphology and metabolism. Trypanosomes such as *T. brucei*, *T. cruzi*, and *Leishmania* spp. that are parasites of vertebrates must differentiate rapidly and specifically upon transmission from vector to host and back. These changes clearly demand a developmental regulation of differential gene expression. Moreover, even proliferating cells of a single life cycle stage require different proteins through the cell cycle. The organization of the trypanosome genome and nucleus places particular constraint on these processes.

Differential Gene Expression through the Life Cycle

Several studies have now addressed the issue of global regulation of genes in different life cycle stages of trypanosome species, using microarrays (for examples, see references 39, 86, 122, 158, and 159) and, recently, through the use of RNA-seq and digital gene expression technologies (163, 183). The most extensively studied stage-specifically regulated genes are those encoding the major surface proteins of the bloodstream form and fly midgut stage of *T. brucei*, VSG and the procyclin. These genes are regulated at several levels, including at the level of transcription initiation from their pol I promoters, transcription elongation, and mRNA stability dictated by sequence elements in their 3′UTRs (14, 24, 157, 181). In sharp contrast to this situation, the vast majority of the pol II-transcribed genome shows little change in expression levels between life cycle stages in *T. brucei* (39, 86, 163, 183). Considering the absence of evidence for control of transcription initiation of these pol II-transcribed protein-coding genes and the fact that DGCs contain genes required at different life cycle stages, it is likely that the regulation of most trypanosomal gene expression takes place at posttranscriptional levels (29, 117). In agreement with this hypothesis, all DGCs produce similar mean levels of mRNA for the contained genes, and there is little clustering of genes according to life cycle regulation (Fig. 4). Some rapidly evolving gene families, such as expression site-associated genes, tend to cluster in subtelomeres and SSRs (15), probably because of weaker purifying selection in these regions, and this in turn results in a greater tendency for differential regulation at the very ends of DGCs (mostly upregulation in the bloodstream form) (183) (Fig. 4B). However, the majority of genes in polycistronic gene clusters appear to not to be ordered by transcriptional regulation. For example, the pol II-transcribed genes *PGKB* and *PGKC* (encoding two isoforms of phosphoglycerate kinase) are directly adjacent within a DGC but oppositely regulated: *PGKB* mRNA is upregulated in procyclic cells, whereas *PGKC* is more abundant in the bloodstream-form parasite (60). Given this level of resolution in gene regulation within the DGCs, it is very unlikely that nuclear positioning or chromatin status is involved in the differential regulation of most pol II-transcribed genes in trypanosomes.

As for several other genes (see reference 28), regulation of the *PGKC* gene has been found to be linked to sequence elements in its 3′UTR (30). Trypanosome genomes encode a large number of proteins with predicted RNA-binding domains, and it has been hypothesized that these bind to the UTRs of mRNAs and influence protein-coding gene expression levels (34). An important example is ZPF3, which binds sequence specifically to the 3′UTRs of mRNAs of some isoforms of the procyclin genes and regulates their translation (185). In *T. cruzi*, UBP-1 binds to U-rich elements in the 3′UTR of SMUG mRNAs, which results in their destabilization (40). Despite being relatively nonspecific RNA-binding proteins in *T. brucei*, UBP-1 and UBP-2 affect the levels of mRNAs from an array of transmembrane protein-encoding genes (73). Thus, while it is clear that posttranscriptional regulation of mRNAs occurs in trypanosomes, it is not yet understood in depth how the interaction of RNA-binding proteins with elements in the 3′UTRs mechanistically results in a particular stability of the mRNA.

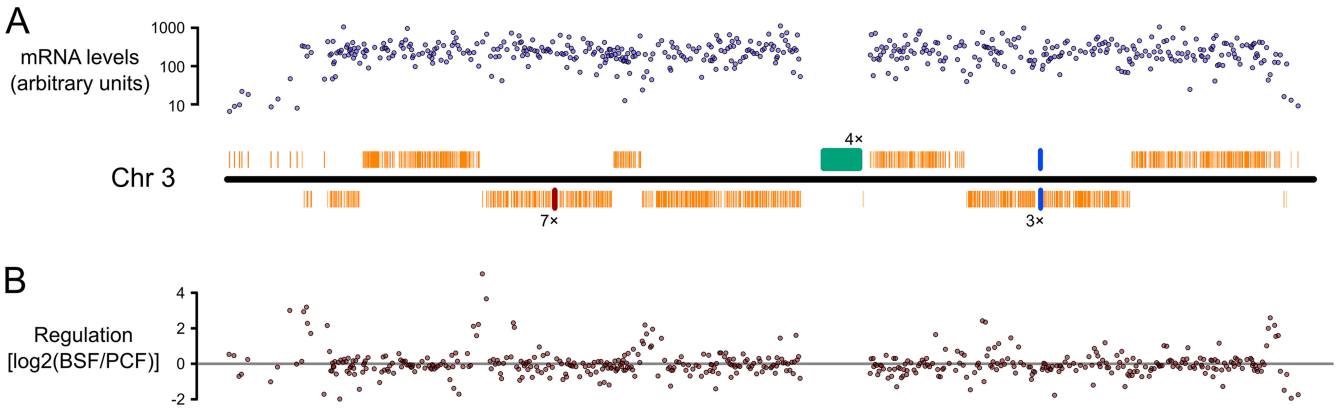

FIG. 4. General lack of gene expression regulation at the level of pol II transcription units in trypanosomes. A map of *T. brucei* chromosome 3 is shown with measurements of total mRNA levels (in cultured bloodstream-form cells) (A) and the fold change in mRNA levels between cultured bloodstream- and procyclic-form cells (B). The map is based on the v4 genome annotation available from GeneDB (www.genedb.org). mRNA measurements are taken from a study by Jensen et al. (86) which has been made publicly available under accession number GSE18049 in the GEO database (www.ncbi.nlm.nih.gov/geo/).

Differential Gene Expression through the Cell Cycle

Another potential need for differential regulation of gene expression presents itself during the cell cycle. However, few examples of cell cycle-regulated transcripts in trypanosomes are known. It is unclear whether this is a consequence of a dearth of cell cycle-dependent control or the lack of established robust synchronization methods for trypanosomes. In *T. brucei*, histone mRNAs fluctuate during the cell cycle, with a peak during S phase (50), and in *Leishmania infantum*, UTRs from histone H2A are sufficient to confer cell cycle regulation to an unrelated coding sequence (1). The levels of mRNA from a Puf domain-containing protein, PUF9, and three mRNAs to which it binds (*LIGKA*, *PNT1*, and *PNT2*) are also regulated in a cell cycle-dependent manner (4). Cell cycle-regulated fluctuations of mRNA levels have been studied perhaps most intensively in *Crithidia fasciculata*, an organism more closely related to *Leishmania* than to *Trypanosoma* species (169). Octamer sequence elements in the 5′UTRs of a topoisomerase gene (145) and a replication protein A gene (*RPA1*) (23) are sufficient for cell cycle-dependent changes in transcript level (112). These octamers are specifically bound by cycling sequence-binding proteins (CSBPs) (111, 112). However, such cycling elements do not necessarily have to be restricted to the 5′UTR, since the elements in the 5′UTR of *RPA1* also function when being moved to the 3′UTR (23). Furthermore, octamers necessary for cycling were also found in the 3′UTR and the downstream intergenic region of a kinetoplast histone H1-like DNA-binding protein gene (*KAP3*) (5). Interestingly, as the downstream intergenic octamer is sufficient for period cycling of the *KAP3* transcript, mechanisms that regulate the transcript levels are probably functioning cotranscriptionally at the nuclear site where the gene is transcribed (5). In *Trypanosoma* and *Leishmania* genomes, however, such octamer sequences have not been identified.

Differential Gene Positioning

A clear correlation between the expression levels of some genes and their nuclear positioning has been described for some eukaryotes (94). However, the number of examples is limited, and universal rules for the relationship of gene expression and positioning are not yet established (142). For instance, the yeast nuclear periphery contains, on the one hand, a repressive compartment containing heterochromatic telomere clusters. On the other hand, the nuclear pore complexes are associated with active genes (126). Furthermore, the peripheral or internal localization of some, but not all, genes in lymphocyte development can be correlated with their repression or activation, respectively (142). The mechanisms of gene expression regulation in trypanosomes described above discourage the idea of the global importance of such positioning effects, since posttranscriptional events such as proteins binding to mRNA UTRs are likely to be spatially independent from the position of the transcribed gene. Moreover, polycistronic transcription and the close chromosomal organization of the pol II-transcribed protein-coding genes in DGCs make a correlation of gene positioning and expression seem highly unlikely. The coding sequences for the differentially expressed *PGK* genes, for example, are just 350 bp apart on the *T. brucei* chromosome 1. With differentially regulated units this close together, even genes regulated by cotranscriptional events, as indicated for *C. fasciculata KAP3*, are unlikely to be associated with particular nuclear positions.

A clear example of a correlation between gene activity and nuclear positioning, however, is provided by the active BES (135). The downregulation of active transcription from the expressed BES as bloodstream-form cells transform into procyclic cells is accompanied by a repositioning of the BES promoter to the nuclear periphery (96). Additionally, it appears that the promoter subsequently adopts a less accessible chromatin state. This is in agreement with levels of BES expression being significantly reduced in procyclic cells. In fully transformed procyclic cells, the inactive BESs also are found at the nuclear periphery (135). It is interesting that the inactive BESs are not at the periphery in bloodstream-form cells. It is possible that they remain more centrally located to be immediately available for *in situ* switching or recombination events.

It is unclear whether an equivalent repositioning takes place

for the procyclin loci (135). The *GPEET-PAG3* locus resides in the periphery of the nucleolus in procyclic cells, colocalizing with pol I (96). Failure to detect *lacI* sites integrated into the *GPEET-PAG3* locus in bloodstream-form cells, which was accessible in procyclic cells, might indicate that its chromatin is less accessible than that in procyclic cells. A BES-like repositioning of the procyclin loci might be less likely as they are chromosome internal. The *GPEET* polycistronic unit is less than 10 kb away from the gene coding for a cathepsin B-like protease essential for bloodstream-form survival in culture (110). The mRNA of this protein is more abundant in bloodstream-form cells than in procyclic cells. Even more striking is the fact that the haptoglobin hemoglobin receptor involved in heme uptake by the bloodstream-form trypanosome is encoded only ~4 kb upstream of the *EP3*/*GPEET* polycistrons (15, 182). It is therefore clear that any silencing of the procyclin genes would have to be carefully insulated from the surrounding genome in order not to interfere with the regulation of adjacent protein-coding genes.

ORGANIZATION OF CHROMATIN IN THE TRYPANOSOME NUCLEUS

The DNA in the eukaryotic nucleus is compacted into chromatin, which is spatially subdivided into domains of euchromatin and heterochromatin. Euchromatin and heterochromatin can be defined either morphologically or functionally, and it is important to clearly specify which of these definitions is being used. Morphologically, the less electron-dense regions of the nucleus are referred to as euchromatin and the more electron-dense, condensed areas as heterochromatin. Functionally, euchromatin is seen as transcriptionally active and well accessible, while heterochromatin is less accessible and transcriptionally silent. In contrast to a commonly held view, the morphology of chromatin and its functional classification do not seem to correlate globally, at least in mammalian cells (127). Instead, euchromatin and heterochromatin appear to reflect gene-rich and gene-poor parts of the genome, respectively, largely independent of the transcriptional state of the genes.

Trypanosome Chromatin

Different techniques indicate the presence of chromatin with distinguishable morphologies in the trypanosome nucleus. Electron microscopy of interphase nuclei of *T. brucei* and *T. cruzi* cells shows that there are clear areas of differential electron density in the nucleoplasm (Fig. 5) (46, 138). Electron-dense material was found to cluster at the nuclear periphery but to be absent from the proximity of nuclear pores. These nuclear pores are distributed approximately evenly across the nuclear surface, and it has been suggested on the basis of analysis of isolated nuclei that there might be as many as 200 to 300 per nucleus (155), although freeze fracture replicas of bloodstream-stage *T. brucei* cells suggests only ~120 pores per nucleus (C. Gadelha, N. Severs, and K. Gull, unpublished data), consistent with the density observed for *T. cruzi* nuclei (52).

Staining trypanosome nuclei with 4′,6-diamidino-2-phenylindole (DAPI) results in a complex pattern of areas with higher and lower signal intensities. The less intensely stained

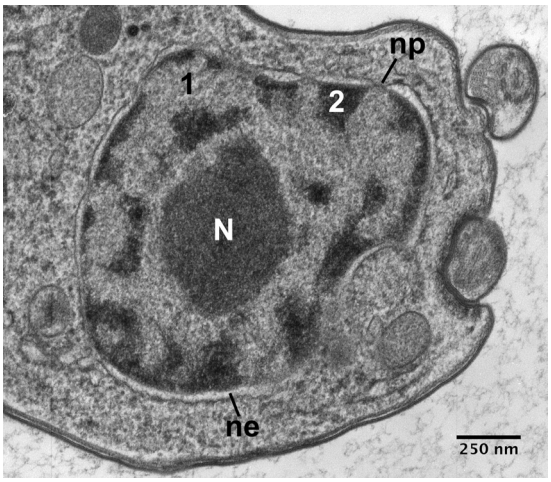

FIG. 5. Ultrastructural compartmentalization of the trypanosome nucleolus. The image shows a transmission electron micrograph of a thin section though a bloodstream-form *T. brucei* nucleus. The most obvious nuclear compartment is the nucleolus (N) in the center of the micrograph, but there is also a clear distinction between electron-lucent (1, "euchromatin") and electron-dense (2, "heterochromatin") regions in the nucleoplasm. The nucleus is surrounded by the nuclear envelop (ne), in which can be seen nuclear pores (np) which are always juxtaposed with electron-lucent nucleoplasm. The sample was prepared by high-pressure freezing, followed by freeze substitution with 1% glutaraldehyde and 2% uranyl acetate in acetone and then 2% osmium tetroxide and 2% uranyl acetate in acetone and embedding in resin. Image courtesy of Catarina Gadelha (University of Cambridge, United Kingdom).

areas correlate with the localization of TFIIS transcription elongation factors (180), suggesting that these more DNA-poor regions are more active transcriptionally, as might be predicted. One should, however, be cautious to refer to these areas as euchromatin or heterochromatin. It is not clear whether, for instance, a low DAPI signal indicates loosely packed chromatin, DNA that is less accessible to DAPI, or interchromatin space. Also, it has to be considered that trypanosomes are not likely to have as much functional heterochromatin as mammals. Trypanosomes have a much higher gene density, and due to the ubiquitous distribution of DGCs, most of the MBCs are believed to be transcribed at very similar rates (the exception being the silent VSG gene arrays). Because of the transcription of noncoregulated protein-coding genes in the same polycistrons (like *PGKB* and *PGKC*), regulation of gene activity at the level of chromatin status seems unlikely. Therefore, on the *T. brucei* MBCs, the telomeric repeats and the subtelomeres (excluding the active MES in metacyclic cells and the active BES in bloodstream-form cells) are the only extended regions with the potential to be heterochromatic from a functional point of view. In addition, the 100 or so MCs (consisting largely of a palindrome of 177-bp repeats [189]) are known to be silent and are likely to be heterochromatic. The electron-dense chromatin at the periphery of the *T. brucei* interphase nucleus has therefore been associated with MCs (138). While telomeric repeats and MCs might well be packed into constitutive heterochromatin, the VSG expression sites in the distal subtelomeres might represent examples of facultative heterochromatin in trypanosomes, as

their transcriptional status changes upon *in situ* switching and throughout the life cycle.

Trypanosome chromatin is generally more loosely packed than mammalian chromatin (12). Trypanosome chromosomes also do not condense into discrete visible chromosomes during mitosis (184). However, the chromatin of trypanosomes is not the same in all stages of the life cycle. Its condensation is different in procyclic and bloodstream-form *T. brucei* (12). In *T. cruzi*, electron-dense chromatin is located mostly in small areas at the nuclear periphery in epimastigotes and extends throughout the volume of the nucleus upon transformation to the trypomastigote form (46). The building blocks of eukaryotic chromatin are nucleosomes, i.e., DNA wrapped around a core histone octamer built from two copies each of H2A, H2B, H3, and H4 (or variant forms). The entry and exit site of the DNA is stabilized by one copy of histone H1. Several genes encoding all of these histone types have also been identified in trypanosomes (76, 84). The N-terminal sequences of H2A, H2B, H3, and H4 are, however, divergent from the yeast and vertebrate sequences, and trypanosome H1 corresponds to just the C-terminal end of human H1 (57). Interestingly, in contrast to the nucleosome core histones, *T. cruzi* H1 seems to be concentrated at the nucleolus in interphase nuclei (70).

Different functional states of chromatin correlate closely with particular posttranslational histone modifications and histone variants. They have been implicated in trypanosome transcription initiation and termination (154, 162, 174, 191), cell cycle progression (70, 85), and telomeric silencing (90). Acetylated histone H4 is distributed in a punctate pattern in the *T. brucei* and *T. cruzi* nucleoplasm (133, 162, 165). In the case of H4K10ac, this most likely reflects the association with pol II transcription start sites (162). Histone variants H2AZ and H2BV are also found at repetitive DNA, such as telomeric repeats and MC DNA (108). These variants do not colocalize with sites of active transcription labeled by BrU incorporation. Chromatin immunoprecipitation combined with sequencing, however, has shown that H2AZ and H2BV, like H4K10ac (and acetyl-H3 in *Leishmania* [174]), are associated with putative transcription start sites (162). In contrast, two trypanosomatid-specific variant histones, H3V and H4V, are enriched at potential transcription termination sites in *T. brucei* (162). H3V is also enriched at telomeric repeats and subtelomeres (107, 162). Since the MCs provide the majority of the telomeres in *T. brucei*, it is not surprising that the H3V distribution in the nucleus largely colocalizes with the MCs (107). The genomic and nuclear distribution of H4V is broader than that of H3V, but it appears to be particularly present in chromatin near the nuclear envelope (162).

The chromatin at the nucleolar periphery displays a high concentration of proteins involved in a variety of nuclear processes. First, pol I transcription takes place at the periphery of the nucleolus (96). Other nucleolar proteins with peripheral localization are NoLP (Fig. 6) (91) and NOPP44/46 (87, 143). Transcription elongation factor TFIIS proteins (Fig. 6) (180), the exosome component RRP4 (72), and the cap hypermethylase Tgs1 (156) are not restricted to but are clearly concentrated at the perinucleolar region. An important point, however, is that except for pol I, it is not clear whether the perinucleolar region is a site of function or merely storage of these proteins. Furthermore, overlay of NoLP and TFIIS2-1 proteins showed that the perinucleolar region is multilayered, as the NoLP and TFIIS signals do not completely colocalize (Fig. 6). At present, it is not clear how many layers there are and which of them are morphologically or functionally inside or outside the nucleolus itself.

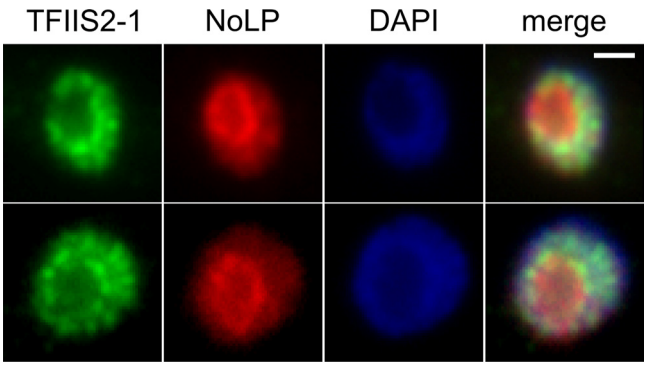

FIG. 6. Concentration of nuclear proteins in the nucleolar periphery. Procyclic cells fixed in 3% formaldehyde are shown. N-terminally TY-YFP-tagged TFIIS2-1 was detected with an anti-TY antibody (BB2, pseudocolored in green) and Nopp140-like protein with an anti-Nopp140-like antibody (pseudocolored in red). DAPI is pseudocolored in blue. Bar, 1 μm. The two proteins concentrate in different parts of the nucleolar periphery, resulting in an incomplete colocalization of signals.

Chromosomal Positioning

Although the arrangement of chromosomes inside the mammalian nucleus is variable to a certain extent from cell to cell, their organization is nonrandom. Radial chromosome location in the nucleus has been found to correlate with gene density and chromosome size (128). Moreover, the relative positioning of chromosomes is nonrandom. Most obviously, 45S rRNA loci on different chromosomes cluster in the nucleolus, but other examples, such as the nonrandom association of chromosomes 12, 14, and 15 in mouse lymphocytes, have been described (127). For trypanosomes, data on the relative positioning of chromosomes are scarce. Interestingly, while the four 45S rRNA locus-containing *T. brucei* chromosomes (per haploid genome) will most likely be clustered in the nucleolus, only one locus is found in the *L. major* haploid genome. Thus, only the two homologs of this chromosome need be clustered in the nucleolus in *L. major*, putting less constraint on chromosome organization. In addition to the chromosomes containing the 45S loci, in *T. brucei* the MCs have a nonrandom distribution in the nucleus. In interphase of procyclic cells, they localize asymmetrically to the nuclear periphery (27, 49). This may be a consequence of their tight clustering during mitosis being passively retained through the rest of the cell cycle, but it cannot be excluded that MCs are actively clustered during interphase.

In mammals, chromosomes show tissue-specific spatial arrangements (141), and chromosome positioning might also be different between trypanosome life cycle stages. MCs and telomeres, which are found at the periphery in procyclic cells, are spread throughout the nucleus in bloodstream-form cells (27, 149). Despite lacking homologous pol I expression sites of pathogenicity-associated genes, a similar repositioning of telo-

meres was observed in *L. major* (42). Here, clusters of telomeres were found to be more centrally distributed in the nuclei of the intracellular stage in humans, compared to the midgut stage in the insect vector. The developmental repositioning of MBC telomeres and telomere-associated genes in *T. brucei* discussed above will most certainly also influence the subnuclear organization of MBC subtelomeres that harbor the silent VSG gene reservoirs. It is not known to what extent homologous recombination between VSG gene sequences also takes place in the procyclic form. It is, however, certain that these recombination events in the bloodstream form are critical for the survival of the parasite in the host. One possibility is that BESs, subtelomeres, and MC telomeres need to be in much closer spatial proximity to the active BES in the bloodstream-form nucleus than in the procyclic nucleus in order to allow efficient homologous recombination to take place.

Chromosome Territories

Mammalian chromosomes are not spread out through the entire nucleus but take up a spatially limited area, their respective chromosome territory (33). In yeast, however, such territories are absent (121). As genetic loci can change their position in the nucleus by 1 μm in several minutes (127), yeast nuclei are possibly too small to stably build up chromosome territories. The same, of course, is likely to apply to the trypanosome nucleus, which has not been extensively studied in this context. In mammals, transcriptional activity of some genes is correlated with their location on loops that exit the chromosome territories (55). These loops are proposed to bring genes into contact with transcriptional factories or juxtapose coregulated genes. Because of the high gene density of trypanosome MBCs and their polycistronic transcription and posttranscriptional mode of regulation, such models are unlikely to be extensible from mammals to trypanosomes.

NUCLEAR DYNAMICS AND NUCLEAR SCAFFOLDS

The Dynamic Interphase Nucleus

Despite high levels of complexity and organization, the interphase nucleus is very dynamic: transcription factors explore the nuclear volume (124), but also nuclear bodies and gene loci move (127). In fact, the dynamic nature of the nuclear structures and constant exchange of their components are a requirement for the models of self-organization (125). The study of intracellular dynamics in most life cycle stages of trypanosomes is complicated by their cellular motility, since movement of the cell resulting from flagellar beating severely restricts the use of live-cell microscopy to follow dynamic processes. However, the highly variable patterns of some nuclear proteins in fixed cells are suggestive of dynamics in trypanosome nuclei. Considering the small size of nuclei in trypanosomes, it is clear that Brownian motion will have a more global impact on nuclear components than in larger nuclei. If the intranuclear mobility of genetic loci of 1 μm in several minutes (127) is also true for trypanosomes, then small chromosomes such as the MCs must be actively clustered for their nonrandom distribution in the interphase nucleus (49). Evidence for a rearrangement of chromosomes in interphase comes from *T. cruzi* (45). Satellite DNA repeats are suggested to reposition from a random distribution in G_1 to the nuclear periphery at the onset of S phase, possibly because of association with a peripheral replication machinery.

Intranuclear mobility of genetic loci is, of course, critical for *in situ* switching of BESs if two expression sites are required to come close together to activate the switch (25). Such movement is also necessary for efficient homologous recombination of the VSG gene in the active BES with VSG gene sequences in other BESs or the silent reservoirs. Class I transcription factor A (CITFA) is a seven-subunit pol I transcription factor in *T. brucei* (22). One of these subunits is the dynein light chain LC8, and it has been hypothesized that it might facilitate the positioning of the BES unit (22). It has to be considered, however, that LC8 has been identified in genomes of organisms that lack dynein heavy chains and thus dynein function altogether, such as higher plants (187). Furthermore, it has been suggested that LC8 functions as a hub in protein networks and that its role as a dynein light chain is just one part of its functional repertoire (7).

Trypanosome Mitosis

Trypanosomes undergo a closed mitosis during which the nuclear envelope does not break down. In *T. brucei* procyclic and bloodstream-form cells, the nucleolus also always stays integral throughout division. The spindle microtubules converge at a small region of fibrous material at opposite poles of the nucleus, and the nucleolus thins and elongates simultaneously with the spindle and in close association with the microtubules (138, 184). One puzzling aspect of trypanosome mitosis is the disparity of kinetochore and chromosome numbers. This is particularly striking for *T. brucei*: despite the presence of ~125 chromosomes (~250 sister chromatids after S phase), only around eight kinetochores can be readily detected (138, 166). Since the MCs are stably inherited in mitosis (188, 196) and clearly associate with the central spindle during their segregation (49), a model of a bifunctional spindle has been suggested in which a kinetochore-independent segregation of MCs on the central spindle is kinetically different from the MBC segregation (51, 66). While MCs move in tight clusters to the spindle poles quite early in mitosis, loci on the MBCs such as 5S rRNA on chromosome 8 segregate later on (49). Also, upon knockdown of SMC3, a component of the cohesion complex involved in the regulated cohesion of sister chromatids, MBC segregation was impaired, while no immediate defects in MC segregation were observed (17).

The Nuclear Envelope and Structural Elements

How are the shape and integrity of the nucleus built up and maintained? In animals, lamina at the nuclear envelope is built up by intermediate filament proteins, the lamins (63). Genes homologous to animal lamins have not been identified in the genomes of plants and yeast, but the presence of a lamina-like structure has been suggested based on field emission scanning EM studies (63). Intermediate filament genes appear also to be absent from the trypanosome genomes (15). This raises the question as to whether the trypanosome nucleus possesses a lamina-like structure and, if so, what molecular components it

is made of. Preparations of nuclear pore complexes from procyclic *T. brucei* nuclei also contain lamina-like structures which contain the protein NUP-1 (155). NUP-1 is an ~350-kDa, repetitive protein that localizes to the inner face of the nuclear envelope (138, 155). It is so far the only putative structural component of a trypanosome nuclear lamina. RNAi-mediated knockdown of RAB23, a small GTPase that colocalizes with NUP-1, resulted in an enlarged nucleolus (38). These observations suggest the existence of a stabilizing structural component in the trypanosome nucleus. However, extensive proteomic analyses of the nuclear envelope and the nuclear pore complex-lamina fraction (35) will be required to enable a thorough understanding of a trypanosome lamina.

CONSTRAINT OF GENOME EVOLUTION THROUGH NUCLEAR ARCHITECTURE

Studies of the evolution of gene and genome sequences have focused mostly on changes in the linear DNA sequence and their effects on functionality of transcripts, coding potential, and gene expression. With the growing awareness that the chromosomes and nuclear functions are compartmentalized and nonrandomly organized, the question arises as to how much the spatial architecture of the nucleus influences the evolution of the genome sequence (6). This includes the influence of gene order on the chromosomes. Indeed, whole-genome analyses provide evidence for clustering of coexpressed genes in eukaryotic genomes (81). In trypanosomes, however, there does not seem to be extensive clustering of related genes. The only exceptions so far identified are a cluster of five genes involved in pyrimidine biosynthesis (58) and clustered paralogs of similar or identical gene copies. The latter are more likely the result of recent duplication events than of selective pressure for clustering. As gene expression regulation appears to be largely posttranscriptional, a clustering of coexpressed genes is perhaps not necessary in the trypanosomes. In spite of this, the trypanosome genomes are highly syntenic (48); 68% and 75% of all genes in *T. brucei* and *L. major*, respectively, have the same genomic context. This is remarkable considering that their ancestors split hundreds of million years ago and that their gene sequences are relatively divergent. It has been suggested that the DGCs act not only as promoters for transcription but also as origins of replication and that thus the codirectionality of transcription and replication is ensured (59). This would select against many chromosomal rearrangements in trypanosomes, as they would disturb this coordination of direction of these nuclear functions. However, if DGCs are reliant on a single upstream promoter and downstream termination site, then these factors provide a strong selective pressure for maintenance of the DGC without the need to invoke other factors, since only rearrangements that keep the promoter linked to the distant termination site (or reconstitute it from sequence elsewhere in the genome) would be permissible.

A striking feature of the *T. brucei* genome organization is the clustering of silent VSG gene sequences in the subtelomeres (9) and the distal subtelomeric position of the BESs. This extensive use of the subtelomeres for antigenic variation systems can be found in a variety of parasitic and pathogenic organisms (10). Since subtelomeres are genomic locations prone to ectopic recombination, this situation is thought to promote mixing of the VSG gene repertoire and also might provide insulation of the silent VSG gene array from transcriptional events on the central parts of the MBCs. Furthermore, genes in subtelomeres could be under the control of telomeric positioning effects, as suggested by evidence that depletion of telomeric protein RAP1 results in derepression of the silent BESs (193). The telomeric location of the expression sites might facilitate their developmental repositioning, as there is a global rearrangement of telomeres during the life cycle stages (149).

CONCLUSIONS AND OUTLOOK

Recent work on the biology of trypanosomes is beginning to unveil the links between the structure of the genome and the function of the nucleus. The synthesis of these two fields, to elucidate what has been termed the "cell biology of the genome," presents a great opportunity to improve our understanding of the mechanisms behind parasitism in these, and other, organisms. Here we propose that many of the concepts of nuclear architecture established through the study of mammalian cells can be usefully applied to the trypanosome nucleus. Nuclear functions, such as transcription and replication, are compartmentalized in the trypanosome nucleus, not homogeneously distributed throughout the nucleus. The most distinctive nuclear compartment, the nucleolus, is substructured into fibrillar and granular components in trypanosomes, as in the vast majority of eukaryotes, and there are indications of the existence of other nuclear bodies. Within the nucleoplasm, domains of different chromatin structure can be detected by either electron microscopy or intercalating DNA dyes. There is also good evidence for a correlation of the transcriptional status of the BESs and their positioning within the nucleus, demonstrating that not all regions with the nucleoplasm are functionally equivalent. Furthermore, in both the interphase and mitotic nucleus, there is a nonrandom arrangement of at least one class of chromosomes (the MCs).

Some aspects of nuclear organization in trypanosomes, however, are different from those in model systems. The ESB is an extranucleolar body harboring pol I-mediated transcription of protein-coding genes, which has so far not been found outside *T. brucei*. Furthermore, the majority of pol II is associated with one or two regions in the nucleus which are the sites of transcription of the SL RNA genes necessary for the processing of all other trypanosomal mRNA. Since transcription in trypanosomes is polycistronic, with the majority of gene expression regulation taking place posttranscriptionally, and genes are generally not clustered according to function or transcript abundance, it is highly unlikely that gene positioning within the nucleus is associated with regulation of the vast majority of pol II-transcribed protein-coding genes.

Although there has been great progress in our understanding of trypanosome genome biology, several outstanding questions remain. Key to some of these is the gap between molecular biology and ultrastructure. For example, which compartment of the nucleolus is responsible for pol I transcription, and how does the location of pol I relate to nucleolar structures such as fibrils? What molecular components define the perinucleolar region, and what cellular processes take place

there? Crucially for African trypanosome biology, how is the extranucleolar ESB assembled, and how does its structure relate to that of the other site of pol I transcription, the nucleolus? No ESB-specific factor has yet been identified, and we do not know how it is that the cell is able to so closely maintain a single ESB. Solving these questions is critical for understanding antigenic variation of *T. brucei* and will require the integration of electron microscopy with molecular techniques. Moreover, for all the trypanosomatids, the mechanisms and spatial organization of pol II transcription of protein-coding genes are also still unclear.

As with many parasites, the life cycle stages of trypanosomes differ dramatically in morphology and metabolism without any genetic change. Evidence exists for a global rearrangement of chromatin and chromosomes during these transformations, and the ultrastructure of the nucleus also changes. To date, we know very little of the extent of these rearrangements and the mechanisms behind them, but they demonstrate that nuclear architecture is not static. The study of the dynamics of the trypanosome nucleus is still in its infancy. The application of live-cell microscopy techniques such as photobleaching or photoactivation is complicated by the rapid motility of most trypanosome cells generated by flagellar beating (56). This problem must be circumvented by constraining cell movement (75, 151) or by the use of motility mutants or naturally immotile life cycle stages, such as the amastigote stage of *Leishmania* species. However, cell types such as the medically important bloodstream stages of African trypanosomes have yet to be effectively immobilized on the time scales necessary for such work (151).

Finally, as we are further able to integrate cell biological data and genome sequence, an opportunity arises for the future design of computational models of the organization of the trypanosome nucleus. These would provide a theoretical framework that could be used to explore the spatial organization of the trypanosome genome in the nucleus, as well as to generate hypotheses that could then be tested *in vivo*. Such a model, and the levels of understanding necessary to build it, must surely be one of the aims of the next few years of research into the cell biology of the trypanosome nucleus.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust. J.-P.D. was also supported by the EP Abraham Trust and the Studienstiftung des deutschen Volkes (German National Merit Foundation).

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