Intraclonal mating in *Trypanosoma brucei* is associated with out-crossing

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To examine whether mating can occur within as well as between clones of *Trypanosoma brucei*, we transformed three *T. brucei* subspecies stocks with heterologous genes conferring resistance to either hygromycin or Geneticin and carried out a series of inter- and intraclone matings in all possible double drug combinations. Double drug-resistant hybrids were recovered from three of the six out-crosses, but not from any of the three intraclone matings. However, further analysis of cloned progeny trypanosomes from one of the out-crosses using RFLP markers, molecular karyotyping and RAPD (random amplification of polymorphic DNA) produced unequivocal evidence that intra- as well as interclone mating had occurred. The progeny of interclone mating were double drug-resistant and heterozygous at 9 of 13 loci examined. In contrast, the progeny of intraclone mating had no demonstrable input of genetic material from the hygromycin-resistant parent and were similar to the Geneticin-resistant parent for most markers, except for five loci which were heterozygous in the Geneticin-resistant parent but homozygous in these clones (aldolase, THT1 glucose transporter, procyclin, tubulin and cDNA 23). In addition, PFGE showed considerable karyotypic rearrangements in these clones and loss of genetic material was evident from RAPD and VSG (variant surface glycoprotein) gene fingerprint analysis. We conclude that intraclone mating can occur in trypanosomes, but only during out-crossing, suggesting that meiosis and/or fusion are triggered by a diffusible factor.

**Keywords**: *Trypanosoma brucei*, genetic exchange, transformation, trisomy, selfing

**INTRODUCTION**

*Trypanosoma brucei* is a protozoan parasite which causes human and animal trypanosomiasis in tropical Africa and is transmitted by bloodsucking tsetse flies (genus *Glossina*). Genetic exchange was first demonstrated by Jenni et al. (1986) after co-transmission of two different trypanosome clones through tsetse flies. In this first successful laboratory cross and several subsequent ones (Gibson, 1989; Turner et al., 1990; Gibson & Garside, 1991; Schweizer et al., 1994; Degen et al., 1995), hybrid trypanosomes were detected by laboriously searching through cloned trypanosomes for those with a non-parental phenotype or genotype. The development of methods for the stable transformation of trypanosomes with heterologous genes (Ten Asbroek et al., 1990; Lee & Van der Ploeg, 1991; Eid & Sollner-Webb, 1991) led to a new approach: incorporation of selectable markers for drug resistance into the parental trypanosome clones and selection of hybrid trypanosomes by double drug resistance (Gibson & Whittington, 1993). This methodology not only facilitates the recovery of large numbers of hybrid genotypes, but also enables rare genetic exchange events to be detected. Using this approach we previously achieved one successful backcross in a series of experimental back and F1 crosses (Gibson et al., 1995) in an attempt to define the limits of genetic exchange in trypanosomes.

**Abbreviations**: *hph*, hygromycin phosphotransferase gene; neo, neomycin phosphotransferase gene; H, hygromycin-B-resistant; N, Geneticin (G418)-resistant; ALD, aldolase; GAPDH, glyceraldehyde phosphate dehydrogenase; GPI, glucose phosphate isomerase; PARP, procyclin acidic repetitive protein; PGK, phosphoglycerate kinase; PLC, phospholipase C; PYK, pyruvate kinase; RAPD, random amplification of polymorphic DNA; RNAP, RNA polymerase; SSC, standard saline citrate; TIM, triose phosphate isomerase; VSG, variant surface glycoprotein.
Extensive comparisons of parental and hybrid clones by a number of genetic techniques have elucidated many aspects of the mechanism of genetic exchange in trypanosomes. The process is non-obligatory, with both parental and hybrid clones represented among the infective salivary gland forms (metacyclics) produced by an individual fly (Schweizer et al., 1988). T. brucei is diploid throughout its life cycle and no haploid gamete stage has been detected (Shapiro et al., 1984; Tait et al., 1989; Kooy et al., 1989), although the fleeting or rare occurrence of a haploid life cycle stage cannot be ruled out. Analysis of isoenzymes and RFLPs indicates that a mitotic division probably occurs during genetic exchange: segregation and reassortment of genetic markers is observed (Jenni et al., 1986; Paindavoine et al., 1986; Wells et al., 1987; Gibson, 1989; Sternberg et al., 1989; Turner et al., 1990; Gibson & Garside, 1991; Gibson et al., 1992, 1995; Gibson & Whittington, 1993; Schweizer et al., 1994; Gibson & Bailey, 1994; Degen et al., 1995) and also a high frequency of chromosomal recombination in hybrids (Gibson et al., 1992; Gibson & Bailey, 1994). However, hybrids with a 3n DNA content have been found in four of five crosses for which DNA contents were measured (Paindavoine et al., 1986; Wells et al., 1987; Gibson et al., 1992, 1995; Gibson & Bailey, 1994), including two early crosses which did not employ selectable markers. These 3n hybrids have been interpreted as triploid, since DNA contents of independent clones clustered at 3n, rather than a range of intermediate values, and in addition, two clones analysed in depth showed trisomy for several chromosomes (Gibson et al., 1992; Gibson & Bailey, 1994). That triploidy is a frequent error of trypanosome genetic exchange indicates a mechanism involving meiosis followed by fusion of multiple haploid nuclei or haploid and diploid nuclei. Analysis of the inheritance of kinetoplast DNA (kDNA), which is the mitochondrial DNA of trypanosomes, has shown that one DNA component (maxicircles) is inherited unparentally, while the other (minicircles) is inherited from both parents (Gibson & Garside, 1990). The generation of hybrid kDNA networks implies that both cellular and mitochondrial fusion occur during genetic exchange. All the above experimental observations have been consolidated into a model in which trypanosomes undergo cell fusion and meiosis (not necessarily in that order), followed by fusion of (normally) two haploid nuclei, the rest disintegrating (Gibson, 1995).

No sex or mating type barriers to genetic exchange in trypanosomes have yet been demonstrated. A three-way cross ruled out the possibility of a simple two sex mating system (Turner et al., 1990) and all subspecies and subgroups within T. brucei, except Type I T. b. gambiense, have been successfully mated (Jenni et al., 1986; Gibson, 1989; Turner et al., 1990; Gibson & Garside, 1991; Schweizer et al., 1994; Degen et al., 1995). Other lower eukaryotes have multiple mating types, determined by loci which encode or control production of specific pheromones; such systems ensure that mating occurs only between organisms of different mating types and hence favour out-crossing. The question remains whether trypanosomes have mating types and can thus recognize ‘strangers’. If incapable of distinguishing self from non-self, then intraclone mating should occur. Experiments in which clones heterozygous for isoenzyme markers were singly transmitted through tsetse provided no evidence of intraclone mating, i.e. conversion of heterozygous markers to homozygosity (Tait et al., 1989). However, putative products of self fertilization were found among hybrid trypanosomes from an experimental cross: five clones, which were identical to parent A for homozygous markers, showed homozygosity at one or more loci where parent A was heterozygous (Tait et al., 1993, 1996). These results tentatively suggested that intraclone mating could occur among out-crossing trypanosomes.

Here we have searched for intraclone mating events by incorporating drug resistance markers into otherwise identical clones of the same trypanosome stock and selecting for double drug-resistant hybrids following cotransmission through the tsetse fly.

METHODS

Trypanosomes. The cloned transformants used for crossing are listed in Table 1. Hygromycin resistance (H) was obtained by transformation with plasmid construct pBnsp-H-T (Lee & Van der Ploeg, 1991) carrying the hph gene and Geneticin (Life Technologies) resistance (N) by transformation with plasmid construct pUCTbneo3 (Ten Asbroek et al., 1990) carrying the neo gene using the procedures previously described (Gibson & Whittington, 1993). Each antibiotic resistance gene was integrated into one of the two allelic tubulin gene clusters by homologous recombination.

Crosses. All pairwise H X N combinations of the six cloned transformants were cotransmitted through tsetse flies as described previously (Gibson, 1989) (Table 2). In addition, the six transformants were singly transmitted during the same experimental series; one batch of cryopreserved bloodstream form stabiles was used for all infective feeds of each clone. Each cage of infected flies (35–45 male Glossina morsitans) was maintained on membrane-fed sterile horse blood, except for a weekly feed on two clean, anaesthetized (Hypnorm, 1:2 ml kg⁻¹ i.p.) mice, commencing 16–17 days after the infective feed. These mice were monitored for parasitaemia; trypanosomes were harvested when numbers were > 10⁷ (ml blood⁻¹) and transformed to procyclics in Cunningham’s medium (Cunningham, 1977) with 20 mM HEPES (CM) and 20 µg gantamycin ml⁻¹; these procyclic populations were then tested for drug resistance as below. Flies were finally dissected 8 weeks after infection. Infected salivary glands were inoculated into immunosuppressed mice (200 mg cyclophosphamide kg⁻¹) and the corresponding infected midguts were cultured in CM with 100 µg gantamycin ml⁻¹. Bloodstream forms from infected mice were harvested when parasitaemia exceeded 10⁷ (ml blood⁻¹) and were transformed to procyclics in CM.

Drug tests. Midgut procyclics were tested for resistance to Geneticin and/or hygromycin at 50 µg ml⁻¹ in CM in a microtitre plate format. Bloodstream forms from mice were transformed to procyclics and tested for drug resistance in 24-well plates in the same way. Plates were generally examined every 1 or 2 days for 2 weeks and individual wells passaged as necessary.
Flow cytometric analysis. Samples of procyclines were fixed, stained and analysed using an EPICS-CS flow cytometer (Coulter Electronics) as described previously (Gibson et al., 1984) using a quartz tip to stabilize the particle stream. The DNA contents of 12 clones from cross B were measured relative to parental clones 058H and TH2N prepared at the same time. Each sample was analysed three times, giving a cumulative total of 90 000 trypanosomes of each clone.

Analysis of trypanosome samples. Samples for PFGE were made by lysing and deproteinizing trypanosomes in situ in agarose blocks as described by Van der Ploeg et al. (1984). Samples of DNA for restriction analysis were prepared by a standard method (Van der Ploeg et al., 1982). Genomic DNAs were restricted and size-fractionated using 0.7 or 1.5% (w/v) agarose gels. Size separation of chromosomal DNAs was carried out using an LKB Pulsaphor system with a hexagonal electrode array and five-phase program (Gibson et al., 1992) or a Biorad CHEF DR II system using a series of ramped pulses from 60 to 1800 s.

Gels were blotted in the standard way (Southern, 1975) and nitrocellulose filters were hybridized as previously described (Gibson et al., 1988) with the following oligo-labelled DNA probes: (1) neo gene amplified from plasmid pg3neo by PCR; (2) Hph gene HindIII–Scal fragment containing the gene from plasmid pBabe; (3) β-tubulin, insert from cDNA clone pTb/T-C2 (Thomashow et al., 1983); (4) variant surface glycoprotein (VSG) 117, 1.5 kb PstI fragment from cDNA clone TCV-117.5 (Bernards et al., 1981); (5) purified 12 kb EcoRI fragment from kinetoplast maxi-circle DNA of T. b. rhodesiense; (6) phosphoglycerate kinase (PGK), 1.3 kb PCR-amplified insert from cDNA pTcPGK8 (Osinga et al., 1985); (7) glucose phosphate isomerase (GPI), 1.9 kb HindIII–BanHI fragment from genomic clone (Marchand et al., 1989); (8) pyruvate kinase (PYK), 1.2 kb PstI–EcoRI fragment from genomic DNA clone pTgPK5 (Allert et al., 1991); (9) cytosolic glyceraldehyde phosphate dehydrogenase (cGAPDH), 1.7 kb BamHI–PstI fragment from genomic DNA clone pTgGAPc6 (Michels et al., 1991); (10) aldolase (ALD), 1.3 kb PCR-amplified insert from cDNA pTcALD7 (Marchand et al., 1988); (11) triose phosphate isomerase (TIM), 460 bp amplified fragment spanning polymorphic KpnI site within gene from genomic clone pTgTIM12 (Swinkels et al., 1986); (12) 760 bp insert from cDNA clone TcV-117.5 region of THTl glucose transporter gene (Bringaud & Baltz, 1994); (13) 650 bp insert from cDNA 23 (De Lange et al., 1984); (14) 180 bp PCR-amplified fragment from clone puc-THT1-Tag spanning 5' region of THT1 glucose transporter gene (König et al., 1991); (15) 2 kb PstI fragment Pap5 cloned from and unique to downstream region of proA procyclin locus (subsequently designated PARP B) (ibid.); (16) 2 kb insert from procyclin (or procyclic acidic repetitive protein, PARP) cDNA clone pPRO2001 (König et al., 1989); (17) 2 kb PstI fragment Pap5 cloned from and unique to downstream region of proA procyclin locus (subsequently designated PARP B) (ibid.).
from clone pDH4 (Mensa-Wilmot et al., 1990); (19) ~ 8 kb insert from clone Trp6 containing the RNA polymerase (RNAP) III gene from T. brucei (Köck et al., 1988). All post-hybridization washes were to 0·1 x SSC (1 x SSC = 0·15 M NaCl, 0·015 M sodium citrate) at 65 °C, except for probe 4 where washes were to 3 x SSC only.

Random amplified polymorphic DNA analysis. Genomic DNAs were used as substrate for random amplification of polymorphic DNA (Welsh & McClelland, 1990; Williams et al., 1990) using eight different arbitrary primers, which produce multi-banded patterns with T. brucei subspecies (Tibayrenc et al., 1993). Primers were as follows 5'-3': 1, CAGGCCCCTTC; 2, TGGGAGCAGCT; 4, AATCGGCGCTG; 5, AGGGGCTGTCT; 3, TGAACGGGTG; 8, GTGACGTAAG; 9, GGTAACGCC; 10, GTGATCGCAG. Amplification was carried out using approximately 25 ng DNA and primers at a final concentration of 0·2 μM using a programme of 94 °C/180 s followed by 35 cycles of 94 °C/60 s, 36 °C/60 s, 72 °C/120 s.

RESULTS

Experimental crosses

Six inter- and three intraclone mating experiments were carried out (Table 2). Three of the six out-crosses (A, B, C) produced double drug-resistant (i.e. hybrid) progeny. Trypanosomes with single drug resistance only (assumed to be parentals) were found in the other six crosses attempted, including the three intraclone matings. In the three successful out-crosses, hybrids were found among trypanosomes derived from the salivary glands but not midguts of individual flies. This brings the total number of crosses in which hybrids have been found only in the salivary glands to five and strengthens the view that the salivary glands rather than the midgut are the site of genetic exchange (Gibson & Whittington, 1993; Gibson et al., 1995).

Flies were dissected at 8 weeks to allow maximum time for hybrid formation (Schweizer et al., 1988), although survival of older flies was poor, with typically 50% mortality by the end of the experiment. To monitor the development of infectivity, flies were fed on clean anaesthetized mice weekly from approximately 2 weeks post-infection. The resultant trypanosome populations from cotransmission experiments were drug tested to ascertain the time of first appearance of double drug-resistant trypanosomes. In the single transmissions, four of the transformants produced infective forms by day 16/17 (Table 1); two transformants were much later however, maturing by day 23 (KP2H) or day 29 (058N). These transformants were also later maturing in the mixed transmissions (KP2H, day 23–36; 058N, day 23–30; other transformants, day 15–23) and neither was successfully crossed, possibly for this reason. Double drug-resistant hybrids were first detected on day 29/30 for crosses B and C, and on day 37 for cross A (Table 2).

From flies dissected at 8 weeks, the majority of midguts had mixed infections of both parental trypanosomes as judged by drug resistance tests (not shown). However, the majority of the corresponding salivary glands produced uniparental infections when drug tested (Table 2). In particular, TH2N was very dominant, coming through by itself in 23 of 27 flies with mature infections in crosses B, F and H. There were no noticeable differences between the parental clones, in terms of infectivity and virulence to mice or ability to transform to procyclics and grow in vitro, which would explain the predominance of one or other parent in the drug tested procyclic populations. The results can therefore be taken to reflect the true composition of the salivary gland populations.

Analysis of progeny

For crosses A–C, procyclics were cloned from cryopreserved aliquots of the original unselected and double drug-selected wells from the drug test plates; these trypanosome populations had been cultivated in mice and subsequently in procyclic culture for a combined period of 18–20 d following fly dissection. Each clone was drug tested and a PFGE sample made to compare the molecular karyotype with those of the appropriate parental clones by PFGE. The double drug-resistant clones had PFGE karyotypes which were composed of chromosome bands from both parents, thus verifying their hybrid status. In Fig. 1, clones 7–12 also have a non-parental band of approximately 1 Mb.

Progeny from cross B were examined further, since it involved a new combination of parental strains; parental transformants TH2N and 058H will be referred to as P1 and P2, respectively, in the following. Twelve clones were isolated in all, six of which from the unselected population were resistant to Geneticin only, the remainder from the selected population being double drug-resistant as expected. Of the six Geneticin-resistant (N) clones, five were found to have non-parental...
karyotypes, only clone 1 being identical to parent P1 (Fig. 1). The six double drug-resistant (H × N) hybrid clones (7–12) also had non-parental karyotypes as expected. However, by comparison, clones 2–6 showed far more alterations in the size and/or number of bands throughout the resolved size range relative to P1 and P2 (Fig. 1). Bearing in mind the low frequency and minor nature of karyotypic rearrangements seen in parental progeny clones, only clone 1 being identical to parent P1 (Fig. 1). The six double drug-resistant (H × N) hybrid clones (7–12) also had non-parental karyotypes as expected. However, by comparison, clones 2–6 showed far more alterations in the size and/or number of bands throughout the resolved size range relative to P1 and P2 (Fig. 1). Bearing in mind the low frequency and minor nature of karyotypic rearrangements seen in parental progeny clones, only clone 1 being identical to parent P1 (Fig. 1). The six double drug-resistant (H × N) hybrid clones (7–12) also had non-parental karyotypes as expected. However, by comparison, clones 2–6 showed far more alterations in the size and/or number of bands throughout the resolved size range relative to P1 and P2 (Fig. 1). Bearing in mind the low frequency and minor nature of karyotypic rearrangements seen in parental progeny clones, only clone 1 being identical to parent P1 (Fig. 1).

**DNA content**

Measurement of DNA content by flow cytometry showed that the six N clones had similar DNA contents to the parents, i.e. 2n, while the six H × N clones were 3n with respect to the parents (Fig. 2). Despite small differences between the DNA contents of the parents and between those of the clones, the clustering of values at either 2n or 3n clearly points to inheritance of DNA in packets of n, i.e. exchange of haploid nuclei. The lack of intermediate values indicates that high DNA contents are not inherently unstable during cloning and subsequent vegetative growth.

**RFLP analysis**

The 12 clones were compared with the parents by analysis of polymorphic restriction sites in genes for six

**Table 3. Analysis of progeny from cross B**

FACS: DNA content, D = 2n (diploid), T = 3n (triploid). Drug*: drug resistance phenotype. R genes: presence of genes conferring resistance to hygromycin (hph) or Geneticin (neo). PFGE: molecular karyotype parental P1 or P2, NP = non-parental. kDNA: type of maxi-circles present.

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* Bold letters indicate homozygous alleles derived from P1.
† Presence of three alleles deduced from band stoichiometry.
glycolytic enzymes (PGK, ALD, TIM, PYK, cGAPDH, GPI), the glucose transporter gene array (THT1), two cDNAs encoding unknown single copy genes (cDNA 8 and 23), the phospholipase C (PLC) gene, the procyclin genes, the RNAP III gene and a repetitive gene of unknown function conserved in *T. cruzi* (116). Complete results were obtained for clones 1-5, 7 and 8 only (Figs 3-6, Table 3). For five loci (GPI, PYK, cGAPDH, PLC, RNAP III) where both parents were homozygous for a given gene, hybrid clones 7 and 8 were heterozygous as expected, while clones 1-6 were identical to P1 with no detectable input of genetic material from P2 (e.g. Fig. 3a, b). Likewise for the PGK and 116 loci, where P1 was homozygous and P2 was heterozygous, hybrid clones 7 and 8 were also heterozygous, while clones 1-6 were indistinguishable from parent P1 (Table 3). The parental origin of alleles in the progeny clones is uncertain for the PGK genes, since both parents have allele B, but is unequivocal for the 116 genes, where clones 1–6 have C alleles from P1, and the triploid hybrid clones have a C allele from P1 and both P2 alleles (Fig. 4a).

For the remaining six loci, P1 was heterozygous. Again clones 1–6 were indistinguishable from P1 for TIM and cDNA 8, but remarkably clones 2–6 showed homozygosity at the other four loci. For the THT1 locus, the B and C alleles of P1 have segregated to homozygous BB in clones 2 and 3, and CC in clones 4–6 (Fig. 4b). The B and C alleles are unique to P1, while P2 is homozygous for a slightly larger fragment, A. The H × N clones inherited A alleles, together with either allele B (clones 9–12) or C (clones 7, 8) from P1 (Fig. 4b). This latter result was the only evidence of heterogeneity among the H × N clones.

For ALD, where P1 was heterozygous with alleles CD, clones 2 and 3 had only the D allele, while clones 4 and
5 had only the C allele (Fig. 5a). The origin of the D allele from P1 is unequivocal, but the C allele could also derive from P2; however, this is unlikely to be the case for clones 4 and 5, since no other input from P2 was demonstrated in this study. For procyclin or PARP, restriction fragments derived from the PARP A and B loci could be distinguished using the downstream Pap5 DNA probe (Konig et al., 1989), revealing homozygosity for clones 4 and 5 at the PARP B locus; it is clear that the 8 kb fragment for PARP B/allele B derives from P1 (Fig. 5b). Clones 1–3 had 8 and 12 kb fragments for PARP B (alleles B and C) like P1, while the H × N clones had alleles A and B, in what looks like a 2:1 ratio. For cDNA 23, the paired fragments of P1 and P2 are taken to represent four different alleles; clones 2 and 3 are therefore homozygous CC, while clones 4 and 5 are CD-
like P1. As for probe 116, the H × N clones clearly have three alleles for cDNA 23: A and B from P2 plus D from P1 (Fig. 5c).

In summary, four heterozygous loci of P1 show homozygosity in progeny clones 2–6. Numerous independent mutations would have been required to produce this result for four separate loci in these two sets of clones. Given the lack of demonstrable input of genetic material from P2 for the five homozygous markers (GPI, PYK, cGAPDH, PLC, RNAP III), the most simple explanation for these results is the segregation and reassortment of P1 alleles alone, i.e. intraclonal mating. Meanwhile, clone 1 was identical to P1 in all respects and there is no reason to believe that this represents anything other than asexual transmission of P1, since sexual reproduction is not an obligatory part of the trypanosome life cycle. In previous crosses, clones of parental genotype have also been transmitted with high fidelity and karyotypic rearrangements are associated far more frequently with meiotic rather than mitotic division (Gibson & Garside, 1991; Gibson et al., 1992, 1995; Gibson & Bailey, 1994).

**Chromosomal linkage of markers**

The ALD and PARP B loci were previously mapped to the same ~5 Mb chromosome (Gottedien et al., 1990) and our results map the THT1 locus to the same chromosomal location (Fig. 6a). Therefore, with the proviso that resolving power is limited in this size range, all three loci are probably on the same chromosome. Looking at Table 3, the results for ALD and THT1 both show homozygosity for clones 2–6, with linkage of alleles ALD D/THT1 B and ALD C/THT1 C. However, for PARP B, clones 2 and 3 are heterozygous like parent P1, while clones 4 and 5 are homozygous. To explain this anomaly, either the PARP B, ALD and THT1 loci are on different, comigrating chromosomes, or a crossing over event took place between the B and C alleles of PARP B during the meiotic division from which clones 2 and 3 were derived.

cDNA 23 maps to much smaller chromosomes of about 1.5–1.8 Mb, which are well resolved by PFGE analysis (Fig. 6b). Assuming that allele C resides on the smaller chromosome (1.5 Mb) of P1, the apparent loss of the larger chromosome (1.8 Mb) in clones 2 and 3 agrees with the observed homozygosity of these clones. Clones 4–6 had alleles CD and we interpret the 1.6 Mb band as a deletion product of the 1.8 Mb chromosome of P1 carrying allele D. The 1.5 Mb chromosome of the H × N clones (7–9) could derive from either parent, but the faint 1.8 Mb band is from P2 (underloaded on this gel) and there is a non-parental band of approximately 1.6 Mb.

Hybridization of a PFGE blot with a probe for the β-tubulin gene is shown in Fig. 6(c). P1 and P2 each have two homologues carrying the tubulin array, which differ slightly in size (N1/N2 and H1/H2, respectively). Judging by size, clones 2 and 3 appear to have inherited N2 only, while clones 1 and 4–6 are identical to P1 with both N1 and N2; triploid H × N clones 7–9 have H1 and probably H2 and N2 comigrating. The hph gene was previously found to be located on H1 of P2 (Gibson & Whittington, 1993), while the neo gene is on N2 of P1 (not shown). The presence of H1 or N2 with their assigned hph and neo genes concur with the drug resistance phenotypes of the clones.

**Fingerprint analysis**

Low stringency hybridization with VSG gene 117 and PCR using eight arbitrary primers (RAPD) were used to fingerprint the parental and progeny clones. These analyses not only confirmed the hybrid status of individual clones if the fingerprints were different to those of both parents, but also established which clones were identical. Hybridization with VSG gene 117 (Fig. 7) divided progeny clones 1–8 into the same groups as PFGE (Fig. 1). It can be seen that while clone 1 is identical to P1, clones 2 and 3 lack two fragments of approximately 3.5 kb relative to P1 and clones 4–6 lack fragments of approximately 2.5 and 3.5 kb (Fig. 7); neither of the prominent bands of P2 at 1.3 or 2 kb has been inherited by clones 1–6. Loss of genetic material was also clearly seen with two of the RAPD primers for clones 2 and 3 (not shown). These results are consistent with the hypothesis that clones 2–6 are products of intraclonal mating of P1, which have effectively lost allelic variation.

**kDNA**

The kDNA maxi-circle type of the 12 clones was assessed by presence (P2 type) or absence (P1 type) of an extra EcoRI site. All clones had P1 type maxi-circles without the extra EcoRI site. Preliminary RFLP analysis of kDNA minicircles of clones 1–3 and 10 did not show major differences between the minicircles of P1 and clones 1–3, although the hybrid clone 10 was shown to have some P2-derived minicircles by hybridization (M. Crow & W. Gibson, unpublished results).

**DISCUSSION**

These experiments were designed to test the hypothesis that intraclonal mating occurs in *T. brucei* subspecies. Segregants explicable as products of intracircle mating were produced in an out-cross, together with authentic double drug-resistant hybrids. However, we failed to detect double drug-resistant hybrids in three attempted matings of single clones transformed with two different drug resistance markers.

The evidence that these segregants were the products of intracircle mating rests on (1) lack of detectable input of genetic material from one parent, P2, in particular for five marker genes for which both parents P1 and P2 had unique homozygous alleles; (2) homozygosity at four loci (ALD, THT1, PARP B, cDNA 23) where P1 was heterozygous; (3) extensive reorganization of molecular karyotype; (4) homozygosity of the chromosomes...
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**Fig. 6.** PFGE size-fractionated chromosomal DNAs hybridized with probes for (a) the THT1 gene, (b) cDNA 23 and (c) the β-tubulin gene. Approximate sizes were estimated from yeast and bacteriophage λ markers.

and RAPD analysis using two primers. The generation of homozygosity at a total of five loci is hard to explain by mutation, since many independent mutations would have been required. The karyotype alterations seen in these clones were far more extensive than seen previously for progeny clones of parental genotype; in four different crosses only a handful of such clones have shown small differences of mobility in at most one chromosome band and the more usual finding is a high degree of karyotype stability, except when meiosis is involved (Gibson & Garside, 1991; Gibson et al., 1992, 1995; Gibson & Bailey, 1994). The DNA contents of these clones were normal (i.e. 2n), ruling out the possibility of wholesale chromosome loss.

Thus the simplest conclusion is that clones 2–6 were the products of mating of parent P1 alone. The hybrid-secreting fly from out-cross B therefore contained a mixture of double drug-resistant hybrids (clones 7–12), products of P1 alone (clones 2–6) and parental (clone 1) trypanosomes. Results along similar lines were reported by Tait and colleagues for another cross, where evidence rested on the isoenzyme product for tyrosine kinase, for which the genetic basis of variability is unknown, RFLP analysis of an unidentified locus and differences for three chromosomal markers in PFGE analysis (Tait et al., 1993, 1996). Taken together, the results strongly suggest that intraclone mating can take place among a mixture of trypanosomes undergoing interclone mating, but not in a single clone, as our three failed attempts to find double drug-resistant trypanosomes in crosses of the same clone bearing different drug resistance markers demonstrate. Tait and colleagues also failed to find evidence of homozygosity in heterozygous clones transmitted singly via the tsetse fly (Tait et al., 1989, 1993, 1996).

The observation that intraclone mating takes place only during sexual reproduction among heterologous trypanosomes has intriguing implications: (1) that trypanosomes distinguish self and non-self, i.e. there is a
system of mating types; and (2) that recognition of non-self triggers mating. The possible occurrence and genetic basis of mating types in *T. brucei* subspecies has not yet been examined in detail. Crosses between all three subspecies except Type 1 *T. b. gambiense*, which is difficult to transmit experimentally, have succeeded (Jenni *et al.*, 1986; Gibson, 1989; Turner *et al.*, 1990). The three-way cross of Turner *et al.* (1990) ruled out a two sex mating system. Control of mating types in a diploid organism presents particular problems (Hodgkin, 1992; Judelson *et al.*, 1993) and it will be interesting to see how this ancient eukaryote has solved them. The mating types of other protists control the secretion of pheromones for self/non-self recognition (Sleigh, 1989). Our results suggest that similar diffusible factors are at work in trypanosomes, as mediators of mating between trypanosomes of different mating types and as triggers of meiosis/fusion in trypanosomes of the same mating type. The observed segregation of alleles is unequivocal evidence of meiosis, while the fact that not all heterozygous markers of P1 became homozygous points to the fusion of haploid gametic nuclei derived from different meioses, i.e. probably fusion of two P1 individuals.

Products of intraclonal mating were not selectable in this experimental set-up, since they had the same drug resistance phenotype as one parent. The homozygosity of deleterious mutations might also be expected to reduce fitness relative to heterozygous clones. It is therefore surprising that two different genotypes of such clones were isolated in this experiment, suggesting either high levels of virulence and/or large numbers in the salivary glands. It is possible that diploid clones might out-compete triploid hybrids during vegetative growth, but doubtful whether this would also apply to the diploid parents. If the pheromone hypothesis outlined above is correct, then all responsive trypanosomes may be triggered into meiosis by diffusible factors, with the result that the majority of salivary gland trypanosomes mate. The intraclonal mating products seen in this experiment might thus simply reflect an imbalance in parental types. Indeed, it is not unreasonable to suggest that haploids might predominate in the salivary glands under these particular circumstances, although epimastigotes and metacyclics are normally diplaid (Shapiro *et al.*, 1986; Gibson, 1989; Turner *et al.*, 1990). The result that the majority of salivary gland trypanosomes was demonstrated for two previously isolated clones (Gibson *et al.*, 1992) and three alleles were demonstrated at several loci in the 3n hybrids from this experiment. Although there are slight but consistent differences in DNA content between clones, perhaps reflecting variation in the numbers of small chromosomes, all clones analysed thus far conform unequivocally to 2n or 3n; intermediate DNA contents, suggestive of random chromosome loss from a tetraploid precursor, have not been found. Therefore, it is reasonable to conclude that 3n hybrids are triploid and most likely derive from fusion of haploid and diploid nuclei, with the important corollary that meiosis must be involved. Triploidy is a commonly encountered but usually lethal problem of eukaryote zygote formation even in man, where nearly 1% of all conceptuses are triploid. Triploid trypanosome hybrids are only unusual in terms of their vigour.

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