Chromosoma (2000) 108:501–513

CHROMOSOMA

Architecture of the *Trypanosoma brucei* nucleus during interphase and mitosis

Emmanuel Ogbadoyi, Klaus Ersfeld, Derrick Robinson, Trevor Sherwin, Keith Gull

School of Biological Science, 2.205 Stopford Building, University of Manchester, Manchester M13 9PT, UK

Received: 9 August 1999; in revised form: 15 October 1999 / Accepted: 10 November 1999

Abstract. The structural basis of mitosis, spindle organisation and chromosome segregation, in the unicellular parasite *Trypanosoma brucei* is poorly understood. Here, using immunocytochemistry, fluorescent in situ hybridisation and electron microscopy, we provide a detailed analysis of mitosis in this parasite. We describe the organisation of the mitotic spindle during different stages of mitosis, the complex ultrastructure of kinetochores and the identification of a potential spindle-organising centre in the mitotic nucleus. We investigate the dynamics of chromosome segregation using telomeric and chromosome-specific probes. We also discuss the problems involved in chromosome segregation in the light of the fact that the *T. brucei* karyotype has 22 chromosomes in the apparent presence of only eight ultrastructurally defined kinetochores.

Introduction

One of the most conserved elements in mitosis is the central role played by microtubules in the segregation of chromosomes (Kubai 1975; McIntosh and Koonce 1989). Although all eukaryotic nuclear genomes are faithfully segregated by the association of chromosomes with a mitotic spindle the details of this association and the subsequent segregation of homologues vary considerably among different organisms (Heath 1980). This is exemplified by the dinoflagellates, which assemble a spindle outside the nucleus and interact with the chromosomes through specialised structures in the nuclear envelope, which remains, as in many protists, intact during mitosis (Ris 1975). Other protists, such as some fungi, assemble a spindle inside the nucleus during the onset of mitosis (Ding et al. 1993; Winey et al. 1995). Although chromosome segregation in most, if not all, eukaryotes is dependent on the formation of a mitotic spindle, the structural basis of chromosome interaction with microtubules varies considerably (Goode 1975; Heath 1980). Interestingly, there seems to be no obvious pattern to the variation of DNA sequence requirements of centromeres or for the structure of kinetochores (Earnshaw 1994; Choo 1997).

The nuclear genome of *Trypanosoma brucei*, a flagellated unicellular parasite and the causative agent of African sleeping sickness, contains approximately 120 chromosomes (van der Ploeg et al. 1984; Ersfeld et al. 1999). Only 22 of these chromosomes are larger than 1 Mb and these 11 homologous pairs carry all actively transcribed genes. The exceptions to this are the variant surface glycoprotein (VSG) genes, which can also be transcribed from one of the 3–5 intermediate chromosomes of 200–500 kb in size. The organisation of the large chromosomes is very different from that of most higher eukaryotes. Genes are arranged in long polycistronic transcription units. No RNA polymerase II-specific promoters have been identified as yet, suggesting the presence of only very few transcriptional start points on each chromosome (Pays 1993). Both observations have recently been confirmed by the analysis of the complete sequence of chromosome I of the related parasite *Leishmania major* (Myler et al. 1999). The chromatin of trypanosomes appears less compact than in higher eukaryotes. Although the DNA is arranged in nucleosomes, they are less resistant to nuclease digestion and the histones are more sensitive to salt extraction (Hecker et al. 1994).

In addition to the large and intermediate chromosomes, *T. brucei* contains approximately 100 minichromosomes of only 50–150 kb in size (Sloof et al. 1983; Weiden et al. 1991). The major portion of these chromosomes consists mainly of a 177 bp repetitive sequence element. In their subtelomeric region, however, minichromosomes contain a silent copy of a VSG gene. It is therefore assumed that an important function of minichromosomes is to act as a reservoir for VSG genes, which can be transposed onto large chromosomes where

E. Ogbadoyi and K. Ersfeld contributed equally to this paper

Edited by: U. Scheer

Correspondence to: K. Ersfeld (e-mail: k.ersfeld@man.ac.uk)

they can be transcribed in one of ca. 20 potential expression sites.

There has been a recent resurgence of interest in the interphase nuclear architecture of *T. brucei*. This is due to work carried out in several laboratories that suggests possible links between gene activity and gene positioning within the nucleus. It has been observed that the activity of a single VSG expression site and the concomitant silencing of all other potential expression sites in bloodstream-form trypanosomes is likely not to be primarily regulated by *cis*-acting elements such as promoters, but by as yet unidentified factors. It has been argued that epigenetic effects, defined as independent of the primary DNA sequence but heritable over many generations, might play a role in gene regulation (Horn and Cross 1995, 1997; Rudenko et al. 1995; Biebinger et al. 1996). Such effects could involve the localisation of a particular gene at specific sites within the nucleus (Chaves et al. 1998). Similar phenomena are well documented in other organisms, e.g. telomeric or centromeric gene silencing in yeast or position effect variegation in *Drosophila* (Ekwall et al. 1995; Csink and Henikoff 1996).

The timing and overall light microscopic appearance of the mitotic spindle during the cell cycle of *T. brucei* have been described (Sasse and Gull 1988; Woodward and Gull 1990). More recently we have used fluorescent in situ hybridisation (FISH) and shown that both large and minichromosomes are segregated via interaction with the mitotic spindle but that the positioning of the latter during mitosis is distinct from that of the large chromosomes (Ersfeld and Gull 1997). This suggests that functionally different components of the spindle could be involved in the segregation of both classes of chromosomes (Gull et al. 1998). In most higher eukaryotes both the spindle and the interphase microtubule cytoskeleton are organised by ultrastructurally defined structures, commonly referred to as microtubule organising centres (MTOCs) (Pickett-Heaps 1969; Compton 1998). Microtubules are nucleated by MTOCs with a defined and uniform polarity. γ-Tubulin is a conserved component of MTOCs and is an essential component for the nucleation and stabilisation of the spindle. γ-Tubulin has been identified in *T. brucei* and co-localises with the poles of the spindle, suggesting, despite the absence of a discrete structural equivalent of a centrosome or spindle pole body, that the principles of microtubular organisation are similar to those described for other organisms (Scott et al. 1997).

However, this renewed interest in the nuclear molecular biology of trypanosomes requires a detailed knowledge of the high order interphase nuclear structure and mitosis. Therefore, we have extended previous studies of the nuclear ultrastructure and dynamics of *T. brucei* (Vickerman and Preston 1970). In order to provide a structural basis for a model of genome segregation and spindle organisation we analysed elements of the fine structure of the nucleus using electron microscopy, immunocytochemistry and in situ hybridisation.

Materials and methods

Cell cultures

Procyclic *T. brucei* 427 was propagated in SDM79 medium as described by Brun and Schönenberger (1979).

Generation of monoclonal antibodies

To prepare the antigen, cells were extracted with 1% Nonidet P-40 in PBS for 5 min on ice, centrifuged for 5 min at 13,000 *g*, and the insoluble fraction was re-extracted with 1 M urea in PBS. After centrifugation as above, the pellet was solubilised with 4 M urea in PBS. This fraction was mixed with an equal volume of Freund's complete adjuvant, emulsified by sonication and injected into the footpad of a Balb/c mouse. Injections were repeated twice at 5 day intervals using incomplete adjuvant. Fifteen days after the first injection the mouse was sacrificed and the poplietal lymph node isolated. A single-cell suspension was prepared by carefully grinding the lymph node between the frosted sections of two glass microscope slides. Fusion was carried out using standard polyethylene glycol-based techniques with P3X63-Ag8.653 myeloma cells. Selection was done in OptiMEM medium containing 5% fetal calf serum and hypoxanthine, aminopterin and thymidine (Gibco-BRL, UK). Supernatants were screened by immunofluorescence microscopy for antigens with a nuclear localisation. The monoclonal antibody (mAb) KMX, which preferentially recognises β-tubulin in the mitotic spindle was generated as described (Sasse and Gull 1988).

Immunofluorescence microscopy

Trypanosomes were allowed to settle on poly-L-lysine-coated slides and fixed with 3.6% formaldehyde in PBS for 10 min at room temperature. After a brief wash in PBS, cells were permeabilised with 0.1% Nonidet P-40 in PBS for 5 min at room temperature and washed three times in PBS, 5 min each time. Primary antibodies were applied as undiluted cell culture supernatants. The secondary antibody was an anti-mouse fluorescein isothiocyanate (FITC)-conjugate raised in rabbit (Dako, UK). All washes were done three times for 5 min each in PBS, 0.1% Tween 20. Cells were embedded in Vectashield (Vector Laboratories, UK) including 100 ng/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma, UK). Cells were analysed on a Leica DMRBE epifluorescence microscope and images captured using a cooled charge-coupled device camera (Photometrics, USA) and IPLab software (Signal Analytics, USA) on a Macintosh computer. Images were pseudocoloured and processed in Adobe Photoshop.

Transmission electron microscopy (TEM)

Whole cells were prepared according to Tooze (1985). Briefly, cells were fixed in 2% v/v glutaraldehyde, 2% w/v paraformaldehyde in 0.1 M sodium cacodylate buffer (SCB), pH 7.2, washed in SCB, post-fixed with 2% w/v osmium tetroxide in SCB, stained with 0.5% aqueous magnesium uranyl acetate, washed in SCB, dehydrated through an ethanol series and gradually embedded in Spurr's resin.

Detergent-extracted cells were prepared by simultaneously fixing and extracting the cells with a 1:1 mixture of the glutaraldehyde:paraformaldehyde fixative as described above and 0.2% Nonidet P-40 in PEM (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$) for 1 hour at room temperature. All other steps were identical to the procedure described above. For detailed visualisation of kinetochore structures, cells were extracted in 1% Nonidet P-40 in PEM for 5 min, then fixed in 4% glutaraldehyde in PEM for 1 h. All other steps were as described above. Blocks were sectioned at 50–70 nm thickness and sections were stained in 5% w/v

uranyl acetate in 1% acetic acid in water and Reynold's lead citrate. Sections were examined on a Philips 420 TEM.

For the immunolocalisation of incorporated 5-bromo-2'-deoxyuridine (BrdU), cells were labelled, processed for TEM analysis and immunolabelled as described (Robinson and Gull 1994). Briefly, cells were labelled for 6 h with 50 µM BrdU and deoxycytidine, respectively, to ensure the completion of at least one cell cycle and consequently one S-phase. Cells were then washed in PBS, fixed in 3.8% paraformaldehyde, 0.6% glutaraldehyde in PBS, dehydrated through a methanol series and embedded in Lowicryl K4 M at –35°C. Sections were denatured with 1.5 M HCl, washed, blocked in 1% BSA in PBS and processed for immunogold detection.

Fluorescent in situ hybridisation

Minichromosomes were detected as described using a biotinylated DNA probe (Ersfeld and Gull 1997). The tubulin locus located on chromosome I of *T. brucei* was detected by using DNA isolated from a P1 plasmid with a 60 kb insert containing the complete α/β-tubulin gene cluster (kindly provided by Dr. S Melville, Cambridge). Telomeres were detected by using the oligonucleotide $(TTAGGG)_5$ end-labelled with digoxigenin-ddUTP using terminal transferase (Roche Diagnostics). Hybridisation was done as described (Ersfeld and Gull 1997) except that the formamide concentration for the oligonucleotide-based detection was decreased to 40% in the hybridisation and washing buffer. Visualisation was done by either using Cy-3 conjugated ExtrAvidin (Sigma) for minichromosomes or sheep anti-digoxigenin Fab fragment (Roche Diagnostics) and FITC anti-sheep IgG (Jackson Laboratories, USA) for telomeric probes. Images were analysed, captured and processed as described for immunofluorescence microscopy.

Results

The interphase nucleus

The overall ultrastructural appearance of the *T. brucei* interphase nucleus is depicted in Fig. 1A. The nucleus has a diameter of approximately 2.5 µm. Areas of differential electron density within the nucleoplasm of interphase cells can be visualised and some of the more electron-dense areas are clustered in close proximity to the nuclear envelope; others are located throughout the nucleus. However, care should be taken in the interpretation of these different regions as hetero- or euchromatin. In contrast to mammalian cells in which less than 5% of the DNA is transcriptionally active at any time, the particular nature of chromosome structure, gene organisation and transcription in trypanosomes makes it likely that large sections of chromosomes are constitutively active, therefore preventing the formation of extensive regions of heterochromatin. Using (FISH) we show that the telomeric regions of the chromosomes are located in a peripheral location close to the nuclear envelope during interphase (Fig. 2A). The 100 or so minichromosomes of *T. brucei* possess telomeres identical to those on the 22 large chromosomes and therefore most of the signal is likely to originate from minichromosomes. Double labelling using a minichromosomal and a telomeric probe reveals a high degree of overlap of both signals (Fig. 2).

The nucleolus of approximately 1 µm in diameter remains intact during the mitotic cycle. Although the nucleolus of *T. brucei* is not as clearly structured into different components as in many higher eukaryotic cells, a

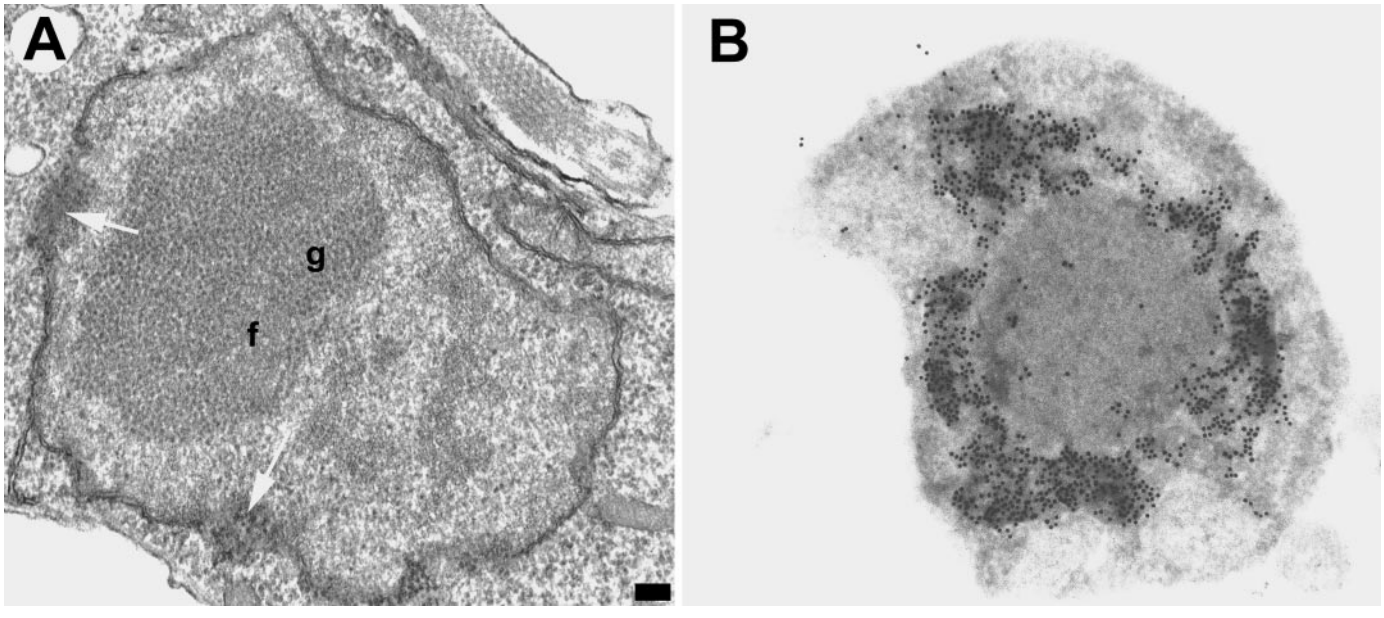

Fig. 1. A Transmission electron micrograph **(**TEM) of a transverse section of a *Trypanosoma brucei* cell, including the interphase nucleus. Note the differential staining of the nucleoplasm with patches of electron-dense material clustered along the nuclear envelope (*arrows*). The nucleolus is differentiated into areas of granular (*g*) and fibrillar (*f*) appearance. *Bar* represents 0.1 µm. **B** TEM of a transverse section through a whole cell grown in the presence of 5-bromo-2'-deoxyuridine (BrdU). Immunogold (10 nm gold) detection of BrdU incorporation into replicated DNA labels most areas of the nucleoplasm but only small clusters within the nucleolus

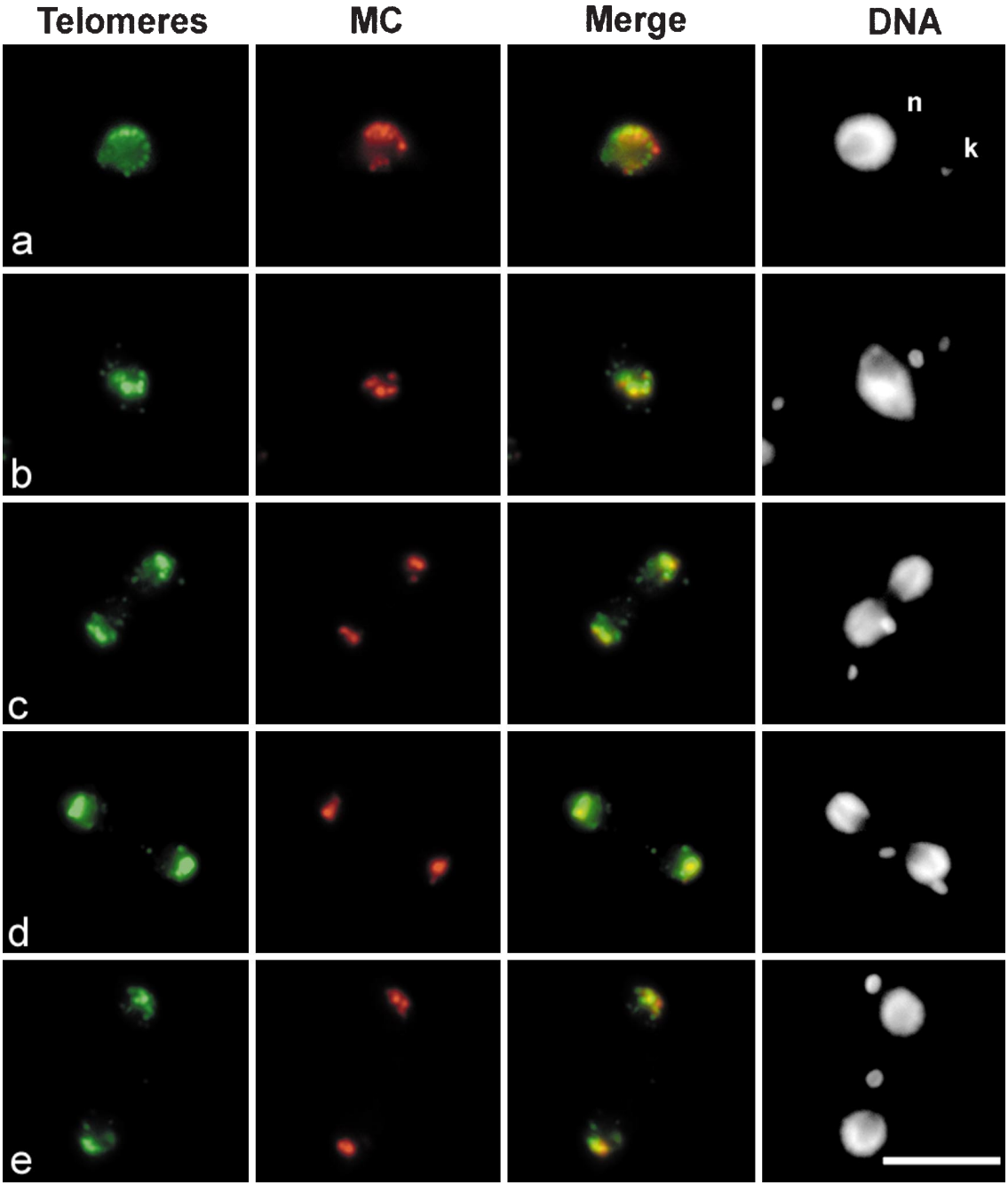

Fig. 2a–e. Fluorescent in situ hybridisation analysis of the distribution of telomeres (*green*) and minichromosomes (*MCs*, *red*) during the cell cycle in *T. brucei*. The position of each cell is determined by the DNA staining pattern of the nucleus (*n*) and the kinetoplast (*k*) using DAPI (4',6-diamidino-2-phenylindole, *white signal*). **a** Interphase cell (spherical nucleus, one kinetoplast). Much of the telomeric and MC signal is localised in a section in close proximity to the nuclear envelope. **b** Metaphase cell (elongated nucleus with DNA concentrated in a central position, two kinetoplasts). Most telomeric signals and all MCs congregate in the nuclear centre. Some telomeres are still located acentrically. **c, d** Later in mitosis, both telomeres and MCs are located near the spindle poles. Some telomeres do not co-localise with MCs and are located further towards the centre of the dividing nucleus. This is more obvious in earlier stages (**c**). **e** Karyokinesis (two spherical nuclei, two kinetoplasts); distribution of both telomeres and MCs similar to interphase cell. Polarity is determined by spindle axis. *Bar* represents 10 µm

substructure is visible by electron microscopy (Fig. 1A). A structural differentiation into granular and more fibrillar areas is observed. A clear distinction between fibrillar (FC) and dense fibrillar (DFC) components could not be made. Using FISH it has been confirmed that rRNA is localised in the nucleolus of trypanosomes (Zomerdijk 1992; Chaves et al. 1998). In order to assess the occurrence and localisation of DNA in the nucleolus, cells were grown in the presence of BrdU for 6 h and processed for TEM immunolabelling using anti-BrdU primary antibodies and gold-labelled secondary antibodies. Most areas of the nucleoplasm are heavily labelled, indi-

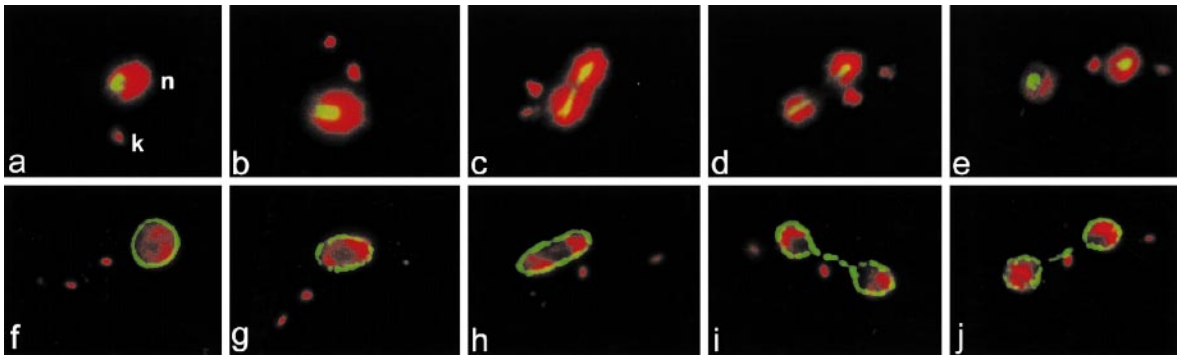

Fig. 3a–j. Localisation of the nucleolus and nuclear envelope during the cell cycle. Formaldehyde-fixed trypanosomes were labelled with the anti-nucleolar monclonal antibody (mAb) NUMAG (**a–e**) or the anti-nuclear envelope mAb NUP (**f–j**) (*green*). DNA was stained with DAPI (*red*). Images in each column represent similar stages of the cell cycle: **a, f** interphase; **b, c, g, h** mitosis; **d, e, i, j** late mitosis and karyokinesis. (*n* nucleus, *k* kinetoplast)

cating the presence of replicated DNA (Fig. 1B). In the nucleolus, however, only a few gold particles are visible in small dispersed clusters. Owing to the different electron microscopy processing conditions used for BrdU-labelled specimens in contrast to standard thin sections it is not possible to correlate, at high resolution, the morphological substructures within the nucleolus with the distribution of BrdU incorporation. However, numerous cells were analysed and higher incorporation levels of nucleolar BrdU labelling were not observed.

The mitotic nucleus

To describe nuclear dynamics and morphology we used immunofluorescence microscopy, electron microscopy and in situ hybridisation. Monoclonal antibodies directed against antigens of the nuclear envelope (mAb NUP-1), the nucleolus (mAb NUMAG) and the mitotic spindle (mAb KMX), respectively, were used to describe the overall appearance of these major structures during the cell cycle. DNA probes against telomeres, minichromosomes and the tubulin gene locus were used to address the question whether discrete stages analogous to those of mammalian mitosis can be observed in trypanosomes. This issue cannot be resolved by general DNA labelling techniques because chromosomes in *T. brucei* do not condense and therefore any reorganisation of chromatin is difficult to visualise. Finally, electron microscopy was employed to address specific questions concerning the existence and ultrastructure of putative kinetochores and their interaction with spindle microtubules, the fine structure of the mitotic spindle, its possible association with MTOCs and the segregation of nucleolar material.

Labelling of fixed cells with NUP-1 reveals the overall appearance of the nuclear envelope at various stages during the cycles (Fig. 3F–J). In interphase cells the nucleus has a spherical shape. After the initial elongation during early mitosis the nuclear envelope forms an isthmus between the two emerging daughter nuclei. Electron microscopy clearly shows that the constriction in the nuclear envelope coincides with the central portion of the mitotic spindle and is uniform (Fig. 4D–E). We do not know whether the final constriction that divides the nucleus occurs at a single site or at multiple sites simultaneously. Our ultrastructural observations show no distinct structure or envelope differentiation that might define a unique site.

In *T. brucei* the mitotic spindle forms within the nucleus. Using KMX, a monoclonal anti-β-tubulin antibody that preferentially stains microtubules of the spindle (Sasse and Gull 1988), we show that the first visible spindle inside the nucleus is often rhomboid and converges into two poles at opposite ends of the nucleus (Fig. 5A). The DAPI staining pattern at this stage does not indicate a change in nuclear organisation. During mitotic progression a prominent central spindle develops and elongates between the spindle poles (Fig. 5C, D). Although there is no prominent structure that might serve to nucleate spindle microtubules we have identified a putative spindle pole structure. Visualisation of these structures was obtained by simultaneous fixation and detergent extraction of whole cells and subsequent TEM analysis. In sections of extracted mitotic nuclei the convergence of individual microtubules into a single focus close to the nuclear envelope was clearly visible (Fig6A–C). These microtubules emanate from a small fibrous structure close to the nuclear envelope (Fig. 6C). This structure is distinct from the surrounding nucleoplasm and appears to be associated with, or an integral part of, the nuclear envelope. A higher magnification of an oblique section of a spindle pole reveals the presence of a ring-like structure of approximately 70 nm within this fibrous material (Fig. 6C'). This appears to be the first evidence of a defined structure at the spindle poles in *T. brucei*. Its positional correlation with the emerging spindle microtubules and the nuclear envelope suggests that this element could be a MTOC.

Initially, the convergence of the spindle into single poles is maintained, but later during mitosis this focal organisation is lost and the spindle, at both ends, becomes bifurcated. This conversion can also be observed by electron microscopy (Fig. 4C–E). The loss of convergence into one defined focus at both ends is accompanied by the progressive segregation of nucleolar material into opposite halves of the nucleus. The nucleolus does

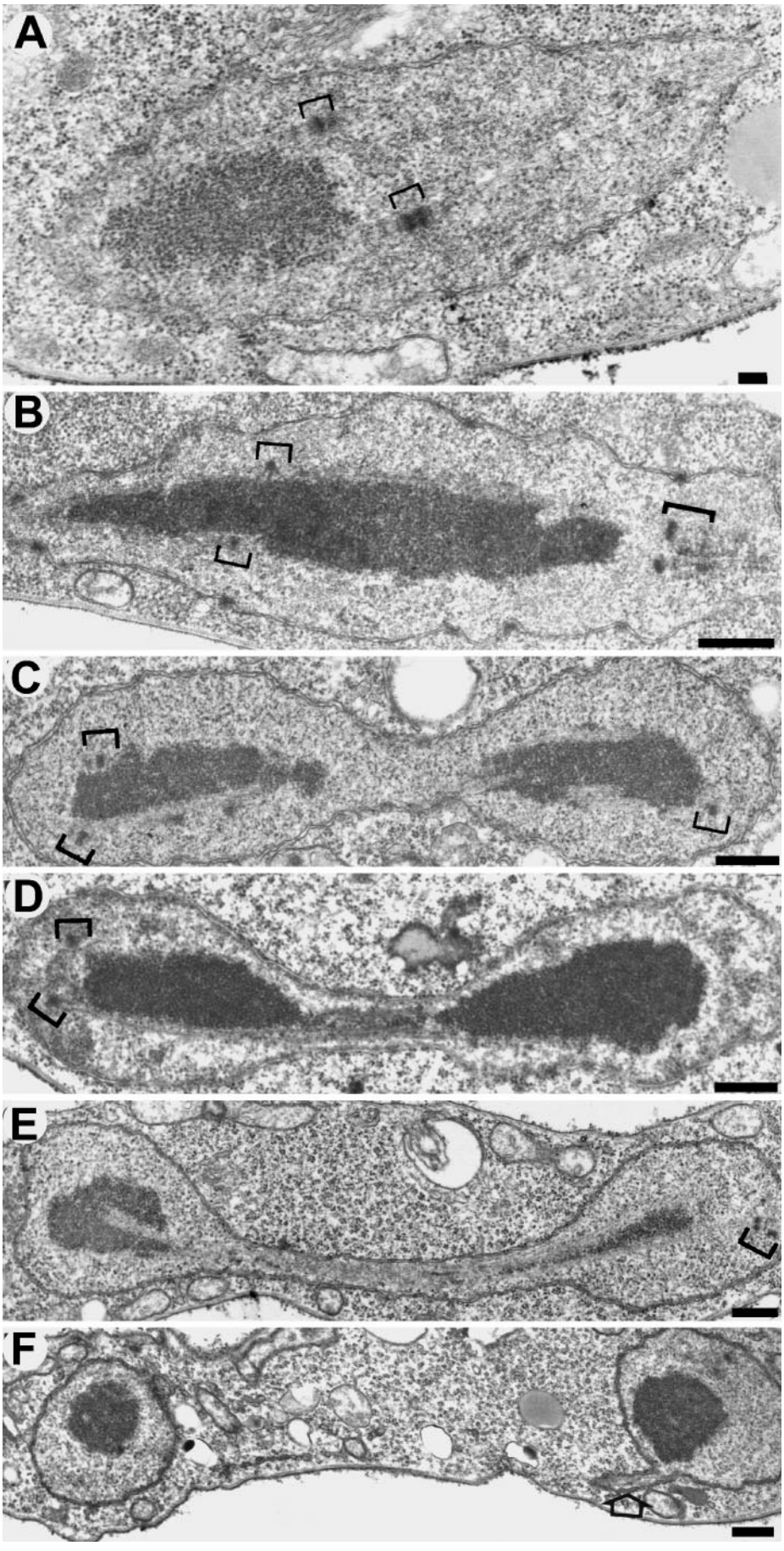

Fig. 4A–F. Series of thin sections through nuclei of *T. brucei* at different stages of the cell cycle. The position of the kinetochores is indicated by *open brackets*. **A** Early stage of mitosis, probably corresponding to metaphase. Kinetochores are not yet segregated and are located in the central plane of the nucleus. Owing to the position of this section only part of the nucleolus is visible. Electron-dense patches of chromatin, which are characteristic of the interphase nucleus, are not detectable. Small darker areas of 50–100 nm in diameter embedded in the nuclear envelope are most likely nuclear pore complexes. **B** At a later stage, paired kinetochores are now segregated. They are positioned within the spindle, which extends alongside the nucleolus. The nucleolus now extends between the spindle poles. **C** This stage of mitosis is characterised by the formation of a constriction in the centre of the nucleus and the division of the nucleolar material. Kinetochores have moved to a position close to the nuclear poles. **D, E** The nuclear constriction extends along the central spindle. Even in late stages of mitosis no morphologically distinct cleavage furrow can be identified within this nuclear constriction. In **E**, kinetochores are still visible at one nuclear pole. **F** Karyokinesis is almost complete. In this section it is not clear whether the remnant of the connecting isthmus (*arrow*) still extends between the daughter nuclei. The nuclear envelope and nucleolus have reacquired a spherical shape. *Bar* represents 0.1 µm in **A**, 0.5 µm in **B–F**

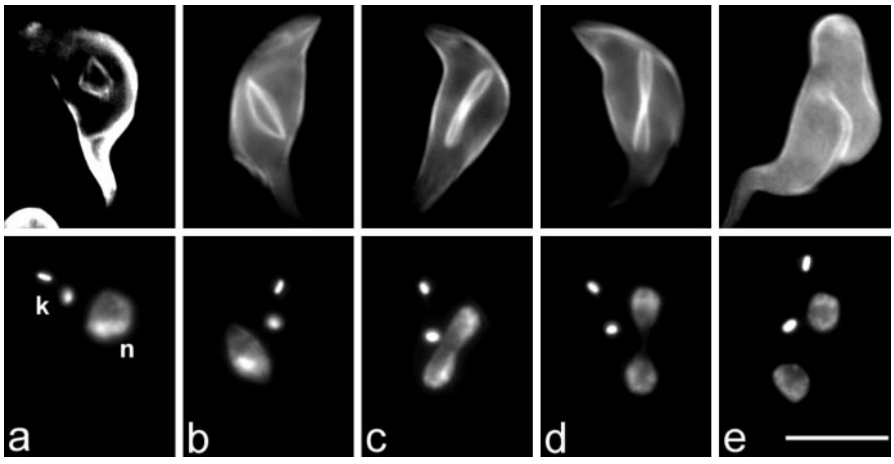

Fig. 5a–e. The spindle of *T. brucei* during mitosis. Cells were labelled with anti-tubulin antibody KMX (*upper row*) and the DNA stained with DAPI (*lower row*, *k* kinetoplast, *n* nucleus). Note the transformation from a closed confirmation with a defined polar origin (**a–c**) to an open bifurcated spindle in late mitosis (**d**). After karyokinesis the spindle is no longer detectable (**e**). *Bar* represents 10 µm

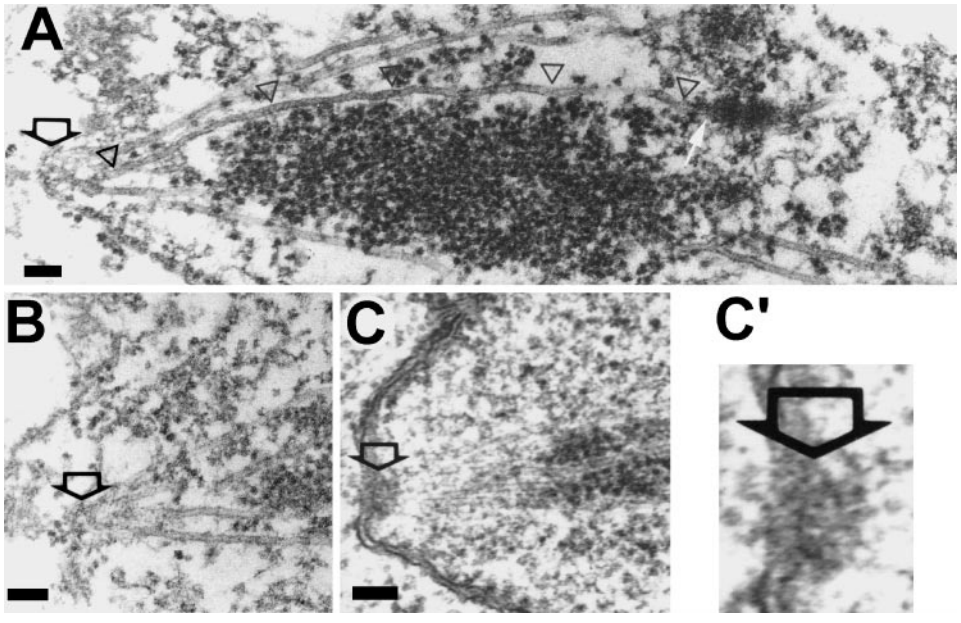

Fig. 6A–C'. TEM analysis of the mitotic spindle in cells that have been simultaneously extracted with detergent (NP-40) and fixed. **A** Microtubules emanating from a zone adjacent to the nuclear envelope (*arrow*) engulf nucleolar material. A single microtubule (*triangles*) can be traced from the spindle pole to a kinetochore (*white arrow*). The kinetochore is not segregated, indicating a metaphase configuration. **B, C** The spindle microtubules emerge from a polar structure on the nuclear envelope. In **C** this structure appears to be part of the nuclear envelope as the bilaminated structure of the nuclear membrane, clearly visible on both sides of the polar structure, is not detectable. Further magnification of the polar structure (**C'**) reveals a ring-like structure (*arrow*) within the fibrous pole

not break down or fragment but remains intact throughout mitosis. We have generated a mAb (termed NUMAG) against an M_r 90,000 nucleolar protein, which provides a general overview of nucleolar segregation by immunofluorescence (Fig. 3A–E). In the interphase cell, the nucleolus is spherical in shape and is usually located in an acentric position within the nucleus. At an early stage of mitosis the nucleolus acquires a bar-shaped form that, during progression of mitosis, splits into two clusters of equal size and re-acquires its spherical appearance preceding karyokinesis. Electron microscopic analysis of nucleolar segregation shows that in early stages of mitosis the nucleolar material resides within the interior of the mitotic spindle (Fig. 4B). Transverse and longitudinal sections of mitotic nuclei show that microtubules surround the nucleolar material, while several bundles of microtubules penetrate the periphery of the nucleolus (Fig. 7A, B).

One of the central problems concerning trypanosome genome segregation is the question of how the cell segregates its unusually large number of chromosomes. Ultrastructural studies on several trypanosomatids have identified electron-dense plaques within the nucleoplasm that occur only in the mitotic nucleus (Vickerman and Preston 1970; Solari 1980b; Urena 1986). Their association with microtubules and their positioning during mitosis suggest that these plaques are kinetochores. However, in all trypanosomatids studied the number of such kinetochores detected appears to be less than the number of chromosomes found in the organism, suggesting either a non-conventional function of these kinetochores or the presence of alternative segregation mechanisms. This conundrum is especially valid for *T. brucei* with its more than 100 chromosomes but observed number of kinetochores that appears not to exceed eight (Solari 1995). In order to determine whether these putative kinetochores are structurally and functionally comparable to conventional kinetochores we investigated their ultrastructure, positioning and interaction with spindle microtubules.

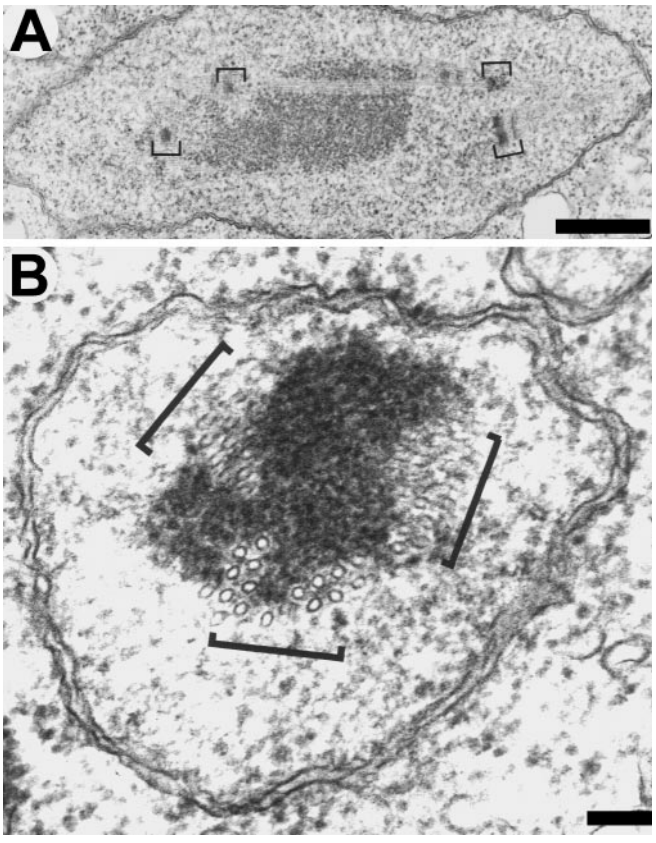

Fig. 7. A TEM of a longitudinal section of a dividing nucleus. Bundles of microtubules traverse the nucleolus. The dense plaques (*brackets*) are interpreted as kinetochores (see Fig. 4 for details). **B** A transverse section showing details of the spatial organisation of the mitotic spindle and the nucleolus. Several bundles of up to 20 individual microtubules (*brackets*) surround and penetrate the nucleolus. *Bars* represent 0.1 µm

In the interphase cell no such electron-dense structures are visible (Fig. 1A). In mitotic cells they are clearly visible as electron-dense plaques with a laminated substructure (Fig. 4A). High magnification shows details of this substructure (Fig. 8A–D). The plaques are approximately 100 by 120 nm in size. These laminated plaques consist of two adjacent electron-dense outer layers of ca. 50 nm in thickness. Each outer layer is separated from a single thin electron-dense mid-zone by an electron-transparent intermediate zone. Up to four microtubules penetrate and terminate at the outer layers. To visualise kinetochore structure and interaction with microtubules in more detail, sections of cells extracted with 0.1% NP-40 prior to fixation were examined. In these preparations the trilaminar organisation of an undivided kinetochore is even more pronounced and two to four microtubules can be seen attached to the outer plates of the kinetochore (Fig. 8C, C'). Also, individual microtubules could be tracked along their entire length from the spindle pole to a kinetochore in extracted cells, providing further evidence that the electron-dense plaques are indeed kinetochores (Fig. 6A). On progression of mitosis the kinetochore pairs segregate and form single kinetochores. Each single kinetochore is composed of a trilaminar structure. A 10 nm dense layer that is orientated proximal to the spindle poles is separated by a 50 nm transparent region from a second electron-dense layer of ca. 50 nm. Up to four spindle microtubules appear to be associated with the 10 nm layer (Fig. 8D). The process of kinetochore segregation is, however, not just a simple splitting of the paired kinetochore along the midlayer. Instead, the unpaired kinetochore has a more complex structure than expected from simple segregation (Fig. 8B, D). During the process of segregation an additional electron-dense layer is located proximal to the nuclear poles. When the kinetochore is still paired, but already elongated before separation, this additional layer is separated from the main layer by only a very thin (<10 nm) opaque layer (Fig. 8B). Only after segregation of the pair is complete is this new layer clearly distinct and well separated by a 50 nm gap from the main electron-dense layer (Fig. 8D). The structure of the kinetochore during different stages of mitosis is schematically depicted in Fig. 9.

Non-segregated kinetochores appear initially in the midplane of the mitotic nucleus. Upon progression of mitosis, indicated by spindle elongation, nucleolar segregation and nuclear envelope constriction, there is a movement of the kinetochores towards the nuclear poles. This stage resembles anaphase A in a standard mitotic model (Fig. 4B–D). During this poleward movement kinetochores are located close to the periphery of the nucleolus. In the final stages of mitosis some kinetochores are still visible close to the poles of the spindle (Fig. 4E).

Segregation of chromosomes during mitosis

We investigated whether the sequence of events described for chromosomal movements of higher eukaryotes could also be observed in trypanosomes. Owing to the large number of chromosomes and their lack of condensation a general DNA staining technique is not helpful in this analysis. We designed a number of different DNA probes that allowed us to visualise defined regions on chromosomes. We used a telomeric oligonucleotide probe in a series of FISH experiments to detect only the ends of chromosomes. This probe serves as a marker for the entire chromosome population, including minichromosomes. To discriminate between the minichromosomal telomeres and those of larger chromosomes we used a minchromosome-specific, repetitive DNA probe in a double-labelling experiment.

In interphase cells the telomeres are located in close proximity to the nuclear envelope. Most, but not all, signals co-localise with minichromosomes (Fig. 2A). At the onset of mitosis, as indicated by nuclear elongation, the telomeres congregate towards the centre of the nucleus (Fig. 2B). This congession of chromosomes in the nuclear centre is similar to metaphase in higher eukaryotes. Much of the staining in the very centre of the nucleus is due to the large number of minichromosomes. A small number of focal points of signal remained slightly outside the main body of staining. These very likely repres-

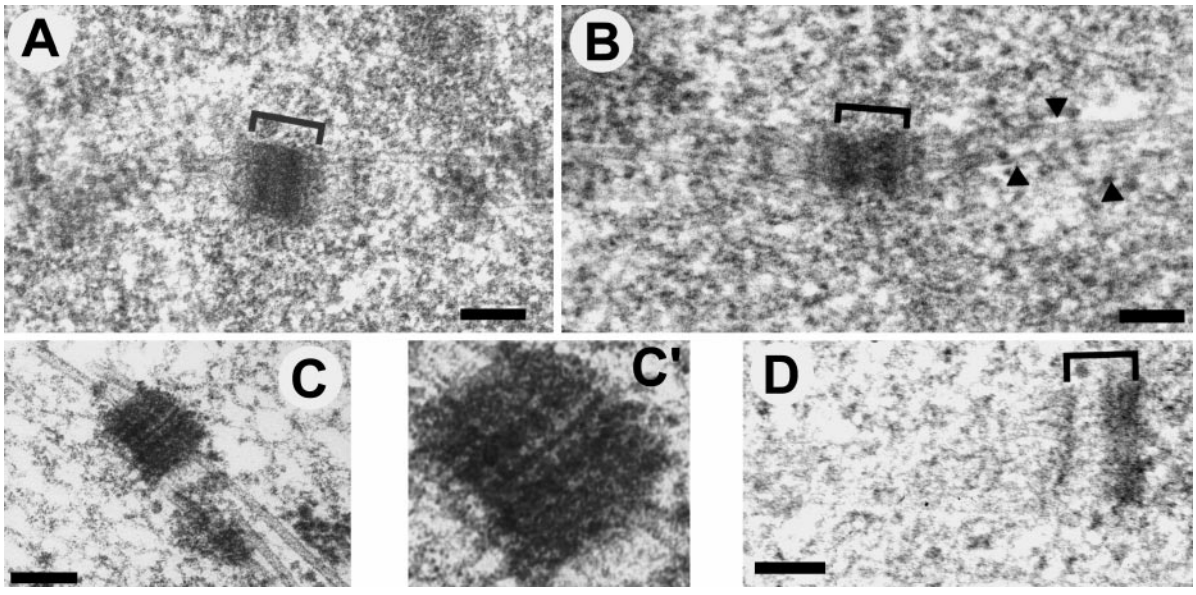

Fig. 8A–D. Details of the ultrastructure of kinetochores in *T. brucei*. **A, B** Non-segregated kinetochore pairs reveal a characteristic trilaminar structure. The plaques are approximately 100 by 120 nm and consist of two adjacent electron-dense outer layers of ca. 50 nm in thickness. Each outer layer is separated from a single thin electron-dense mid-zone by an electron-opaque intermediate zone. Microtubules (*arrows* in **B**) are attached to the outer layer. Detergent extraction of cells prior to fixation allows a more detailed analysis of the substructure of kinetochores and spindle microtubules (**C, C'**). The outer dense layer consists of three components: a very dense layer of about 15 nm in thickness lying immediately adjacent to an opaque layer of similar thickness, followed by a less dense outermost layer of approximately 35 nm in thickness. In this section two and four microtubules, respectively, terminate in each of the opposite outer layers. **D** Detailed ultrastructure of a single kinetochore. It consists of a very thin (<10 nm) electron-dense layer facing the nearest nuclear pole and 50 nm thick electron-dense layer facing towards the centre of the nucleus. Both layers are separated by a ca. 40 nm opaque mid-region. Microtubules can be seen to terminate in the thin layer facing the spindle poles. *Bars* represent 0.1 μm

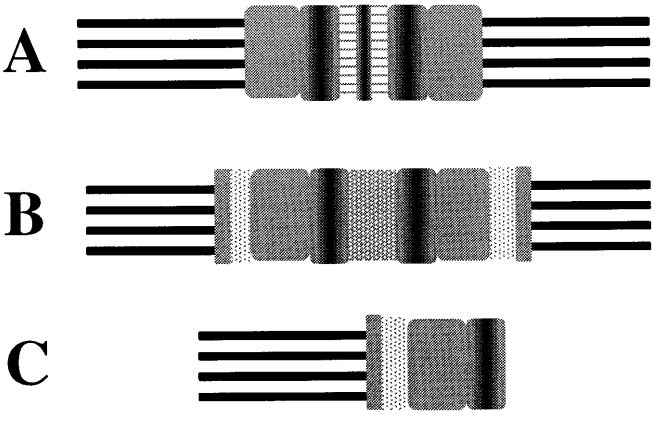

Fig. 9A–C. Schematical representation of *T. brucei* kinetochores during mitosis. **A** Early mitosis, metaphase-like configuration. **B** Metaphase-anaphase transition; kinetochores are elongated and ultrastructure is altered. **C** Single kinetochore at an anaphase stage in mitosis. *Different grades of shading* represent areas of differential electron density within the kinetochore; kinetochore microtubules are depicted as *black bars*

ent the telomeres of some of the large chromosomes, assuming that centromeres are facing towards the nuclear centre with telomeres facing towards the nuclear periphery. In the next stages chromosomes segregate into two clusters. Subsequently, most of the staining is concentrated near the nuclear poles. The double-labelling experiment suggests that these telomeres are of minichromosomal origin. A small number of signals are located further distal to the spindle poles (Fig. 2C–E). They are more likely to be telomeres of larger chromosomes.

In the above-described set of FISH experiments we examined chromosome populations or, in the case of the minichromosomes, subpopulations. In order to assess the segregation pattern of an individual chromosome we used a DNA probe specific to only a subregion of a single diploid chromosome pair. Using FISH we were able to trace the segregation pattern of this chromosome pair during mitosis. The DNA probe is targeted to the locus containing the coding region of tubulin. This gene occurs in 16–18 tandem repeat copies on one chromosome, spanning a distance of 45 to 50 kb, and is therefore easy to detect by FISH. The tubulin gene locus is on chromosome I, which belongs to the megabasepair-sized population of chromosomes. Homologous chromosomes in *T. brucei* are known often to differ considerably in length and in this case the two homologues are 1 and 3 Mb.

G1/S-phase and G2-phase in *T. brucei* can be morphologically distinguished by the presence of a single nucleus and one or two kinetoplasts, respectively. In these stages the FISH signals of the two tubulin loci are well separated from each other within the nucleoplasm (Fig. 10A). Upon entry into mitosis the chromatin distribution begins to change as seen by the non-uniform DAPI staining pattern of the nuclear DNA. The bulk of the DNA is concentrated in a more central position with-

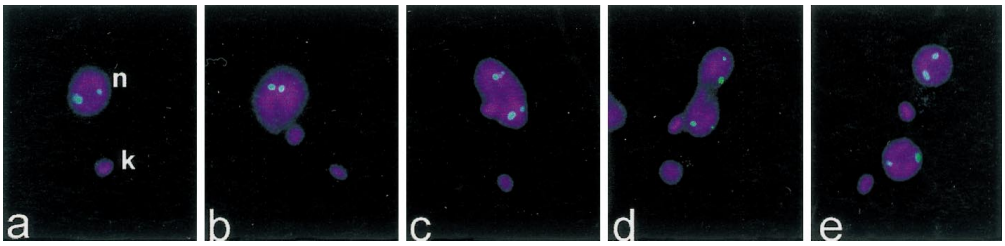

Fig. 10a–e. In situ hybridisation analysis of the localisation of chromosome I during the cell cycle. Trypanosomes were hybridised to a probe containing the complete tubulin gene locus and covering approximately 60 kb of this diploid chromosome (*green signals*). Total DNA was stained with DAPI (*blue*). (*n* nucleus, *k* kinetoplast)

in the nucleus and the FISH signal is located within this prominent mass of DNA (Fig. 10B). At the next stage of mitosis, characterised by nuclear elongation, the sister chromatids are clearly separated from each other as four distinct signals are now detectable (Fig. 10C, D). In later stages of mitosis the chromosomes are segregated towards the opposite halves of the dividing nucleus (Fig. 10E).

Discussion

In 1970, Vickerman and Preston stated that there had been little progress in the field of mitosis in *T. brucei* since the light microscope study of Noble, McRary and Beaver in 1953 (Noble et al. 1953). There has now been another long interlude since the work of Vickerman and Preston on the structure of the dividing nucleus of *T. brucei rhodesiense* and the related *Trypanosoma raiae*. Major advances have, however, been made in understanding nuclear function and the structural and molecular requirements that ensure faithful chromosome segregation in a wide range of organisms from yeast to human. It now seems pertinent to provide a modern cell biological view of nuclear organisation in the trypanosome. Also, a major focus of trypanosome research involves questions of how this parasite regulates the expression of the VSG genes and how these VSG expression sites are activated or inactivated to ensure the survival of the parasite population in the blood of the host by allowing antigenic surface coat changes in individual cells.

The nucleolus of *T. brucei* does not dissociate during mitosis. This is different from the situation in some trypanosomatids (e.g. *Blastocrithidia, Trypanosoma cruzi*) in which the nucleolus fragments or disperses during mitosis (De Souza and Meyer 1974; Heywood and Weinman 1978; Solari 1983) but similar to *Leishmania* ssp. In the euglenoids, a group of organisms closely related to trypanosomatids, the nucleolus also persists during mitosis. This has been taken as a indication of a closer relationship between the euglenoids and *T. brucei* than to most other trypanosomatids (Leedale 1968; Chaly et al. 1977; Triemer and Farmer 1991; Cavalier-Smith 1993). The biology of the nucleolus of *T. brucei* has gained additional significance through speculation that it might be involved in VSG gene transcription. VSG genes are transcribed by a RNA polymerase that is resistant to α-amanitin, a potent inhibitor of RNA polymerase II. This observation and other evidence has led to the hypothesis that VSG genes are transcribed by RNA polymerase I, or by a yet unidentified form of RNA polymerase II, which is, however, less likely (Rudenko et al. 1992). The main function of RNA polymerase I in eukaryotic organisms is the transcription of rRNA genes and the enzyme is typically localised in the fibrillar zone of the nucleolus (Shaw and Jordan 1995). If the trypanosomal RNA polymerase I transcribes VSG genes it has been speculated that VSG transcription sites may be localised at or in the nucleolus and that nucleolar organisation and its DNA composition might be different or unusual in comparison with other lower eukaryotes. However, recent results localising nuclear transcripts of active VSG expression sites by FISH do not support this hypothesis (Chaves et al. 1998). It is now generally accepted that rDNA resides in or around the FC of the nucleolus. Our BrdU incorporation studies revealed sparse nucleolar labelling. The labelling pattern is consistent with the view that the FC does not contain large amounts of DNA in addition to the expected rDNA genes and is very similar to the levels of nucleolar DNA detected by BrdU, anti-DNA antibody and radiolabelling in plant nucleoli (Risueno and Testillano 1994). Therefore, our observation of the ultrastructure and the DNA content of the nucleolus indicates no evident features that distinguish the trypanosome nucleolus from nucleoli described in many other eukaryotes.

As in many protozoan organisms the nuclear envelope of trypanosomes persists during mitosis. During interphase most of the chromatin remains dispersed throughout the nucleoplasm. Some of the chromatin aggregates in patches close to the nuclear envelope. A similar distribution has been reported for *T. brucei rhodesiense* (Vickerman and Preston 1970). Interestingly, the localisation of these chromatin patches is very similar to the distribution of minichromosomes in *T. brucei* interphase cells as observed by FISH (Chung et al. 1990; Ersfeld and Gull 1997). Chromosomes in trypanosomes do not visibly condense during prophase. This might be a consequence of the unusual linker histone 1A, which differs from other eukaryotic linker histones (Burri et al. 1993, 1995). The protein is approximately 40% shorter than conventional histone H1 proteins owing to a large deletion of the globular domain near its N-terminus. Furthermore, the N-terminal sequences of the core histones, which are highly conserved in most organisms, vary be-

tween trypanosomes and other organisms (Galanti et al. 1998).

The main structural feature of a dividing *T. brucei* nucleus is its prominent mitotic spindle. The spindle extends from the poles of the nucleus. It is not known whether there are continuous microtubules stretching between opposite poles or whether microtubules terminate between the poles. A three-dimensional reconstruction of the spindle in *T. cruzi* failed to detect any continuous microtubules (Solari 1980a). No structural equivalents of spindle organising centres, such as spindle pole bodies or centrosomes, have been found in trypanosomes. However, at early stages of mitosis the spindle poles clearly emanate from a small area close to the nuclear envelope that differs in ultrastructural detail from its surroundings. This suggests the existence of a spindle-organising centre. In this context it is interesting to look at organelle segregation in *T. brucei* from an evolutionary point of view. Obviously, trypanosomes possess typical centrioles in the form of basal bodies at the base of the flagellum. The primary function of centrioles is the organisation of the flagellum or cilium in motile cells (Pickett-Heaps 1974). In trypanosomatids the centriole/basal body is, in addition to its role in spindle organisation, also directly involved in the partitioning of the mitochondrial genome, the kinetoplast (Robinson and Gull 1991). Since trypanosomes possess only a single mitochondrion, faithful segregation of this organelle is essential for cell survival, and therefore the involvement of an organelle normally associated with flagellar organisation and segregation seems to be a logical consequence in its evolutionary development.

The positioning and structure of the laminated plaques and their exclusive occurrence in mitotic nuclei suggest that these structures are kinetochores, as suggested previously (Vickerman and Preston 1970; Solari 1995). Nevertheless, an unequivocal association with chromatin has yet to be demonstrated. In a review on this subject Solari (1995) suggests that there are no more than eight kinetochores in a metaphase cell. A calculation based solely on the 22 Mb chromosomes of the diploid genome would suggest the presence of 22 kinetochores. None of the estimates of partial or completely reconstructed three-dimensional analyses of mitotic *T. brucei* nuclei gives numbers approaching this figure. It seems unlikely that most of the kinetochore complement has been missed in these electron microscopy studies. Moreover, this problem is compounded by the large number of intermediate and minichromosomes. To explain this discrepancy Solari (1995) proposed a model in which the kinetochore plaques are linked to DNA replication forks and that each half of the plaque segregates a pair of replicated chromosomes, attached to each other by a short segment of unreplicated DNA. The topology of his model, however, would prevent the universally accepted and observed mechanism of chromosome segregation by which each daughter cell receives a copy of each pair of homologues as a result of equal segregation of sister chromatids. It contradicts the result of many genetic experiments in which a single marker gene insertion event, as the result of genetic recombination by homologous insertion, was propagated throughout the entire cell population, not just half the population (Ten Asbroek et al. 1990). It would also prevent the stable inheritance of the monoallelic VSG expression sites. One might explain this apparent lack of kinetochores in relation to chromosome numbers by other means. It could be that these kinetochores represent complex aggregates of different chromosomes or even paired and replicated homologues. A different geometry of microtubule connection to that suggested by Solari (1995) would then be needed to ensure segregation of homologues from the complex. Alternatively, only some of the large chromosomes may form visible kinetochore structures and other chromosomes could possess small kinetochores that remain undetected by electron microscopy. Finally, we have suggested that they are segregated by kinetochore-independent mechanisms. It has long been argued that the necessity to develop dedicated structures on chromosomes to ensure faithful segregation by a highly regulated and specific interaction with spindle microtubules emerged only with the increasing length and complexity of chromosomes (Pickett-Heaps 1974; Pickett-Heaps and Tippit 1978).

It is interesting that not only in *T. brucei* does the number of kinetochores not match chromosome number. In *Leishmania* and *T. cruzi*, which possess 36 and 32 chromosomes each, only six and ten kinetochores, respectively, have been identified (Solari 1995; Donelson 1996; Zingales et al. 1997). The size of chromosomes in both organisms varies between 4 and 0.45 Mb. Moreover, the complete DNA sequence of the the 0.45 Mb chromosome of *L. major* reveals no sequences that resemble the centromere sequences described in other organisms (Myler et al. 1999). On the other hand, in *Saccharomyces cerevisiae*, which has a similar chromosome number and size range to the megabase pair chromosomes of trypanosomes, each of the chromosomes is equipped with small, but functionally defined and biochemically identical kinetochores, although their ultastructure is not visible by electron micoscopy (Peterson and Ris 1976; Hyman and Sorger 1995).

It seems likely that progress in this area of trypanosome biology will require an integration of information from the genome projects and future molecular cell biology experiments.

Acknowledgements. The work in our laboratory is supported by a Programme and Equipment Grant from The Wellcome Trust. D.R. is supported by a Wellcome Trust Career Development Fellowship. This investigation received financial support from the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases.

References

Biebinger S, Rettenmaier S, Flaspohler J, Hartmann C, PenaDiaz J, Wirtz LE, Hotz HR, Barry JD, Clayton C (1996) The PARP promoter of *Trypanosoma brucei* is developmentally regulated in a chromosomal context. Nucleic Acids Res 24:1202–1211

Brun R, Schönenberger M (1979) Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop 36:289–292

Burri M, Schlimme W, Betschart B, Kampfer U, Schaller J, Hecker H (1993) Biochemical and functional characterization of histone H1-like proteins in procyclic *Trypanosoma brucei brucei*. Parasitol Res 79:649–659

Burri M, Schlimme W, Betschart B, Lindner H, Kampfer U, Schaller J, Hecker H (1995) Partial amino acid sequence and functional aspects of histone H1 proteins in *Trypanosoma brucei brucei*. Biol Cell 83:23–31

Cavalier-Smith T (1993) Kingdom Protozoa and its 18 phyla. Microbiol Rev 57:953–994

Chaly N, Lord A, Lafontaine JG (1977) A light- and electron-microscope study of nuclear structure throughout the cell cycle in the euglenoid *Astasia longa* (Jahn). J Cell Sci 27:23–45

Chaves I, Zomerdijk J, Dirks Mulder A, Dirks RW, Raap AK, Borst P (1998) Subnuclear localization of the active variant surface glycoprotein gene expression site in *Trypanosoma brucei*. Proc Natl Acad Sci U S A 95:12328–12333

Choo KHA (1997) The centromere. Oxford University Press, Oxford

Chung HMM, Shea C, Fields S, Taub RN, Van Der Ploeg LHT, Tse DB (1990) Architectural organization in the interphase nucleus of the protozoan *Trypanosoma brucei*: location of telomeres and mini-chromosomes. EMBO J 9:2611–2619

Compton DA (1998) Focusing on spindle poles. J Cell Sci 111: 1477–1481

Csink AK, Henikoff S (1996) Genetic modification of heterochromatin association and nuclear organization in *Drosophila*. Nature 381:529–531

De Souza W, Meyer H (1974) On the fine structure of the nucleus in *Trypanosoma cruzi* in tissue culture forms. Spindle fibers in the dividing nucleus. J Protozool 21:48–52

Ding R, McDonald KL, McIntosh JR (1993) Three-dimensional reconstruction and analysis of mitotic spindles from the yeast, *Schizosaccharomyces pombe*. J Cell Biol 120:141–151

Donelson JE (1996) Genome research and evolution in trypanosomes. Curr Opin Genet Dev 6:699–703

Earnshaw WC (1994) Structure and molecular biology of the kinetochore. In: Hyams JS, Lloyd CW (eds) Microtubules. Wiley-Liss, New York, pp 393–412

Ekwall K, Javerzat J-P, Lorentz A, Schmidt H, Cranston G, Allshire R (1995) The chromodomain protein swi6: a key component at fission yeast centromeres. Science 269:1429–1431

Ersfeld K, Gull K (1997) Partitioning of large and minichromosomes in *Trypanosoma brucei*. Science 276:611–614

Ersfeld K, Melville SE, Gull K (1999) Nuclear and genome organisation of *Trypanosoma brucei*. Parasitol Today 15:58–63

Galanti N, Galindo M, Sabaj V, Espinoza I, Roro GC (1998) Histone genes in trypanosomatids. Parasitol Today 14:64–70

Goode D (1975) Evolution of mitosis in protozoa: the association of chromosomes, nuclear envelope, kinetochores and microtubules. BioSystems 7:318–325

Gull K, Alsford S, Ersfeld K (1998) Segregation of minichromosomes in trypanosomes: implications for mitotic mechanisms. Trends Microbiol 6:319–323

Heath IB (1980) Variant mitoses in lower eukaryotes: indicators of the evolution of mitosis? In: Bourne GH, Danielli JF (eds) Int Rev Cytol 64:1–80

Hecker H, Betschart B, Bender K, Burri M, Schlimme W (1994) The chromatin of trypanosomes. Int J Parasitol 24:809–819

Heywood P, Weinman D (1978) Mitosis in the hemoflagelate *Trypanosoma cyclops*. J Protozool 25:287–292

Horn D, Cross GAM (1995) A developmentally regulated position effect at a telomeric locus in *Trypanosoma brucei*. Cell 83: 555–561

Horn D, Cross GAM (1997) Position-dependent and promoter-specific regulation of gene expression in *Trypanosoma brucei*. EMBO J 16:7422–7431

Hyman AA, Sorger PK (1995) Structure and function of kinetochores in budding yeast. Annu Rev Cell Dev Biol 11:471–495

Kubai DF (1975) The evolution of the mitotic spindle. In: Bourne GH, Danielli JF (eds) Int Rev Cytol 43:167–227

Leedale GF (1968) The nucleus in *Euglena*. In: Buetow DE (ed) The biology of Euglena, vol 1. Academic Press, New York pp 185–242

McIntosh JR, Koonce MP (1989) Mitosis. Science 246:622–628

Myler PJ, Audleman L, deVos T, Hixson G, Kiser P, Lemley C, Magness C, Rickel E, Sisk E, Sunkin S, Swartzell S, Westlake T, Bastien P, Fu G, Ivens A, Stuart K (1999) *Leishmania major* Friedlin chromosome 1 has an unusual distribution of protein-coding genes. Proc Natl Acad Sci USA 96:2902–2906

Noble ER, McRay WL, Beaver ET (1953) Cell division in trypanosomes. Trans Am Microsc Soc 72:236–248

Pays E (1993) Genome organization and control of gene expression in trypanosomatids. In: Broda PMA, Oliver SG, Sims PFG (eds) The eukaryotic genome: organisation and regulation. Cambridge University Press, Cambridge, pp 127–160

Peterson JB, Ris H (1976) Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. J Cell Sci 22:219–242

Pickett-Heaps JD (1969) The evolution of the mitotic apparatus: an attempt at comparative ultrastructural cytology in dividing plant cells. Cytobios 1:257–280

Pickett-Heaps JD (1974) The evolution of mitosis and the eukaryotic condition. BioSystems 6:37–48

Pickett-Heaps JD, Tippit DH (1978) The diatom spindle in perspective. Cell 14:455–467

Ris H (1975) Primitive mitotic mechanisms. BioSystems 7:298–304

Risueno MC, Testillano PS (1994) Cytochemistry and immunocytochemistry of nucleolar chromatin in plants. Micron 25:331–360

Robinson DR, Gull K (1991) Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle. Nature 352:731–733

Robinson DR, Gull K (1994) The configuration of DNA replication sites within the *Trypanosoma brucei* kinetoplast. J Cell Biol 126:641–648

Rudenko G, Lee MGS, Van der Ploeg LHT (1992) 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

Rudenko G, Blundell PA, DirksMulder A, Kieft R, Borst P (1995) A ribosomal DNA promoter replacing the promoter of a telomeric VSG gene expression site can be efficiently switched on and off in *Trypanosoma brucei*. Cell 83:547–553

Sasse R, Gull K (1988) Tubulin post-translational modifications and the construction of microtubular organelles in *Trypanosoma brucei*. J Cell Sci 90:577–589

Scott V, Sherwin T, Gull K (1997) γ-Tubulin in trypanosomes: molecular characterisation and localisation to multiple and diverse microtubule organising centres. J Cell Sci 110:157–168

Shaw PJ, Jordan EG (1995) The nucleolus. Annu Rev Cell Dev Biol 11:93–121

Sloof P, Bos JL, Konings A, Menke HH, Borst P, Gutteridge WE, Leon W (1983) Characterization of satellite DNA in *Trypanosoma brucei* and *Trypanosoma cruzi*. J Mol Biol 167:1–21

Solari AJ (1980a) The 3-dimensional fine structure of the mitotic spindle in *Trypanosoma cruzi*. Chromosoma 78:239–255

Solari AJ (1980b) Function of the dense plaques during mitosis in *Trypanosoma cruzi*. Exp Cell Res 127:457–460

Solari AJ (1983) The ultrastructure of mitotic nuclei of *Blastocrithidia triatomae*. Z Parasitenkd 69:3–15

Solari AJ (1995) Mitosis and genome partitioning in trypanosomes. Biocell 19:65–84

Ten Asbroek A, Quellette M, Borst P (1990) Targeted insertion of the neomycin phosphotransferase gene into the tubulin gene cluster of *Trypanosoma brucei*. Nature 348:174–175

Tooze J (1985) Blocked coated pits in AtT20 cells results from endocytosis of budding retrovirions. J Cell Biol 101:1713–1723

Triemer RE, Farmer MA (1991) An ultrastructural comparison of the mitotic apparatus, feeding apparatus, flagellar apparatus and cytoskeleton in euglenoids and kinetoplasts. Protoplasma 164:91–104

Urena F (1986) Three-dimensional reconstruction of the mitotic spindle and dense plaques in three species of *Leishmania*. Z Parasitenkd 72:299–306

Van der Ploeg LHT, Schwartz DC, Cantor CR, Borst P (1984) Antigenic variation in *Trypanosoma brucei* analyzed by electrophoretic separation of chromosome-sized DNA molecules. Cell 37:77–84

Vickerman K, Preston TM (1970) Spindle microtubules in the dividing nuclei of trypanosomes. J Cell Sci 6:365–383

Weiden M, Osheim YN, Beyer AL, Van der Ploeg LHT (1991) Chromosome structure: DNA nucleotide sequence elements of a subset of the minichromosomes of the protozoan *Trypanosoma brucei*. Mol Cell Biol 11:3823–3834

Winey M, Mamay CL, O'Toole ET, Mastronarde DN, Giddings TH, McDonals KL, McIntosh JR (1995) Three-dimensional ultrastructural analysis of the *Saccharomyces cerevisiae* mitotic spindle. J Cell Biol 129:1601–1615

Woodward R, Gull K (1990) Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of *Trypanosoma brucei*. J Cell Sci 95:49–57

Zingales B, Rondinelli E, Degrave W, da Silveira JF, Levin M, Le Paslier D, Modabber F, Dobrokhotov B, Swindle J, Kelly JM, Aslund L, Hoheisel JD, Ruiz AM, Cazzulo JJ, Pettersson U, Frasch AC (1997) The *Trypanosoma cruzi* genome initiative. Parasitol Today 13:16–22

Zomerdijk J (1992) Subnuclear localization of transcripts from ribosomal RNA genes and VSG gene expression sites in *Trypanosoma brucei*. PhD Thesis, University of Amsterdam