The identification of circular extrachromosomal DNA in the nuclear genome of *Trypanosoma brucei*

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Summary

Nuclear extrachromosomal DNA elements have been identified in several kinetoplastids such as *Leishmania* and *Trypanosoma cruzi*, but never in *Trypanosoma brucei*. They can occur naturally or arise spontaneously as the result of sublethal drug exposure of parasites. In most cases, they are represented as circular elements and are mitotically unstable. In this study we describe the presence of circular DNA in the nucleus of *Trypanosoma brucei*. This novel type of DNA was termed NR-element (*Mal*II *r*epeat element). In contrast to drug-induced episomes in other kinetoplastids, the *T. brucei* extrachromosomal NR-element is not generated by drug selection. Furthermore, the element is stable during mitosis over many generations. Restriction analysis of tagged NR-element DNA, unusual migration patterns during pulsed field gel electrophoresis (PFGE) and CsCl/ ethidium bromide equilibrium centrifugation demonstrates that the NR-element represents circular DNA. Whereas it has been found in all field isolates of the parasites we analysed, it is not detectable in some laboratory strains notably the genome reference strain 927. The DNA sequence of this element is related to a 29 bp repeat present in the subtelomeric region of VSG-bearing chromosomes of *T. brucei*. It

has been suggested that this subtelomeric region is part of a transition zone on chromosomes separating the relatively stable telomeric repeats from the recombinationaly active region downstream of VSG genes. Therefore, we discuss a functional connection between the occurrence of this circular DNA and subtelomeric recombination events in *T. brucei*.

Introduction

One of the distinguishing features of the genome of the unicellular parasite *Trypanosoma brucei*, the causative agent of African sleeping sickness, is the presence of a very large number of chromosomes that are extremely heterogeneous both in size and composition. The nuclear genome of the protozoan parasite *T. brucei* (for review see Ersfeld *et al.*, 1999) consists of mini (MCs, 30–150 kbp), intermediate (ICs, 200–300 kbp) and megabase chromosomes (MBCs, 1–6 mbp). Although these classes of chromosomes differ greatly in size, gene content and overall composition they are all linear molecules terminating with a canonical telomeric repeat [GGGTTA]$_n$ (van der Ploeg *et al.*, 1984a). Minichromosomes are transcriptionally inactive and exclusively found in trypanosomes capable of antigenic variation. The presence of silent telomeric VSG genes on these chromosomes suggests that they serve as a reservoir to expand the number of telomeric sites available for recombinational events (van der Ploeg *et al.*, 1984b; Robinson *et al.*, 1999).

Naturally occurring nuclear extrachromosomal DNA has not yet been described in *Trypanosoma brucei*. This is in contrast to other kinetoplastids where both linear and circular DNA elements have been identified (Beverley, 1991). In *Leishmania* and *Trypanosoma cruzi* extrachromosomal DNA can arise spontaneously or as result of gene amplification during drug selection. One of the best characterized examples is the generation of extrachromosomal DNA after exposure of *Leishmania* cells to methotrexate during the development drug resistance (Beverley *et al.*, 1984; Grondin *et al.*, 1998). Extrachromosomal DNA can also arise independent of drug selection, such as the circular 75 kbp LED in *Trypanosoma cruzi* that contains multiple copies of the 18S ribosomal RNA gene, spliced leader sequences and copies of a 196 bp repeat (Wagner and So, 1992).

Despite the diversity of extrachromosomal DNA found in kinetoplastids a unifying feature is that in each case the origins of these elements lie in regions on endogenous chromosomes. The most plausible models suggest that extrachromosomal DNA is generated by over-replication and is maintained either as a linear or closed circular molecule (Beverley, 1991). As in mammalian cells, extra-chromosomal DNA tends to be mitotically unstable and upon removal of drug selection, the amplicons are rapidly lost from a population (Olmo *et al.*, 1995). However, it has also been described that, after prolonged drug exposure, circular extrachromosomal DNA became mitotically stable without alteration in size and sequence (Garvey and Santi, 1986). Exposure of *Leishmania donovani* to the enzyme inhibitor mycophenolic acid resulted in a linear extrachro-mosomal 290 kbp – amplicon containing multiple copies of the gene encoding the targeted enzyme inosine mono-phosphate dehydrogenase and was shown to be stable even in absence of drug selection (Dubessay *et al.*, 2001). In this case, it could be argued, however, that the DNA was an additional 'chromosome' rather than an extrachro-mosomal element because it arose as a result of an amplification of a large subtelomeric region of a chromo-some and also contained telomeres. These chromo-somal regions, in addition to uncharacterized internal sequences, could have provided the necessary compo-nents for replication and segregation. Integration into a chromosome by recombination also renders extrachro-mosomal DNA stable in *Leishmania* (Beverley *et al.*, 1984). Alternatively, a process of neo-centromerisation, described for yeast and mammalian chromosomal frag-ments (Choo, 2000), could provide extrachromosomal DNA, particularly segments containing repetitive, non-transcribed regions, with centromeric properties by epige-netic mechanisms.

In this study we describe the first identification of naturally occurring nuclear extrachromosomal DNA in *T. brucei*. Our data suggest that the majority of this DNA exists in a closed circular form and is mainly composed of an imperfect repeat unit of 41, 40 and 32 bp. Because this repeat can be cut with the restriction enzyme *Nla*III, it was termed the *Nla*III-repeat element (NR-element). It has been identified in all field isolates we investigated and in most laboratory strains of *T. brucei*. It was, however, not detected in some particular laboratory strains, most nota-bly the genome project reference strain TREU 927. It was also not detected in *T. congolense* and *T. vivax* isolates.

Results

Characterization of the NR-element sequence

Originally, the NR-element-containing clone pCA15 was isolated from a cDNA library created from a multidrug resistant strain of bloodstream form *T. brucei brucei* CP547. The association of this DNA with the resistance phenotype, however, turned out to be fortuitous and there was no correlation with drug resistance (H. R. Jamnadass and N. B. Murphy, unpublished data). In addition we have been unable to detect transcripts of the NR-element and it appears likely that the clone constitutes a genomic con-tamination of the cDNA library. All further characterization of the NR-element was done with procyclic *T. brucei* 427 unless indicated otherwise.

The NR-element lacks recognition sites for most of the commonly used restriction enzymes with the exception of *Nla* III (CATG′). Partial digestions of *T. brucei* 427 procy-clic genomic DNA with the restriction endonuclease *Nla*III and detection of the restriction analysis after Southern blotting using the cDNA clone pCA15 revealed a highly repetitive DNA pattern (Fig. 1). Our estimates indicate that the repetitive regions cover at least 4 kb of continuous DNA (Fig. 1A). The size differences between adjacent bands are somewhat irregular with either approximately 40 bp or 70 bp steps. In completely *Nla*III digested DNA two major bands of 120 and 160 bp are visible, but also minor signals of greater length (0.5 kbp, 0.75 kbp, 0.88 kbp) (Fig. 1B). This indicates either an interruption of the repetitive sequence by other sequence elements or a certain degree of degeneracy within the repeat, leading to the abolition of the *Nla*III restriction site. Sequence analysis of the pCA15 plasmid, that contains approxi-mately 400 bp of the NR-element, revealed the molecular basis for this characteristic restriction pattern. The
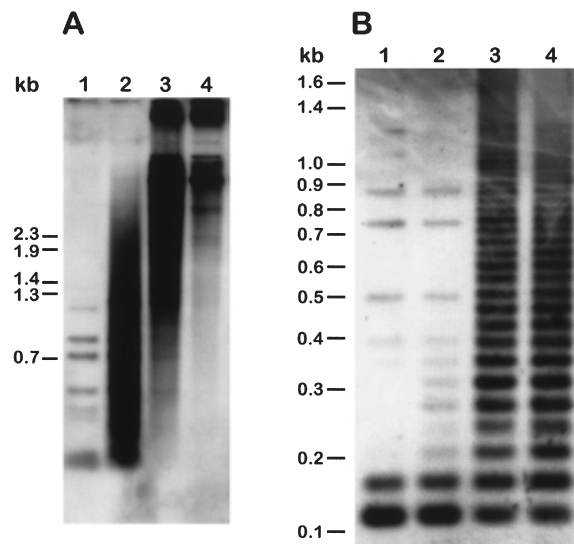

Fig. 1. Partial digestion of *T. brucei* genomic DNA with *Nla*III. 2 µg of DNA were digested with decreasing amounts of enzyme (20, 2, 0.2, 0.02 units). The restricted DNA was separated either on a 1% (A) or 1.7% (B) agarose gel, blotted and probed with digoxigenin-labelled NR-element DNA.

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NR     ACATGAACCTGATAACACCCTATCCCCCACTAACCCAAAGCACATGAACCCGAAAACACA
       AC  GAACC G TAAC CC  A C CCC  TAACCC AA C    T AACCCGAAA CAC
29bp   ACCCGAACCCGCTAAC-CCGAAACACCC--TAACCCGAACCCGCT-AACCCGAAA-CACC

NR     CTAACCCGAAACACCTGAAACCGAAAACACCATATCCC---CCA-CTAACCCGAAACACC
       CTAACCCGAA C CT AA CCGAAA CACC TA CCC   CC  CTAACCCGAAACACC
29bp   CTAACCCGAACCCGCT-AACCCGAAA-CACCCTAACCCGAACCCGCTAACCCGAAACACC

NR     TGAAACCGAAAACAC--ACT--AACCCCCACTAACCCAAAGCACATGAACCTGATAACAC
        AA CCGAA C C AC   AA C CC CTAACCC AA C   TGA C TGA AACAC
29bp   CTAACCCGAACCCGCTGACCCGAAACACC-CTAACCCGAACCCGCTGATC-TGA-AACAC

NR     CCTATCCC---CCA-CTAACCCAAAGCACATGAACCCGAAAACACACTAACCCGAAACAC
       CCTA CCC   CC CTAACCC AA CAC   AACCCGAA C C CTAACCCGAAACAC
29bp   CCTAACCCGAACCCGCTAACCCGAAACACCCTAACCCGAAC-C-CGCTAACCCGAAACAC

NR     CTGAAACCGAAAACAC-A--CT-AACCCCCACTAACCCAAAGCACATGAACCTGATAACA
       C  AA CCGAA  C C A  C  AA C CC CTAACCC AA C   T AACC GA AACA
29bp   CCTAACCCGAACCCGCTATCCCGAAACACC-CTAACCCGAACCCGCT-AACCCGA-AACA

NR     CCCTATCCC----CCACTAACCCAAAGCACATGAACCCGAAAACACACTAACCCGAAACA
       CCCTA CCC     CC CTAACCC AA CAC   AACCCGAA C C CTAACCCGAAACA
29bp   CCCTAACCCGAACCCGCTAACCCGAAACACCCTAACCCGAAC-C-CGCTAACCCGAAACA

NR     CCTGAAACCGAAAACACCATATCCCCCACTAACCCAAAGCGGAA
       CC  AA CCGAA  C C A ACC  AC ACCC A C GAA
29bp   CCCTAACCCGAACCCGCTA-ACCCGAAAC--ACCCTGACCCGAA
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Fig. 2. Comparison of the NR-element nucleotide sequence of clone pCA15 with the subtelomeric 29 bp repeat found on megabase chromosomes of *T. brucei*. The tandemly arranged 29 bp repeat is underlined with an alternating dashed and solid line. The *Nla*III restriction sites in the NR-element are marked bold and underlined, the degenerate *Nla*III-sites are marked bold. The telomere signature contained within the 29 bp repeat is boxed. The 29 bp sequence was obtained from the *T. brucei* Genome Project at the Sanger Centre, sequence identification no. is Tryp1.0.17923. The NR-element sequence is listed as the reverse complement of the database entry AJ427450.

sequence can be dissected into unit repeats of 41, 40 and 32 bp (Fig. 2), but owing to a single nucleotide replacement the *Nla*III recognition site is not present in each repeat. This sequence feature explains the irregular banding patterns observed in partial digests. Database searches revealed significant (~70%) sequence similarities with the 29 bp repeat DNA which is found in subtelomeric locations of megabase chromosomes bearing VSG expression sites and also on minichromosomes carrying silent subtelomeric VSG genes (van der Ploeg *et al.*, 1984b; Weiden *et al.*, 1991). In contrast to the 29 bp repeat, whose characteristic motif is the occurrence of one copy of a telomeric repeat unit (CCCTAA) per unit repeat, the NR-element contains only imperfect copies of telomeric repeat units (Fig. 2). Quantification of comparative Southern blots of cloned and genomic *Nla*III-digested NR-element indicates the presence of approximately 1.5×10^5 copies of the NR-element repeat unit (H. R. Jamnadass and N. B. Murphy, unpublished data). This corresponds to approximately 6% of the diploid genomic DNA content of *T. brucei*.

Genomic organization of the NR-element

Intact chromosomes of trypanosomes can be resolved according to their size by pulsed-field electrophoresis. We therefore employed this technique to characterize the relation of the NR-element to the well-characterized karyotypic distribution of megabase-, intermediate- and minichromosomes. Previous work has shown that an exact description of karyotypic patterns requires the establishment of clonal cell lines of trypanosomes (Alsford *et al.*, 2001). Older populations, that have been maintained in culture for longer periods of time, develop a considerable size heterogeneity of individual chromosomes as a result of loss or gain of non-coding regions within internal regions of chromosomes or expansion or contraction of telomeric sequences. Hence, statements about chromosomal maintenance and mitotic stability are difficult to make if based on chromosomal banding patterns in nonclonal populations.

Hybridization of the NR-element to *T. brucei* 427 chromosomes, which had been separated by contour-clamped homogeneous electric field pulsed field gel electrophoresis (CHEF-PFGE), confirmed *in situ* hybridization observations (see below) that the repeat was not present on the MCs (Fig. 3). Instead, the NlaIII repeat is located on DNA that migrates in the megabase/intermediate region during CHEF-PFGE.

All six clones analysed shared the same MBC molecular karyotype, as revealed by ethidium bromide staining and telomere oligonucleotide hybridization (Fig. 3A and B). In contrast, hybridization of the NR-element probe to these clones produced six unique hybridization patterns (Fig. 3C). Intriguingly, several of the bands produced by hybridization with the NR-element probe could not be aligned with either megabase- or intermediate chromosomes that hybridized with the telomeric probe. Furthermore, these hybridizing bands were not visible on ethidium bromide stained gels. This surprising observation could potentially be explained by the possibility of an unusual configuration of the NR-element leading to an aberrant separation pattern during PFGE. Such a phenomenon has been described for the migration behaviour of circular DNA relative to linear DNA during PFGE. Circular DNA does not co-migrate with similar sized linear
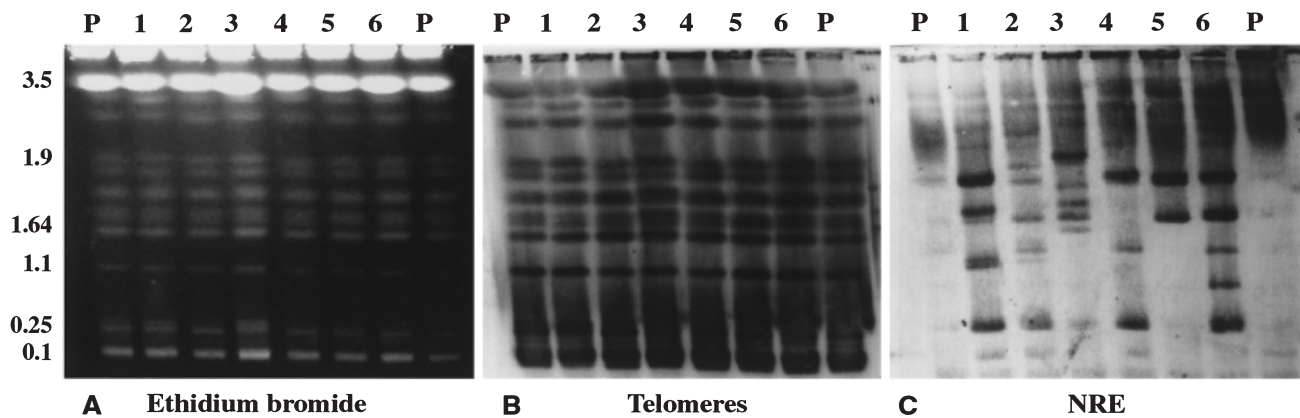
Fig. 3. PFGE-analysis of genomic DNA of individual clones (lanes 1–6) of a parental *T. brucei* population (lanes P). The gel was stained with ethidium bromide (A), blotted and probed with a telomeric oligonucleotide (B) or NR-element DNA (C). Note the heterogeneity of the NR-element hybridization pattern between clones that contrasts with the uniform pattern obtained with the telomeric probe. Separation was in 1% agarose at 2.4 V cm^{-1} in 1xTB(0.1)E at 12°C, 1400–700 s switching time ramped linearly over 144 h. Size standards are in megabasepairs (Mbp).

DNA (Beverley, 1988). Instead, circular DNA is retained much more efficiently during electrophoresis. This could explain the absence of NR-element-corresponding bands in ethidium bromide stained gels as the mass ratio of chromosomal to circular NR-element DNA would be extremely uneven and the sensitivity of the ethidium bromide dye would be insufficient to stain the much smaller circular DNA that apparently co-migrates with much larger linear chromosomal DNA bands. Also, the circular nature of the NR-element would explain why it is undetectable by the telomeric hybridization probe and hence the absence of telomeres from the NR-element.

To investigate further the possible circular nature of this DNA element, as suggested by the abnormal migration in PFGE, we tagged individual NR-elements with a reporter cassette. The plasmid used for stable transformations contained two head-to-tail sequences of the pCA15 insert, separated by a unique restriction site (*Bam*HI) that allowed the linearization of this plasmid to enable insertion into endogenous NR-elements by homologous recombination. As a reporter gene we chose the hygromycin phosphotransferase gene that allowed for drug selection of transformants but also represented a unique probe for hybridization analysis of recombinant trypanosomes. The transcription of the reporter gene was driven by the procyclin promoter (Fig. 4C). We obtained several hygromycin-resistant clones and Southern blotting analysis, using the hygro-DNA as a probe, showed that insertions had occurred into genomic DNA that co-migrated with the bulk of NR-element-hybridizing DNA on PFGE gels (Fig. 4A and B). These recombinant trypanosome clones were used for further analysis. We exploited the lack of restriction sites (except *Nla*III) within the NR-element to analyse its structure. If the NR-element that had the reporter cassette (containing unique restriction sites) inserted was

circular, characteristic hybridization patterns using the hygro probe would be expected: the single *Nde*I restriction site within the hygro gene should result in a single band on a Southern blot after complete restriction because the DNA molecule would not split in two separate fragments, as would be the case if the DNA was linear. In this experiment only the hybridization pattern indicative of circular DNA was observed with five independent clones (Fig. 4B). The size range of the tagged linearized NR-element DNA was between 20 and 50 kbp. Given the observation that the tagged circular DNA was located within the bulk of the NR-element-hybridizing region we expect this to be the average size range of this extrachromosomal DNA. However, at this point we could not exclude that the tagging experiment had targeted only a circular subset of NR-element DNA, leaving linear elements undetected.

To determine the proportion of the NR-element representing this type of circular DNA within the genome, we used asymmetric PFGE that had been developed to separate circular from linear DNA. We chose electrophoresis conditions that led to a drift of linear DNA to the right of a straight line down a gel whereas the migration of circular DNA followed the straight line or skewed slightly to the left. Experiments were performed with DNA from both wild-type and tagged cell lines. Ethidium bromide staining of gels run under these conditions showed that the stainable DNA shifted to the right (Fig. 5A). Hybridization of corresponding blots with either the hygro- or NR-element probe revealed that the entire hybridizing DNA migrated straight or even slightly to the left, indicative of circular DNA (Fig. 5B and C). Hybridization of the blot with a telomeric probe showed that linear chromosomes containing telomeres migrated to the right of the gel (Fig. 5D). Importantly, telomere-containing minichromosomes that
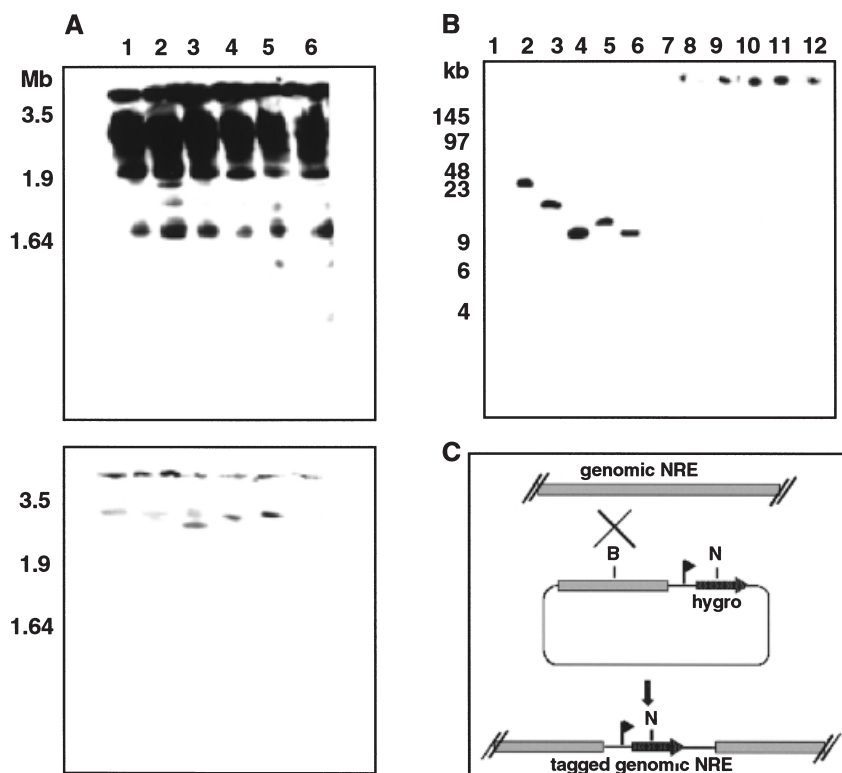
Fig. 4. Molecular tagging reveals the circular character of the NR-element.
A. PFGE analysis of transformed *T. brucei* clones. DNA of five clones is separated by PFGE and blotted and successively probed with an NR-element probe (upper panel, lanes 1–5) and a *hygro*-probe (lower panel, lanes 1–5). Lane 6 represents DNA of non-transformed *T. brucei* as a control.
B. Analysis of transformed clones after restriction of total genomic DNA with *Nde*I (lanes 1–6). The gel was blotted and probed with a *hygro*-probe. Lanes 7–12 on the gel have not been digested and lanes 1 and 7 contain DNA from non-transformed cells.
C. Schematic representation of the vector used to tag endogenous NR-element. The targeting sequence NR-element sequence containing an engineered *Bam*HI-site for linearization) is indicated by a solid grey bar. The marker gene hygro contains a *Nde*I-restriction site (N). The PARP-promoter driving the transcription of the *hygromycin*-resistance gene is indicated as a black flag. Size standards are in Mbp.

have a highly repetitive internal structure do not show the unusual migration pattern exhibited by the NR-element.

As an additional line of evidence to support the concept that NR-element is predominantly circular DNA, we analysed total cellular DNA by CsCl/ethidium-bromide equilibrium centrifugation. If the NR-element was indeed closed circular it should migrate at a higher density in the gradient. Fractionated gradients were analysed by
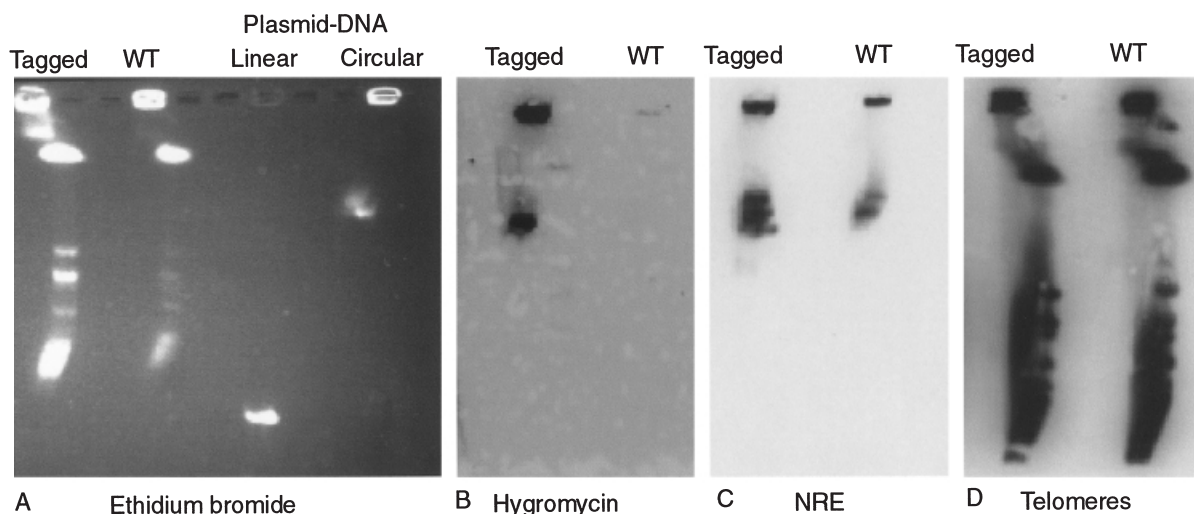

Fig. 5. Analysis of tagged and non-tagged NR-element by asymmetrical PFGE to allow separation of linear and circular DNA. Intact genomic DNA was separated as described in *Experimental procedures*. The gel was stained with ethidium bromide (A), blotted and successively probed with a *hygro*-probe (B), a NR-element probe (C) and a telomere oligonucleotide probe (D). To illustrate the different running characteristics of circular and linear DNA, plasmid DNA was separated on the same gel, either as a circular or a linearized molecule (only shown on the ethidium bromide panel). WT, wild-type (non-transformed) genomic DNA. Note that both the tagged and the total NR-element positive DNA drift to the left of the gel (B, C), whereas the linear chromosomes (telomere-containing) move to the right (D).
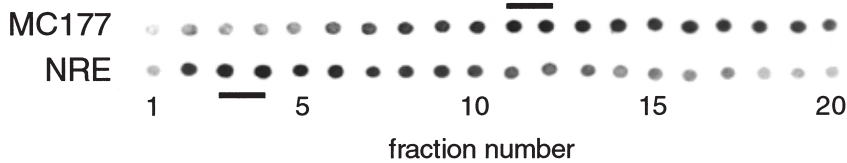
Fig. 6. Dot blot hybridization analysis of the fractionated CsCl-gradient of total genomic DNA. The density of the gradient decreases from left to right (fractions 1–20). Only the DNA-containing fractions out of the entire gradient are shown. Spots in the top row have been hybridized to the minichromosomal 177 repeat (MC177), spots in the lower row to NR-element DNA (NRE). Spots of maximum hybridization signal intensity for each probe are marked.

DNA hybridization using a NR-element probe and a minichromosomal-derived 177 bp-repeat probe. The 177 bp-probe was chosen because it represents a target on linear chromosomes whose abundance is comparable with our estimate of the proportion of NR-element DNA in the genome (approximately 5–6%). The result of this hybridization clearly showed that the NR-element DNA migrated in the gradient at a higher CsCl-concentration than linear DNA (Fig. 6). A parallel gradient, spiked with bacterial plasmid DNA, showed that the position of NR-element DNA corresponded to closed circular DNA (data not shown). The 177 bp probe hybridized to the fractions of the gradient representing the lighter, clearly visible band of the gradient that contains linear chromosomal DNA.

Taken together, these results strongly suggest that the NR-element occurs exclusively, within levels of detectability by a variety of techniques, as closed circular DNA.

Cytological characterization of the NR-element

We inferred from the intensity of hybridization signals in various Southern blotting experiments that the NR-element is an abundant sequence found in the genome of *T. brucei*. This allowed us to use fluorescent *in situ* hybridization (FISH) to characterize the distribution of this novel DNA element on a single cell level. The NR-element probe (pCA15) did not hybridize with the kinetoplast, the DNA-containing structure of the single mitochondrion in trypanosomes (Fig. 7). A strong hybridization signal, however, was observed within the nucleus. The intensity of the signal was comparable to that of minichromosomal 177 bp-FISH. As this 177 bp element represents approximately 6% of the diploid genomic DNA content, the equivalent intensity of the NR-element-FISH confirms data of quantitative Southerns blots (see above) indicating that the NR-element also represents a similar proportion of the nuclear DNA.

Fluorescent *in situ* hybridization also allowed us to monitor the distribution of this DNA during the cell cycle. During G1, S and G2 periods of the cell cycle, characterized by the occurrence of a single or double kinetoplast and one nucleus, the NR-element was distributed throughout the nucleus, excluding the nucleolus. The staining was not even, but occurred in several small clusters that showed a tendency to localize in the periphery of the nucleus (Fig. 7A–D). With the onset of mitosis these clusters condensed initially into few larger clusters (Fig. 7E) and eventually into one mass of DNA near the centre of the nucleus (Fig. 7F and G). During the process of DNA segregation, indicated by a clear elongation of the nucleus, this single mass of DNA split into two entities of approximately equal size (as judged by signal intensity) and was distributed into the emerging daughter nuclei. This staining pattern, particularly during mitosis, was very reminiscent of the pattern we had described for the distribution of minichromosomal DNA during the cell cycle (Ersfeld and Gull, 1997). We have previously shown that small linear chromosomes (the minichromosomes) have a rather different distribution during the cell cycle than the megabase chromosomes. Minichromosomes are, however, associated with the mitotic spindle and mitotic segregation depends on an intact spindle apparatus. To compare the distribution of both unusual DNA elements during the cell cycle we analysed the distribution of the minichromosomal 177 bp repeat and the NR-element simultaneously using double-labelling, two colour FISH in combination with confocal microscopy (Fig. 8). In interphase nuclei the two signals clearly did not overlap. In contrast, the territories of the two elements were almost mutually exclusive (Fig. 8A–D). Optical sectioning and 3D analysis of signal distribution in representative nuclei confirmed this result (Fig. 8, D1–4). To analyse the dynamics of the NR-element during mitosis in more detail, we first used a combined immunolabelling and FISH technique to detect the mitotic spindle and the NR-element. The mitotic spindle was stained using the monoclonal antibody KMX, recognising β-tubulin. After the immunostaining and a post fixation step to stabilize the antigen–antibody complex, FISH was used to detect the NR-element. Similar to what we have described for minichromosomes previously, the NR-element was closely associated with the spindle throughout mitosis (Fig. 8E–H). However, a detailed analysis of mitotic configurations of minichromosomal- and NR-element by double-labelling FISH we observed that during the spindle elongation phase (anaphase B) the two signals clearly did not overlap to any great extent, although a co-distribution along the spindle axis was evident (Fig. 8K). Even during early and very late stages of mitosis, when both DNAs are
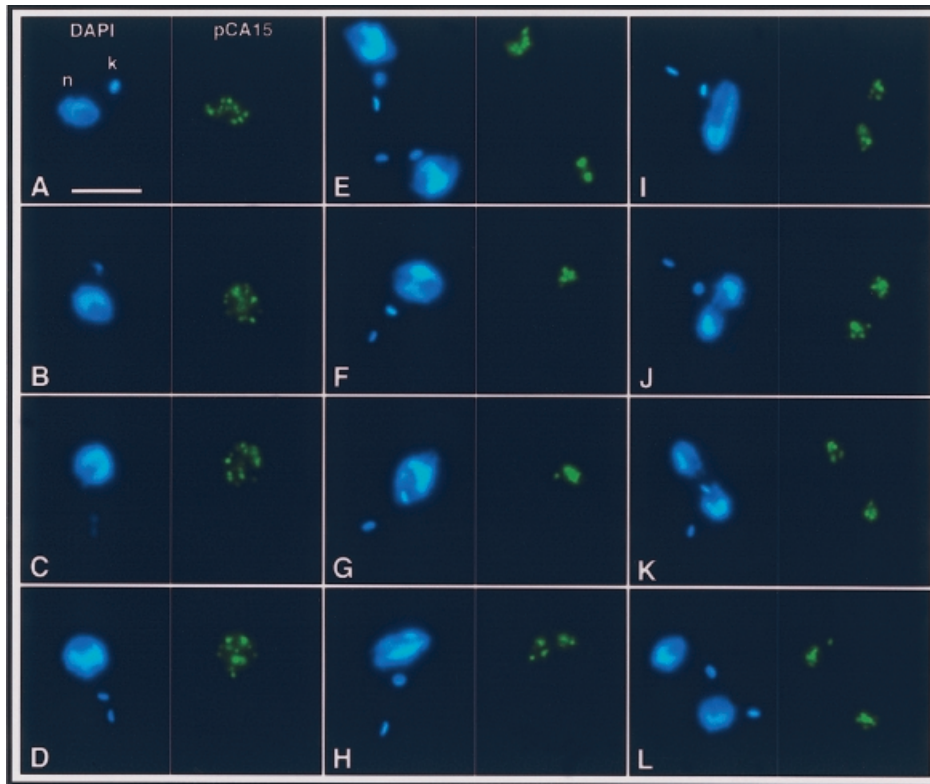
Fig. 7. FISH-analysis of the distribution of the NR-element (pCA15, green) in interphase and mitotic nuclei (blue). Nuclei (n) and the mitochondrial kinetoplast DNA (k) are counterstained with DAPI. The elongating shape of the nucleus (in E) indicates that cells are entering mitosis. Chromosomes of *T. brucei* do not condense during mitosis. A–D, interphase cells; E–F, prophase; G, metaphase; H–K, anaphase; L, telophase. Scale bar, 2 μm.

concentrated in single clusters, only a partial, albeit considerable overlap was detected (Fig. 8I, J and L). These sets of data demonstrated that minichromosomes and NR-element are distinct elements within the nuclear genome. They also provided cytological evidence for a non-random, spindle-dependent segregation mechanism of the NR-element. This suggested a high degree of stability of the NR-element. It was subsequently confirmed by PFGE karyotyping that the NR-element hybridization patterns are stable over at least 120 days (or approximately 360 generations) of continuous cell culture (data shown in Alsford, 1999).

The NR-element in field isolates and laboratory stocks of T. brucei

Finally, we investigated the distribution of the NR-element in various field isolates and several culture-adapted lab strains of *T. brucei*. Genomic DNA from trypanosomes recovered from cattle infections in Uganda, Kenya and Zambia (kindly provided by Dr G. Hide, University of Salford) was digested with *Nla*III, Southern-blotted and hybridized with NR-element DNA. Patterns similar to that observed for *T. brucei* 427 were observed for each isolate (Fig. 9). Human serum sensitive and resistant isolates were included in this analysis but no correlation between serum sensitivity and occurrence of NR-element DNA was observed. Included in this analysis was also DNA isolated from *T. brucei* strain TREU 927, a culture adapted cell line. Surprisingly, the NR-element was not detected in this strain. We confirmed this result by FISH analysis (data not shown).

We expanded the analysis of different laboratory stocks of *T. brucei* by analysing PFGE-separated DNA from various stocks (the blots were kindly provided by Dr S. Melville, University of Cambridge) (Fig. 10). This confirmed that the NR-element is undetectable in TREU 927, and also in STIB 386 and ISTat 1.1. In addition to s427, the NR-element is present in STIB 247, WRATat 1.24, EATRO 795 and EATRO 2340. Although the NR-element is absent from isolates of *T. vivax* and *T. congolense* we analysed, however, we cannot, at this stage, exclude its presence from these trypanosomes in general. Also, the presence of similar elements with a non-crosshybridizing sequences is possible.
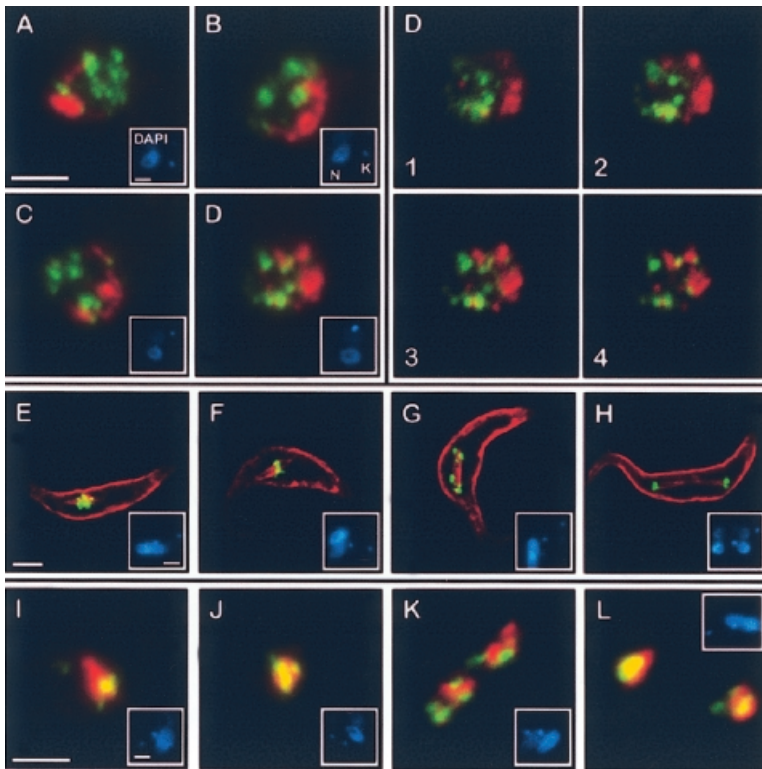
Fig. 8. A–D, dual labelling FISH showing the distribution of the minichromosomal 177 bp-repeat (red) and the NR-element (green). Maximum intensity projections of four cells after optical sectioning by confocal microscopy are shown. The DAPI staining pattern of total nuclear and kinetoplast DNA is shown in the inserted box. To demonstrate the differential distribution of minichromosomes and NR-element throughout the nucleus four single optical Z-axis sections of the nucleus projected in D are also shown (D1–4).
E–H. Combined FISH and immunofluorescence showing the association of the NR-element with the mitotic spindle. Cells were immunostained using a monoclonal against tubulin (red) and then subjected to FISH using the NR-element probe (green). The tubulin staining reveals the subpellicular cytoskeleton of the cells and the position of the microtubules of the spindle. Mitotic stages from metaphase (E) to late anaphase (H) are shown.
I–L. Dual labelling FISH demonstrating the differential distribution of the NR-element (green) and the 177 bp DNA of minichromosomes (red). Although a partial overlap of signals is detected during stages when both DNA elements congress either in the centre of the nucleus at metaphase (I, J) or at the spindle poles in late anaphase (L) are clear distinction between the signals can be seen during the segregation process in early anaphase (K). Scale bar, 2 μm.

Discussion

We have identified a novel type of extrachromosomal DNA in the parasite *Trypanosoma brucei*. It represents the first report of nuclear extrachromosomal DNA in this particular kinetoplastid. The NR-element represents a new class of extrachromosomal DNA. Its occurrence is widespread both in culture-adapted laboratory strains of *T. brucei* but also in all field isolates analysed so far. There appears to be no obvious correlation with any phenotype in the parasite, in particular it is not associated with gene amplification events caused by drug exposure. Furthermore, the NR-element is mitotically stable over many generations under non-selective culture conditions. Other types of extrachromosomal DNA, such as the dihydrofolate reductase amplicon in *Leishmania*, are rapidly lost after removal of methotrexate as a selective drug.

No sequence elements other than the repetitive elements containing the *Nla*III-restriction site have been identified within the NR-element. The repeat itself is unique to this element and shows a PFGE-hybridization pattern that is distinct from the karyotype of the linear chromosomes that have been described for *T. brucei* 427 (Melville *et al.*, 2000). This was particularly apparent under PFGE conditions that separate circular from linear DNA. We have applied five criteria leading us to the conclusion that the NR-element exists as closed circular DNA. First, the band-

ing pattern of NR-element hybridization of PFGE blots does not match that of known chromosomal markers, such as telomeric DNA. Second, these aberrant bands on PFGE gels cannot be stained using ethidium bromide, suggesting that the NR-element-hybridizing bands represent much smaller DNAs in comparison to co-migrating linear DNA markers. This is typical for the separation characteristics of circular DNA relative to linear DNA. Third, using PFGE conditions which exploit the differences in migration behaviour of circular and linear DNA in alternating electrical fields we showed that the NR-element pattern follows the migration characteristics of circular DNA. Fourth, diagnostic restriction analysis of tagged genomic NR-element showed the restriction patterns predicted for covalently closed circular DNA molecules. Fifth, the NR-element exhibits a typical migration pattern for closed circular DNA in CsCl/ethidium-bromide equilibrium centrifugation.

Given our experimental evidence, it is of interest to note that over 20 years ago Riou and co-workers noticed the presence of heterogeneous circular DNA molecules when they analysed naturally occurring dyskinetoplastic isolates of *Trypanosoma equiperdum* (a species related to *T. brucei*) using gradient centrifugation (Riou and Pautrizel, 1977; Riou and Saucier, 1979). It was assumed at that time that this DNA was an unidentified component of the mitochondrial genome. Importantly, these authors
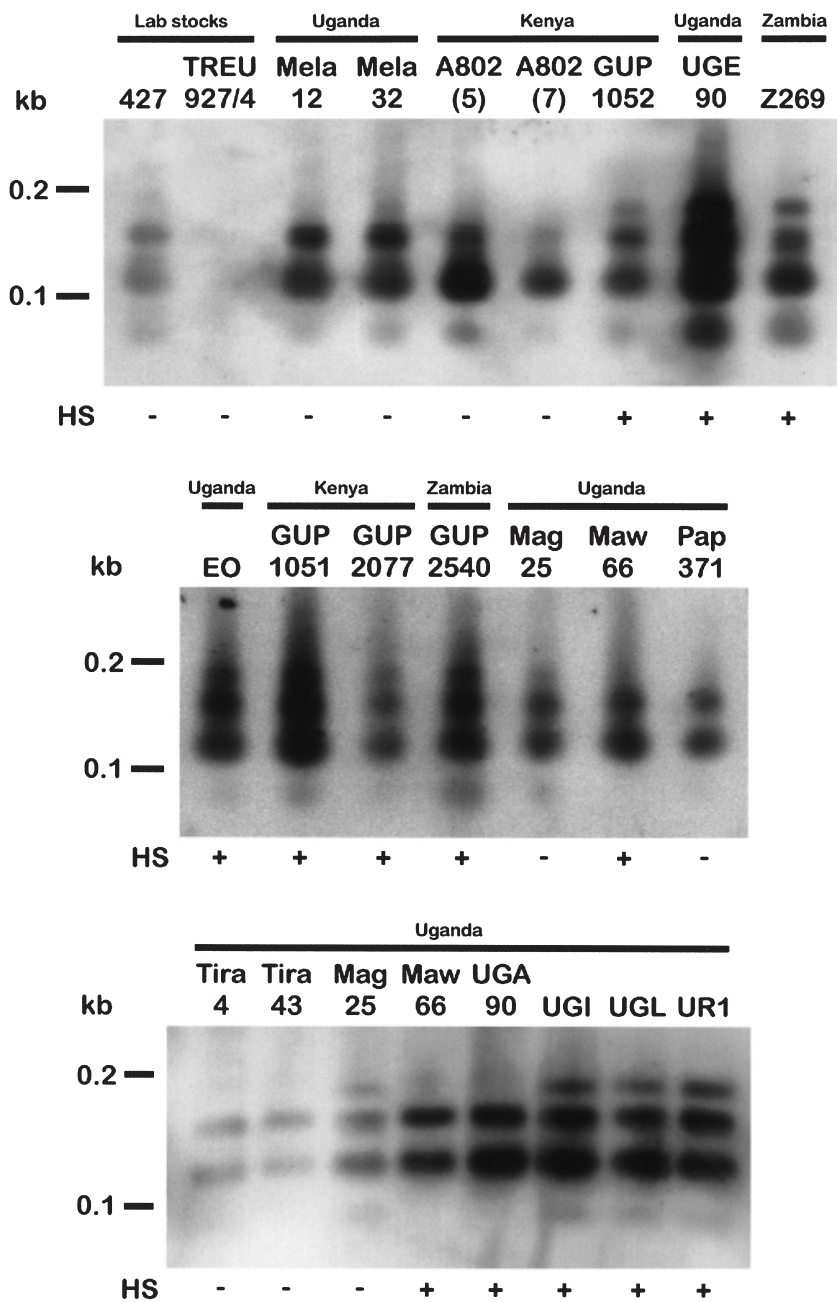
Fig. 9. Presence of the NR-element in field isolates of *T. brucei*. Genomic DNA was restricted with *Nla*III, separated, blotted and hybridized with an NR-element probe. The sensitivity of individual isolates against lysis by human serum is indicated below the blots (HS±).

reported that it was not cut by a series of restriction enzymes (*Nla*III was not tested) and they estimated that this DNA represented about 6% of the total genomic DNA. In the light of our findings, however, it appears plausible that these DNA molecules were the equivalent of the *T. brucei* NR-element in *T. equiperdum*.

In *T. cruzi* circular extrachromosomal DNA containing ribosomal RNA genes and spliced leader sequences have been identified. Over the last years, particularly since the establishment of the *T. brucei* genome project in 1997, a wealth of karyotype data for different strains of Trypano-

somes based on hybridization with a large number of well characterized genes and other DNA sequences have been accumulated and published (Melville *et al.*, 1998; 2000; Hope *et al.*, 1999). All previous studied probes, including rRNA and spliced leader genes, have given hybridization signals that could be assigned to one of the known chromosomes of *T. brucei*. Therefore the presence of the NR-element cannot simply be explained by circularization of chromosomal segments. An intriguing observation is the absence of the NR-element from some laboratory strains of *T. brucei*. Particularly significant is the
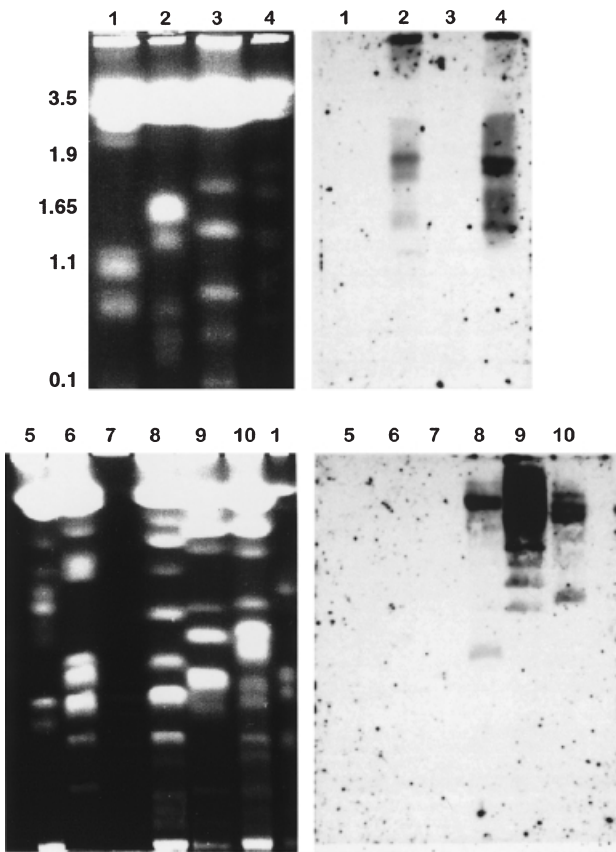
Fig. 10. Distribution of the NR-element in laboratory strains of *T. brucei*, *T. vivax* and *T. congolense*. Lanes 1–5 and 8–10, *T. brucei* TREU 927, STIB 247, STIB 386, 427, ISTat 1.1, WRATat 1.24, EATRO 795, EATRO 2340; lane 6, *T. vivax* ILDat 1.2; lane 7, *T. congolense* ILNat 3.1. Genomic DNA was separated by PFGE, gels stained with ethidium bromide, blotted and hybridized with labelled NR-element DNA. Size standards are in Mbp. Separation conditions are as in Fig. 3.

absence in strain TREU 927. This strain has been chosen as the reference cell line for the *T. brucei* genome project. Consequentially, our inability to find any NR-element sequences in the databases is likely to be explained the fact that the vast majority of sequence data is derived from strain 927. In particular, the abundant presence in field isolates points to a role of the NR-element in the dynamics of the trypanosome genome and it might also be a contributing factor to the relatively large variations of DNA content observed for different isolates of *T. brucei* (Kanmogne *et al.*, 1997). The discovery of the NR-element emphasises the necessity of thorough genomic characterization of diverse trypanosome isolates to avoid a biased view based on the genome of just a single culture-adapted, laboratory strain of *T. brucei*.

Using FISH we established that the cytological basis for the NR-element stability is a close association with the spindle during mitosis. The distribution during mitosis resembles that of minichromosomes but a detailed anal-

ysis showed that NR-element and minichromosomal DNA occupy distinct territories during most stages of the cell cycle. The presence of long stretches of repetitive DNA is, however, a common feature shared with minichromosomes (Weiden *et al.*, 1991). Although different in sequence and unit repeat length it might be possible that both repeats are involved in segregation and represent a kind of holocentric centromeric sequence sharing the same mitotic segregation mechanism. Alternatively, a hitchhiking mechanism, akin to that recently proposed for Rep20-containing plasmids in *Plasmodium falciparum* could explain mitotic stability. The insertion of the *Plasmodium falciparum* Rep20 subtelomeric repeat into a plasmid vector leads to a significant increase in mitotic stability of this circular episome after transfection into the parasite (Scherf *et al.*, 2001; O'Donnell *et al.*, 2002). A possible explanation for this improved stability is the physical tethering of the plasmid, via the Rep20 DNA, to the subtelomeric regions of chromosomes during the cell cycle, as demonstrated by FISH experiments. This phenomenon resembles the close association of the NR-element with minichromosomes during mitosis. The cytological data show that the NR-element uses the mitotic spindle for segregation. However, this stability over time not only requires faithful segregation but also a functioning replication mechanism. The occurrence in wild-type trypanosomes and the mitotic stability does not necessarily indicate that this DNA is advantageous for the parasite. Other well characterized extrachromosomal elements, such as the 2 μm-plasmid in yeast, do not appear to confer any selective advantage. The 2 μm-plasmid is also mitotically stable by exploiting the host segregation machinery (Velmurugan *et al.*, 2000; Mehta *et al.*, 2002).

The significant similarity with the *T. brucei* chromosomal subtelomeric 29 bp repeats could indicate a functional relation between the circular NR-element and the ends of linear chromosomes. Repeats of similar length have been identified in a variety of organisms in a subtelomeric location. Recent data obtained for possible functions of subtelomeric regions in *Plasmodium falciparum* suggests an important role of these repeats in clustering of chromosomal regions involved in antigenic variation (Scherf *et al.*, 2001). Their position coincides with a boundary separating the stable telomeric region of chromosomes against recombinationally active subtelomeric regions. The NR-element could have originated as by-products of recombinational events that are frequent at the terminal regions of trypanosomal chromosomes. Over time, such products may accumulate in the genome.

Experimental procedures

Culture and limiting dilution cloning of procyclic T. brucei

Procyclic stage cells of the culture-adapted *T. brucei* 427

were grown at 28°C in SDM79 supplemented with 10% (v/v) fetal calf serum (FCS) (Brun and Schönenburger, 1979). For cloning by limiting dilution, cells were diluted to 1 cell ml^{-1} and plated in 200 µl aliquots into 96-well plates.

DNA probe preparation

Digoxigenin or biotin-labelled DNA probes were produced from pBluescript (Stratagene) clones containing a 408 bp fragment of the NlaIII repeat or a single 177 bp minichromosomal repeat unit, respectively, by PCR using plasmid-specific primes as described (Ersfeld and Gull, 1997). Telomere-specific probes were produced by end-labelling the [GGGTTA]$_6$ telomere oligonucleotide with digoxigenin-ddUTP (Roche) using terminal transferase. Fluorescein-labelled DNA probes were generated by random priming (Gene Images kit, Amersham).

Restriction digestion of DNA

Genomic DNA was prepared as previously described (Medina-Acosta and Cross, 1993). Approximately 1 µg genomic DNA was digested with the NlaIII restriction endonuclease (New England Biolabs). Digest products were fractionated on 1 or 1.7% agarose gels in 90 mM Tris-borate, 2 mM EDTA (1 ×TBE), and stained in 2.5 µg ml^{-1} ethidium bromide for 15 min.

Pulsed field gel electrophoresis (PFGE)

Standard PFGE. Chromosome blocks were prepared as previously described (Alsford et al., 2001). Contour-clamped homogeneous electric field pulsed field gel electrophoresis (CHEF-PFGE) was carried out in a CHEF DRIII apparatus (Bio-Rad). Chromosome separation was performed in 1% agarose (pulsed field grade, Amersham-Pharmacia Biotech) in 90 mM Tris-borate pH 8, 0.2 mM EDTA (1xTB[0.1]E) at 12°C. The precise separation conditions are given in the figure legends. Following separation chromosomes were stained in 2.5 µg ml^{-1} ethidium bromide for 30 min and destained in distilled water for 30 min.

PFGE designed to distinguish circular from linear DNA. Conditions were developed to allow the separation of circular from linear DNA by asymmetric PFGE using a CHEF Mapper apparatus (Bio-Rad). This technique is based on the different mobility of circular versus linear DNA under alternating currents of different intensities and pulse lengths during PFGE. Whereas the migration of linear DNA deviates from a straight line following the loading well, circular DNA molecules show only a moderate skew under these conditions (Beverley, 1988). During the initial phase of electrophoresis chromosomes were separated using an alternating electric field angle of 120° (±60°) at 6 V cm^{-1} electrode distance with a linear ramp time of field switch of 20–100 s for 16 h. To separate circular from linear DNA, the +60° electric field was run at 6 V cm^{-1} with a linear ramp of 2.5–10 s and the −60° electric field was run at 2 V cm^{-1} with a linear ramp of 10–80 s for a further 16 h in TB(0.1xE) at 12°C.

CsCl-equilibrium gradient centrifugation

DNA was isolated from 3×10^8 trypanosomes by lysis in 5 ml 10 mM Tris-Cl, pH 8, 0.1 M EDTA, 0.5% SDS. After digestion with 100 µg ml^{-1} proteinase K at 50°C for 2 h, CsCl was added to a final density of 1.55 g ml^{-1} and ethidium bromide was added to a final concentration of 740 µg ml^{-1}. The solution was centrifuged for 22 h at 60 000 r.p.m. in a Beckman Ti70.1 rotor at 15°C. The gradient was fractionated into 100 µl fractions and analysed by DNA hybridization (dot blotting).

Southern blot analysis

Fractionated digest products and separated chromosomes were transferred to nylon membranes (Roche) by standard procedures. DNA probes were hybridized to the transferred DNA according to the manufacturer's instructions with the following amendments. The NlaIII and 177 bp repeat-specific probes were hybridized at 40°C overnight and the final washes were carried out in 15 mM NaCl, 1.5 mM sodium citrate (0.1 × SSC)/0.1% (w/v) SDS at 50°C. The telomere specific probe was hybridized at 37°C for 3 h and the final washes were carried out at 50°C in 0.5 × SSC/0.1% (w/v) SDS.

Dot blot analysis

One microlitre of DNA from the CsCl-gradient fractions was spotted on a nylon membrane, denatured with 0.4 N NaOH, neutralized and hybridized with either fluorescein-labelled NR-element DNA or 177 bp DNA. Blots were developed using a chemiluminescence based technique (Gene Images kit, Amersham).

Plasmid constructs and stable transfection of T. brucei

The plasmid pCA15 is a pBluescript plasmid containing 408 bp of the NlaIII repeat. The insert was released by digestion with SacI and HindIII and cloned into a pGEM-based vector containing the hygromycin phosphotransferase gene as a selectable marker and under the control of the trypanosome-specific procyclin promoter. A second copy of the pCA15 insert, released with EcoRV and SmaI, was cloned head-to-tail upstream of the first NlaIII-copy. The resulting construct was termed pSAM4 (see Fig. 6). A unique BamHI restriction site between the two NlaIII copies was used to linearize purified pSAM4 plasmid DNA for stable transfection of procyclic trypanosomes by electroporation using standard techniques (Beverley and Clayton, 1993). Details of the plasmid constructs can be obtained from the authors on request.

DNA–DNA fluorescence in situ hybridization (FISH)

In situ hybridization and combined immunofluorescence/FISH was exactly done as described (Ersfeld and Stone, 1999).

Epifluorescence and laser scanning confocal microscopy

Following DNA–DNA FISH and immunofluorescence, cells

were analysed in a Leica DMRB epifluorescence microscope linked to a cooled CCD camera (Photometrics), or a Leica TCS-NT laser scanning confocal microscope equipped with Kr/Ar and UV lasers. Epifluorescence images were digitally captured using the IPLab Spectrum (Signal Analytics) software package, whereas confocal images were captured using the Leica Scan Electronics and Leica TCS-NT software. All images were subsequently processed and pseudo-coloured using Adobe Photoshop 5.0.

Nucleotide sequence data

Nucleotide sequence data reported in this paper have been submitted to DDJB/EMBL/GenBank™ with the accession number AJ427450

References

Alsford, N.S. (1999) *Gene expression and genome organisation in* Trypanosoma brucei. PhD-thesis, University of Manchester, UK.

Alsford, S., Wickstead, B., Ersfeld, K., and Gull, K. (2001) Diversity and dynamics of the minichromosomal karyotype in *Trypanosoma brucei. Mol Biochem Parasitol* **113:** 79–88.

Beverley, S.M. (1988) Characterization of the 'unusual' mobility of large circular DNAs in pulsed field-gradient electrophoresis. *Nucleic Acids Res* **16:** 925–939.

Beverley, S.M. (1991) Gene amplification in *Leishmania. Annu Rev Microbiol* **45:** 417–444.

Beverley, S.M., Coderre, J.A., Santi, D.V., and Schimke, R.T. (1984) Unstable DNA amplifications in methotrexate-resistant *Leishmania* consist of extrachromosomal circles which relocalize during stabilization. *Cell* **38:** 431–439.

Beverley, S.M., and Clayton, C.E. (1993) Transfection of *Leishmania* and *Trypanosoma brucei* by electroporation. In *Methods in Molecular Biology of Parasites*. Hyde, E. (ed). Totowa: N.J. Humana Press, pp. 333–348.

Brun, R., and Schönenburger, M. (1979) Cultivation and in vitro cloning of procyclc forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Tropica* **36:** 289–292.

Choo, K.H. (2000) Centromerization. *Trends Cell Biol* **10:** 182–188.

Dubessay, P., Ravel, C., Bastien, P., Lignon, M.F., Ullman, B., Pages, M., and Blaineau, C. (2001) Effect of large targeted deletions on the mitotic stability of an extra chromosome mediating drug resistance in *Leishmania. Nucleic Acids Res* **29:** 3231–3240.

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

Ersfeld, K., and Stone, E. (1999) The simultaneous detection of proteins and DNA in cells. In *Localisation by Fluorescence Microscopy: a Practical Approach*. Allan, V. (ed.). Oxford: Oxford University Press, pp. 51–66.

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

Garvey, E.P., and Santi, D.V. (1986) Stable amplified DNA in drug-resistant *Leishmania* exists as extrachromosomal circles. *Science* **233:** 535–540.

Grondin, K., Kundig, C., Roy, G., and Ouellette, M. (1998) Linear amplicons as precursors of amplified circles in methotrexate-resistant *Leishmania tarentolae. Nucleic Acids Res* **26:** 3372–3378.

Hope, M., MacLeod, A., Leech, V., Melville, S., Sasse, J., Tait, A., and Turner, C.M. (1999) Analysis of ploidy (in megabase chromosomes) in *Trypanosoma brucei* after genetic exchange. *Mol Biochem Parasitol* **104:** 1–9.

Kanmogne, G.D., Bailey, M., and Gibson, W.C. (1997) Wide variation in DNA content among isolates of *Trypanosoma brucei* ssp. *Acta Tropica* **63:** 75–87.

Medina-Acosta, E., and Cross, G.A.M. (1993) Rapid isolation of DNA from trypanosomatid protozoa using a simple 'mini-prep' procedure. *Mol Biochem Parasitol* **59:** 327–330.

Mehta, S., Yang, X.M., Chan, C.S., Dobson, M.J., Jayaram, M., and Velmurugan, S. (2002) The 2 micron plasmid purloins the yeast cohesin complex: a mechanism for coupling plasmid partitioning and chromosome segregation? *J Cell Biol* **158:** 625–637.

Melville, S.E., Leech, V., Gerrard, C.S., Tait, A., and Blackwell, J.M. (1998) The molecular karyotype of the megabase chromosomes of *Trypanosoma brucei* and the assignment of chromosome markers. *Mol Biochem Parasitol* **94:** 155–173.

Melville, S.E., Leech, V., Navarro, M., and Cross, G.A. (2000) The molecular karyotype of the megabase chromosomes of *Trypanosoma brucei* stock 427. *Mol Biochem Parasitol* **111:** 261–273.

O'Donnell, R.A., Freitas-Junior, L.H., Preiser, P.R., Williamson, D.H., Duraisingh, M., McElwain, T.F., *et al.* (2002) A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. *EMBO J* **21:** 1231–1239.

Olmo, A., Arrebola, R., Bernier, V., Gonzalez-Pacanowska, D., and Ruiz-Perez, L.M. (1995) Co-existence of circular and multiple linear amplicons in methotrexate- resistant *Leishmania. Nucleic Acids Res* **23:** 2856–2864.

van der Ploeg, L.H.T., Liu, A.Y.C., and Borst, P. (1984a) Structure of the growing telomeres of trypanosomes. *Cell* **36:** 459–468.

van der Ploeg, L.H.T., Cornelissen, A.W.C.A., Barry, J.D., and Borst, P. (1984b) Chromosomes of kinetoplastida. *EMBO J* **3:** 3109–3115.

Riou, G., and Pautrizel, R. (1977) Isolation and characterization of circular DNA molecules heterogeneous in size from a dyskinetoplastic strain of *Trypanosoma equiperdum. Biochem Biophys Res Commun* **79:** 1084–1091.

Riou, G.F., and Saucier, J.M. (1979) Characterization of the molecular components in kinetoplast-mitochondrial DNA of *Trypanosoma equiperdum*. Comparative study of the dyskinetoplastic and wild strains. *J Cell Biol* **82:** 248–263.

Robinson, N.P., Burman, N., Melville, S.E., and Barry, J.D. (1999) Predominance of duplicative VSG gene conversion in antigenic variation in African trypanosomes. *Mol Cell Biol* **19:** 5839–5846.

Scherf, A., Figueiredo, L.M., and Freitas-Junior, L.H. (2001) Plasmodium telomeres: a pathogen's perspective. *Curr Opin Microbiol* **4:** 409–414.

Velmurugan, S., Yang, X.M., Chan, C.S., Dobson, M., and Jayaram, M. (2000) Partitioning of the 2-microm circle plasmid of *Saccharomyces cerevisiae*. Functional coordination with chromosome segregation and plasmid-encoded rep protein distribution. *J Cell Biol* **149:** 553–566.

Wagner, W., and So, M. (1992) Identification of a novel large extrachromosomal DNA (LED) in the Trypanosomatidae. *Mol Microbiol* **6:** 2299–2308.

Weiden, M., Osheim, Y.N., Beyer, A.L., and Van der Ploeg, L.H.T. (1991) Chromosome structure: DNA nucleotide sequence elements of a subset of the minichromosomes of the protozoan *Trypanosoma brucei*. *Mol Cell Biol* **11:** 3823–3834.