GENETIC EXCHANGE IN TRYPANOSOMES

SUMMARY PROCEEDINGS OF AN INTERNATIONAL WORKSHOP

HELD IN NAIROBI, KENYA
6 -- 8 OCTOBER 1986

ILRAD

INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES

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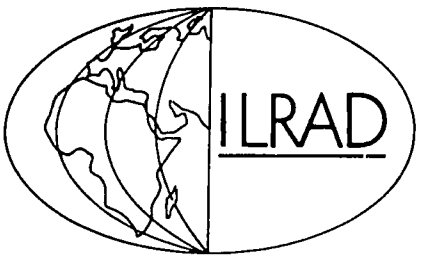

INTERNATIONAL LABORATORY FOR RESEARCH
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FOREWORD

The existence of genetic exchange, by sexual or other processes, in trypanosomes would have profound implications for aspects of the epidemiology of trypanosomiasis, especially in terms of the development of antigenic diversity and the spread of drug resistance. In 1980, Dr. J.A. Tait of the University of Edinburgh (UK) obtained indirect evidence from isoenzyme analysis of field isolates that sexual recombination might be occurring among *Trypanosoma brucei* parasites in the field. More recently, Professor L. Jenni and colleagues at the Swiss Tropical Institute in Basel succeeded in obtaining hybrid trypanosomes from two *T. brucei* stocks after simultaneous infection of the tsetse fly vector in the laboratory. This has allowed intensive investigation of the hybrid trypanosomes in several laboratories. Most of those working in this field were invited to report and discuss their most recent results at a 'Workshop on genetic exchange in trypanosomes', held at ILRAD from 6 to 8 October 1986.

In order to place these new results in an appropriate context, speakers were invited to review processes of genetic exchange in other protozoa. Workshop participants then reviewed the field evidence relating to sexual recombination in trypanosomes and current knowledge of the structure of the trypanosome genome within which exchange may occur.

Many investigators have recognized the potential value of a trypanosome transfection system for artificial genetic exchange in studies of all areas of trypanosome biology. Presentations of work relevant to the development of such systems were therefore included in the meeting.

The Organizing Committee would like to thank all the invited speakers, as well as other participants, for their contributions to the workshop. In addition, we are grateful for the help of Mr K. Nguli for projection of slides and recording the discussions, to Ms S.B. Westley for editing this report and to Ms L. Thairo, Ms M. Kanyungo, Ms C. Munyua and Ms H. Walji for typing the text. The Chairman would like to add his thanks to fellow members of the Organizing Committee, particularly to Dr J.K. Lenahan for the administrative arrangements, and to those who agreed to act as rapporteurs for the discussions.

Dr J.R. Young, Chairman
Dr V.M. Nantulya
Dr G. Knowles
Dr S.Z. Shapiro
Dr S.K. Moloo
Dr J.K. Lenahan, Administration

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SUMMARY

The first day was devoted to the presentation of background and contextual information. After discussion of genetic processes in ciliates, malaria parasites and the kinetoplastid *Crithidia*, evidence relating to the occurrence of genetic exchange in trypanosomes in the field was reviewed. During discussion, the consensus was that, while the available evidence was consistent with the occurrence of mating in trypanosomes, it was insufficient for determination of the frequency with which it occurs or the definition of interbreeding populations. Laboratory work in progress towards understanding the mechanisms of genetic exchange and developing genetic markers should make possible a more informed analysis of field data. Nevertheless, much more comprehensive analysis of carefully chosen field material would be required before the laboratory results could be incorporated into an accurate description of the process of genetic exchange in natural populations of trypanosomes.

The genomes of trypanosomes are composed of a small number of large chromosomes and many smaller chromosomes whose main function may be in the elaboration of the phenomenon of antigenic variation. The distinction between these components was emphasized in the final presentations of the day. Professor M. Steinert of the Université Libre de Bruxelles (Belgium) showed that there were a limited number of kinetochores in dividing nuclei of *T. brucei* which could account for segregation of the larger chromosomes, but that different mechanisms must operate in the segregation of the many small chromosomes. Dr M.G.-S. Lee of Columbia University (USA) then described the remarkable susceptibility of the smaller chromosomes to a variety of recombination processes that could occur during antigenic variation.

As with other parasitic protozoa, genetic exchange in trypanosomes is expected to occur at a specific stage of the life cycle. The day ended with two films on the trypanosome life cycle, in culture and in the tsetse fly, in advance of the following day's discussion on the identification of the life cycle stage(s) where exchange might occur.

The second day was devoted to detailed presentations of laboratory data concerning the production and analysis of laboratory-derived hybrids and the search for haploid trypanosomes, which would be expected if ordinary meiotic division was involved. While one report suggested that metacyclic forms of *T. brucei* might be haploid gametes, most investigators who had measured the DNA content of metacyclic trypanosomes did not concur with this finding. Trypanosomes of haploid DNA content, which might be the product of meiosis, have not been identified in any other life-cycle stages. The search needs to be continued, but may be fruitless if a classical sexual cycle is not involved.

Most hybrid analysis has been conducted with a single hybrid trypanosome clone, and all with the same parental pair. The results obtained, using several markers, showed that the hybrids were not the result of a simple meiosis/fusion cycle. The DNA content of

hybrid trypanosomes was greater than the diploid content of the parents, but less than double that amount. The DNA of the original hybrids may have been unstable, falling to the observed values while the hybrids were expanded into sufficiently large numbers to be analysed. Inheritance of kDNA appeared to be uniparental. Small chromosomes did not segregate, and their inheritance was nearly additive, an observation that could go some way to explaining the increased DNA content of the hybrids. A further problem for the simple meiosis model was provided by the observation that apparently heterozygous markers did not segregate on further fly passages although hybrids could be obtained at high frequency in the original cross.

Instead of a classical sexual cycle, these observations could be indicative of a fusion between diploids in the fly, followed by loss of part of the resulting genetic complement. Whether this was the case or not, the mechanism of this particular genetic cross was clearly complex. It was generally accepted that many crosses need to be studied, preferably between trypanosomes from geographically closer isolates, before any general conclusions are reached concerning the mechanisms of genetic exchange that might occur in the field.

On the third day, participants described studies relating to the development of artificial genetic exchange by transfection. An alternative approach to the investigation of genetic exchange, by the identification of sexual stage-specific gene expression was also discussed.

Work towards the development of a transfection system for kinetoplastidae is being carried out in several laboratories using varlous approaches. While some progress has been made and it has been shown that it is possible to introduce DNA into these organisms, many problems remain. The requirements for the stable establishment of introduced sequences have yet to be determined. Peculiarities of these organisms, for example the trans-spliced leader sequence on all mRNAs, suggest that these requirements may have to be met in unique ways.

Conclusion

Much work is still needed to determine the mechanisms of natural genetic exchange, its frequency in wild populations and its significance in the epidemiology of trypanosomiasis. Recent work has established the existence of genetic exchange and has generated the interest necessary for the required data to be obtained. This information, as well as the development of artificial genetic exchange, will produce significant advances in our understanding of trypanosome biology and will contribute towards the development of methods for the control of trypanosomiasis.

OPENING ADDRESS

A.R. Gray

When I was asked to say a few words of welcome and introduction to this meeting on genetic exchange in trypanosomes, I found it very easy to accept because I find myself repeatedly marvelling at the sudden burst of activity and progress in an area which has been a nagging problem for protozoologists for so many years. When I joined my first protozoology research institute in West Africa in the late 1950s, my colleagues there, Desowitz, Williamson, Stephen and Godfrey (to say nothing of Bill Trager who was visiting), were even then racking their brains as to how to get at the trypanosome chromosomes or whatever the organisms used to regulate and perpetuate their existence. Godfrey was forever citing Wolbach and Bingham who had reported a complement of three chromosomes visualized by traditional cytochemical methods about 1910, and Williamson was soon to start work with Amrein and Fulton which failed to demonstrate exchange of genetic material between drug resistant and sensitive trypanosomes. These far-sighted scientists recognized, even then, the importance of more knowledge in this area.

We have had to wait 20 years until the 1980s when suddenly the techniques of enzymology, molecular biology, biophysics and electron microscopy have been combined and allowed us to look directly into the composition and function of the trypanosome genome. Within the space of 5 years, we have had reports of exchange of genetic material between trypanosomes from examinations of naturally occurring and laboratory-generated materials, direct measurements of the nucleic acid content of individual parasites, the first description of DNA organized as minichromosomes, the separation and visualization of chromosomes of different sizes and classes by pulsed-field gradient gel electrophoresis (PFG) and orthogonal-field alternation gel electrophoresis (OFAGE), to say nothing of genes for every job! A year ago, I was privileged to see a scientist in Belgium painstakingly counting chromosomes by drawing condensations of chromatin possibly representing junctions in photographs of serial EM sections of trypanosome nuclei. The progress is fantastic! Some mild confusion and uncertainty is understandable.

Now, this is not a research area where ILRAD has played a major role or where we have particularly special expertise, except perhaps in some of the molecular biology and aspects of parasite transmission, but it *is* an area in which we have to be very interested, since recombination events have major implications for immunization, parasite identification and speciation, and chemotherapeutic programs.

When Dr. Doyle and I were considering possible topics for seminars and workshops at ILRAD last October, genetic exchange in trypanosomes came naturally to mind, but I do not think either of us had any idea that the eventual timing of the workshop would be so appropriate. It follows closely on the publication of a major work on the topic by Professor Jenni and colleagues. Controversy is rife about the mechanism and the site of exchange in fly or mammal is open to informed and uninformed speculation. It is little wonder

that Professor Vickerman has said that it is high time somebody set down the incontrovertible evidence for sex in trypanosomes in black and white.

This meeting has rather optimistically been called a workshop, but I doubt if it is going to be a real workshop as defined in current scientific understanding. I think you can hold a conventional workshop when there is a body of background information on a subject, when there are recent new findings to consider, and when there is a firm idea of a relevant objective. In this situation, a workshop can indicate the appropriate path to achieve the objective.

The situation we are considering here is more diffuse and we have no rigorously defined objectives. The meeting has therefore been organized as a small conference with a number of aims which can be summarized as follows:

1. to air new information on genetic exchange in trypanosomes and put it into context with knowledge of events in other protozoa
2. to inform scientists from ILRAD and elsewhere more adequately about what is going on in this research field
3. to provide an opportunity for scientists holding diverse views to discuss their experiments and results at leisure, and possibly resolve some of their differences
4. and this may be more difficult, to provide some assessment of the validity and importance of recent findings from an epidemiological and disease control viewpoint
5. possibly, to give some ideas on how work in this area might proceed in the future.

I hope there will be brief addresses, active discussions and just a little writing to provide a record of the meeting.

I now offer my thanks to John Young and Jim Lenahan and the Organizing Committee who have done most of the work so far and my encouragement to the chairpersons, speakers and rapporteurs who must now take over. I wish you all a useful and successful meeting and, to those from overseas, an enjoyable visit to Kenya.

Session 1: GENETIC EXCHANGE IN PROTOZOA

Genetic mechanisms in ciliates and their relationship to surface proteins

J.R. Preer

Differences that persist when cells are cultured under constant environmental conditions are due to heredity, those that disappear are due to environment and those that vary in a cyclical fashion are due to development. Three ways to study hereditary differences are: uniparental genetics, molecular genetics and biparental genetics. Each is useful, but each has its limitations.

The first studies on protozoan genetics utilized only uniparental genetics. Uniparental genetics has been used primarily to study mutation. Mutation enables us to identify and determine the nature of the different elements controlling a given system. Uniparental genetics has played a major role in the study of viruses and bacteria, as well as those eukaryotes which are haploid or have a haploid stage in which characters can be identified. An example is the analysis of photosynthesis in the asexual alga *Chlorella*. In diploids or polyploids its use is severely limited because new mutations are generally recessive and hence do not change the phenotype.

The advent of molecular genetics has made it possible to study the fine structure and transcription of individual genes in virtually all organisms, sexual or asexual. The numbers of genes of a given kind can be counted and similar ones compared. A transformation system is of great importance; if one is available it makes it possible to introduce variant genes and study their effects.

Nevertheless, there are many features of the genetic system that cannot be approached without the use of the sexual cycle or some other means of obtaining genetic recombination between distant elements. Crosses make it possible to obtain recombinants and learn about the overall structure of the genome--the numbers of chromosomes and linkage groups, the organization of genes and groups of genes into different regions of the chromosome. Much can also be learned about gene action by studying the ways that mutations are expressed when placed into the genome in various combinations and determining whether they can complement or recombine. In many organisms where very large numbers of segregants can be examined (by plating them onto agar or by other means), it has been possible to probe genetic fine structure. Moreover, the study of mutations in diploid or polyploid organisms is made possible by taking advantage of the sexual cycle, and mutagenesis is the most powerful technique available to explore the complete repertoire of elements controlling a given trait. Finally, mutation and recombination can serve as a partial substitute for transformation in molecular studies.

In bringing an organism under experimental control for genetic studies, however, one needs much more than the discovery of a mating system. Some of the essential elements are so trivial that their importance is easily overlooked. First, satisfactory means of culture must be found. Second, it must be possible to make large

numbers of isolations and establish clonal cultures with ease so that rare mutants and rare recombinants can be found. Third, the life cycle must not only be established, but it must be brought under laboratory control so that the different stages can be induced at will. Fourth, a means of controlling promiscuous mating is of considerable advantage, so that the desired crosses can be obtained. Finally, if the peculiarities of the life cycle of particular organisms are discovered they can often be utilized to considerable advantage.

The establishment of *Paramecium* as a useful protozoan for genetic studies on surface antigens illustrates many of these principles. The alternative serotypes found in *Paramecium* persist under constant environmental conditions, but all cells in a culture can be induced to switch to another type by suitable treatments. Nevertheless, crosses with appropriate markers show that the differences in expression are determined by differences in the cytoplasm. Our ability to control and measure the amount of cytoplasm exchanged at conjugation has led to experiments that confirm this conclusion. Strains from different geographical locations usually show differences in serotype proteins and crosses reveal a series of independent genetic loci. The analysis was aided by using the process of autogamy which renders all loci homozygous at once, and whose induction is under control of the investigator. Molecular studies reveal that only one of the serotype genes is expressed at a time. The isolation of mutants has also contributed to our knowledge of the control of expression; in addition to the gene coding for the A protein, at least two other loci are necessary for expression. X-ray induced deletions of the A gene have produced no-A mutations, and further X-irradiation of them has produced no-A, no-B mutations. The lack of DNA sequences in the no-A mutants capable of hybridizing to isolated genomic DNA clones thought to be the A gene shows unambiguously that there is only one A gene in the genome and that it has been cloned and identified.

One group of deletions of the A and B genes show the genes in their micronuclei but have defective macronuclei. New macronuclei, forming under control of the defective macronuclei at conjugation and autogamy, not only lack the A (or B) gene(s), but, like the old macronuclei, are defective in their ability to support normal processing at the next conjugation or autogamy. These conclusions are supported by the experiments of Harumoto who transformed mutants permanently into wild type by transferring wild-type macronuclear material into mutant macronuclei. Similar mechanisms have been found for other traits by Sonneborn using heterokaryon formation induced by the process of macronuclear regeneration. Recently Doerder has obtained virtually identical results for certain mutations unable to produce the H serotype protein in *Tetrahymena*.

Finally, transformation has been shown to be a practical method of studying gene expression in *Paramecium*. Godiska and others have just discovered up to 25% transformation to serotype A in an A-deleted strain by microinjecting a cosmid with the A gene into the macronuclei of the deficient cells. The trait persists until the next autogamy. Expression of A in the transformants appears to be under normal cellular and environmental control.

Points arising during discussion

During macronuclear regeneration if autogamy is induced in a heterozygote Aa, half of the progeny will be AA and half aa in the new macronucleus. However, all will be Aa for their old macronucleus. Any cell which arises from a fragment of the old macronucleus, i.e. from macronuclear regeneration, will be Aa and will show the dominant chracteristic, whereas all of those which arise from the new macronucleus will be AA or aa. Half of these will show the recessive phenotype and one can follow cytologically to tell which was derived from which. Thus each fragment which arises from a previous macronucleus is just like the old macronucleus. This applies not just to this gene but to the entire macronuclear content. The ploidy level reaches about 1,000 and the number of fragments is about 30 to 40.

Although the chromosomes are difficult to work with, there may be about 45 chromosomes in the haploid condition in *Paramecium*. *Tetrahymena* has five chromosomes (like *Drosophila*) as a haploid number. Autosomal strains have been produced and genes have been mapped to individual arms of chromosomes. The hypotrich ciliates have polytene chromosomes like *Drosophila*. The DNA content of *Tetrahymena* is about 10^8 base pairs. *Paramecium* is about the same. Two reports of pulsed field electrophoresis have shown, as expected, that chromosomes are broken into fragments when the macronucleus is formed. There is extensive remodelling of the whole genome. The average size of the 'chromosomes' is of the order of 300 kb for *Paramecium* and greater than 600 kb in *Tetrahymena*.

In *Paramecium* and *Tetrahymena* the mating factors are insoluble surface proteins. However, this is not true of other ciliates where soluble factors are released, and these have been isolated in some cases. There are many mating types. In *P. tetraurelia* there are as many as 14 from different localities: 1 mates with 2, 3 with 4 etc. Selfing sometimes occurs. The mating type of the progeny can be affected in mating in a complex way. Sometimes it is simply genic, i.e. A and a. At other times, as in the d48 type, there is a cytoplasmic contribution and macronuclear differentiation occurs.

Despite the large aggregates that form during mating in *Paramecium*, there are only rarely other than biparental fusions, largely because the mating factors are insoluble. In hypotrichs, selfing and multiple fusions are more common. Selfing occurs because both mating types are present transiently. It should be noted that the surface-antigen and mating-type factors are both surface molecules but separate from one another.

It is not known if there is loss of DNA during maturation of the macronucleus in *Paramecium*, but it is probably not very much. In *Tetrahymena* there is only about 10% loss, although in hypotrichs nearly 90% may be lost. Bruns has suggested that mating type is inherited through the presence of two loci within the micronucleus with mating type depending on whether one or other of these gets into the macronucleus. The d48 mutation does not affect mating-type determination and, like other mutations, is quite independent of mating type.

Genetics of the human malaria parasite, *Plasmodium falciparum*

D. Walliker

Malaria parasites (*Plasmodium* spp.) undergo cycles of asexual division and gametocyte production in their vertebrate host, while fertilization of gametes takes place in the mosquito vector. Malaria parasites are analysed genetically by feeding mosquitoes on a mixture of gametocytes of two cloned lines differing in a number of genetically determined characters, to permit cross-fertilization of gametes of each clone. The sporozoites which develop are then used to infect a new host, and the resulting blood forms are examined for the presence of parasites exhibiting non-parental combinations of characters.

Early studies on the genetics of rodent malaria species, *P. yoelii* and *P. chabaudi*, made use of characters such as isoenzymes, drug-resistance and antigens as strain markers. These studies showed that (1) the malaria parasite appears to undergo a normal Mendelian pattern of inheritance of such characters, (2) the blood forms are haploid, meiosis probably occurring during early division of the zygote in mosquitoes, (3) resistance to drugs such as pyrimethamine and chloroquine is due to gene mutation, and (4) recombination occurs readily following cross-fertilization.

Genetic studies on the human parasite *P. falciparum* have been started recently. In the first cross, two clones were chosen which differed in pyrimethamine resistance, in electrophoretic forms of adenosine deaminase (ADA), in epitopes of two blood-form antigens detected by monoclonal antibodies, in certain proteins detected by two-dimensional polyacrylamide gel electrophoresis (PAGE), in patterns of hybridization of a repetitive DNA probe, and in chromosome sizes as revealed by pulse field electrophoresis. Gametocytes of each clone were grown in culture, then mixed together and fed to mosquitoes (*Anopheles freeborni*). Sporozoites were used to infect a chimpanzee, and the resulting blood forms were established in culture. Clones were isolated from the progeny of the cross and examined for each parental line marker. The principal findings of this study were:

1. Recombination between each parent-line character was detected. The inheritance patterns of enzyme, antigen and pyrimethamine-resistance markers confirmed the haploid nature of the blood forms.
2. The chromosome studies showed that considerable genome rearrangements take place following cross-fertilization. The two parent lines differed in the size of chromosomes 3 and 4. Certain progeny clones exhibited a chromosome 4 of intermediate size between that of each parent. In other clones, chromosome 2 was larger than in either parent. Changes in some other chromosomes were also apparent.
3. Recombination appeared to occur at a remarkably high frequency, suggesting that cross-fertilization was favoured over self-fertilization. Among 14 clones examined, 12 were recombinants; whereas if fertilization had occurred randomly between gametes, it would have been predicted that at least 50% of the progeny would be parental types.

In view of the fact that malaria patients are frequently infected with more than one genetically distinct parasite, these results show that novel genotypes are likely to arise frequently following mosquito transmission. This finding has important implications for measures designed to control *P. falciparum* by chemotherpy or vaccination.

Points arising during discussion

During presentation of the paper, the importance of the timing of cloning to determine recombinant progeny was suggested to the speaker and readily agreed. Because of experimental constraints, the cloning step had been carried out only once in the reported experiments on *P. falciparum*. However, cloning proved relatively easy by the method of limiting dilution although occasional mixtures of progeny types had resulted.

The apparent non-appearance of parental types bearing the histidine rich protein (HRP)-negative phenotype was difficult to explain unless cross-fertilization was favoured. Speculatively, if the HRP-negative phenotype is associated with loss of the knobbed phenotype, it might then find difficulty in establishing in the mammalian host (chimpanzee). However, the speaker felt that published work on the disappearance and reacquisition of the knobbed phenotype was unconvincing.

In the case of rodent malarias, it is relatively easy to achieve mating between strains, though occasionally it is not possible between geographically separated isolates. In the case of *P. falciparum*, it was only achieved once. Although the existence of mating types remains a possibility, recombination was obtained between virtually every pair of strains tested except for the HRP types.

Evidence of recombination in the bloodstream has not been sought in *P. falciparum*. In rodents, workers deliberately mixed sporozoites or blood forms, infected mice and looked for recombination. Although only two drug markers were used, no recombination was found.

If a phenomenon occurs in malaria similar to parasex in fungi, in addition to the sexual cycle, an excess of recombinants might arise. However, such a situation is not known to exist in malaria.

Genetic recombination in the trypanosomatid *Crithidia fasciculata*

J. Glassberg

Mutants of *Crithidia fasciculata* were isolated, following mutagenesis, and fell into the following classes: auxotrophic, drug-resistant and colony-morphology. The single auxotrophic mutant found required cysteine for growth. This requirement was satisfied by cystathione but not by homocysteinethiolactone. Drug-resistant mutants were found resistant to actinomycin D, azauracil, azauridine and 5-fluorouracil. These mutants showed no cross-resistance. Azauracil and fluororacil mutants were uptake mutants.

Crossing an actinomycin D-resistant strain with an azauracil-resistant strain resulted in the appearance of doubly drug-resistant colonies. These colonies remained resistant to both drugs even after growth on non-selective plates.

Crossing an actinomycin D-resistant, azauridine-resistant strain with an azauracil-resistant, fluorouracil-resistant strain resulted in a complex assortment of colonies exhibiting both parental and recombinant phenotypes. Most of the recombinants were unstable but some stable wild-type and azauridine-resistant clones were found. These data suggest that *Crithidia* undergoes some type of genetic recombination and must be diploid at some time during the process.

Points arising during discussion

In the case of resistance to azauridine, repeated 'picking' showed the same phenotype, but it is not known how long this stability would be maintained. However, each colony contains many generations. The hypothesis concerning the existence of unstable diploids which resolve into stable haploids was questioned on the ground that the wild-type chromosomes seemed to survive the drug pressure.

Session 2: GENETIC EXCHANGE IN TRYPANOSOMES--EPIDEMIOLOGICAL DATA

Genetic variation in populations of *T. brucei* spp: evidence for diploidy and genetic exchange

A. Tait

The question of whether Africa trypanosomes have a system of gene exchange has a long history, with a number of claims relating to the morphological observation of chromosomes, gametes and mating trypanosomes. Over the past 10 years, the use of enzyme electrophoresis as a means of characterizing these organisms (Godfrey & Kilgour 1976; Gibson et al. 1980) has led to evidence for a sexual cycle based on the nature of the enzyme variants and their frequencies in discrete populations of isolates (Tait 1980).

The central arguments for a sexual cycle are as follows:

1. the nature of the enzyme variant patterns
2. the frequency of the phenotypes observed which fit those predicted on a model of random mating
3. the frequency of all possible combinations of variants when two loci are examined in a single population or in clones from a single fly isolate
4. the frequency of variants observed when compared to asexual populations of other organisms.

The speaker reviewed this evidence by reference to specific examples and discussed its strengths and weaknesses, including cases where deviation from expected phenotype frequencies is observed. He briefly discussed the effect of a sexual cycle on various aspects of epidemiology, such as the definition of non-interbreeding populations and of subspecies.

Sexual processes in natural populations of *T. brucei* and the epidemiology of trypanosomiasis

R.E. Cibulskis

Isoenzyme electrophoresis provides a convenient means of exploring the genetic composition of a population over several loci. Data resulting from an isoenzyme survey can be subjected to various forms of analysis to yield information on sexual processes in natural populations of trypanosomes.

One approach has been to consider whether the diversity observed in a population could have been produced through an acceptable pattern of mutation. Previous arguments about mutation have been confined to the range of genotypes observed at a single locus, but they can be extended to consider several loci simultaneously by using a cladistic method. The method provides evidence that the diversity observed in three East African populations of *T. brucei* could not have been produced by mutation alone, but does not indicate to what extent genetic exchange currently operates.

Another approach has considered the relative frequencies with which different genotypes occur, i.e. whether or not they conform to Hardy-Weinberg expectations. In general, this analysis requires large sample sizes before it is of much value. A more powerful analysis is provided by considering loci in combination and using a randomization procedure to overcome problems of small sample sizes. It reveals significant associations between genotypes from different loci, suggesting a potential for distinct strains of *T. brucei* to evolve within a population.

Analysis of population infrastructure in Lambwe Valley, Kenya, indicates heterogeneity over comparatively short distances. A significant association is observed between host species and the multiple-locus genotype of *T. brucei* variants. The association appears to be stable over a 3-year period, suggesting that sex has little influence on the genetic structure of trypanosome populations, at least over this time scale.

The status of *T.b. gambiense* and *T. evansi*

W.C. Gibson

Among the diversity of *Trypanozoon* stocks found in nature, two types stand out on both behavioural and biochemical grounds, namely *T. evansi* and *T.b. gambiense*. Not confined to tropical Africa by dependence on a tsetse fly vector, *T. evansi* has been able to spread widely throughout tropical and subtropical regions of the world. Despite this wide distribution, *T. evansi* for the most part is remarkably uniform in terms of isoenzyme and kinetoplast DNA variation. In particular, all *T. evansi* stocks so far examined lack kinetoplast maxicircles and have lost the minicircle heterogeneity characteristic of *Trypanozoon* stocks in general. Only two biochemically distinct types of *T. evansi* have so far been identified, suggesting that *T. evansi* may have arisen from *T. brucei* on as few as two independent occasions and that subsequent mutations then created the minor variations observed within the two types. Since no genetic exchange is possible between *T. evansi* and *T. brucei* or among *T. evansi* stocks in the tsetse fly, *T. evansi* remains a small group of isolated mutant strains.

If *T.b. gambiense* is defined as all trypanosome isolates from man in areas of Gambian sleeping sickness, then such trypanosomes fall into two groups. Group 1 is easily and clearly demarcated and corresponds to the classical definition of *T.b. gambiense*. The trypanosomes have low infectivity and virulence for rodents, high resistance to human serum, a limited antigenic repertoire and particular isoenzyme and DNA markers. Group 2 is less easily defined and shares none of the above characters; its place in the epidemiology of Gambian sleeping sickness has yet to be investigated. The lack of variability among Group 1 stocks, even at the nucleotide level, is remarkable considering that they originate from several regions of Africa. It suggests that Group 1 *T.b. gambiense* consists of a handful of related trypanosome strains which have spread rapidly in recent times and so have not yet diverged noticeably. Neither do they appear to be changing genetically by recombination with *T.b. brucei* strains in the invaded areas. Thus both *T. evansi* and Group 1 *T.b. gambiense* would seem to be small, easily defined groups because each, although widespread, consists of only a handful of mutant *T.b. brucei* strains which do not exchange genetic information with the main body of *T.b. brucei* and thus evolve at a slow rate.

Points arising during discussion

The lack of substantial evidence as to whether *T. evansi* evolved from *T.b. brucei* or whether both evolved from a common ancestor was noted. Another point raised was whether genetic exchange occurs between *T.b. gambiense* and *T.b. brucei* or *T.b. rhodesiense*. At the population level, certain enzyme characters specific to *T. gambiense* have been encountered in areas of *T.b. gambiense* and *T.b. rhodesiense* overlap, namely along Lake Victoria and *T.b. gambiense* does hybridize with *T.b. rhodesiense* from this area, yet classical *T.b. gambiense* and *T.b. rhodesiense* should not occur together in the same area. The more convincing approach should be one of experimental co-transmission through tsetse.

Characterization of the *T.b. gambiense* mVSG genes L2 and U1 and their possible use in the differentiation of *T.b. gambiense* substrains and differentiation of *T.b. gambiense* from other *Trypanosoma* spp.

D.A. Barnes, J.C. Mottram and N. Agabian

The *T.b. gambiense* metacyclic variable surface glycoprotein (mVSG) genes L2 and U1 were characterized and their genomic environment physically mapped. The L2 VSG gene in an L2-expressing, substrain-B organism is present in three telomere-linked copies. In a 30-day L2 relapse, there is one telomere-linked copy. One of the copies in the L2 expressor appears to be a bloodstream-like expression-linked copy (ELC) in that it contains a very large barren region. This copy is not expressed. The other two copies appear to be highly related and are more metacyclic-like in that they have little or no S' 'barren region'. One of these two copies is identical to the one remaining copy in the L2 relapse. A PVU II polymorphism exists in all of the unexpressed copies of the L2 gene.

The U1 VSG gene in a U1-expressing, substrain-A organism was present in two copies. One copy appeared to be a bloodstream-like ELC and the other a metacyclic-like ELC. It is believed that the bloodstream-like ELC copy is the one that is transcribed because it is linked to an expression site-associated gene (ESAG) which was cloned from an exreession library. This ESAG is highly homologous to the two ESAG sequences published by Cully et al. (1985). It hybridizes to a 1.2 Kb transcript on northern blots of total U1 RNA. This clone does not hybridize to any transcript in L2 total RNA.

The two cDNA clones of U1 and L2 were used to probe Southern blots of genomic DNA from *T.b. brucei*, *T.b. gambiense*, *T.b. rhodesiense*, *T equinum* and *T. vivax*. The U1 gene is only found in *T.b. gambiense* substrain-A organisms. A U1-related gene fragment hybridizes to the U1 cDNA probe. This fragment is found in many *T. brucei* subspecies, but neither the U1 gene nor the U1-related gene fragments were observed in the substrain-B organisms. The L2 gene is found only in *T.b. gambiense* substrain-A and -B isolates and was not observed in any other *Trypanosoma* species.

Long-term intentions are, first, to develop markers which would be useful in the identification and genetic manipulation of *T.b. gambiense* isolates for epidemiological and/or diagnostic purposes and, second, to provide a basic research agenda describing and characterizing the metacyclic antigens and regulatory regions associated with expression of *T.b. gambiense*-like specific VSGs.

Genetically distinct groups within *T. congolense* from the Kenya coast

P.A.O. Majiwa

Chromosome-sized DNA molecules from different clones of *T. congolense* have been size-fractionated by OFAGE in order to obtain molecular karyotypes. The trypanosome clones used were deliberately chosen for purposes of comparison to represent: (1) isolates from different geographic areas, (2) distinct VAT repertoires (serodemes), (3) different VATs within a single antigenic repertoire.

The chromosomes of *T. congolense* were observed to fall into three broad size categories: (1) minichromosomes which are approximately 50 kb and constitute the smallest trypanosomal chromosomes observed so far; (2) medium-sized chromosomes which have a size range of 0.4 to 1 Mb and appear to vary in both size and number depending on the cloned isolate examined; and (3) large chromosomes greater than 1 Mb.

Molecular karyotypes obtained appeared to differ among clones from different antigenic repertoires, but were identical for variant clones from the same antigenic repertoire. Furthermore, sequences encoding VSGs could not be detected by Southern blot hybridizations in the genomes of *T. congolense* clones derived from heterologous antigenic repertoires.

All clones derived from *T. congolense* isolates from Kilifi so far examined by OFAGE appear to have distinctly different molecular karyotypes, quite unlike those observed among the other *T. congolense* clones described above. In these trypanosomes, the minichromosomes are generally bigger, about 50 kb, and the chromosomes in the region of 0.2 to 0.4 Mb are more numerous than in the other *T. congolense* clones. These trypanosomes have subsequently been compared with the others by molecular and biochemical techniques, and shown to differ in the following ways: molecular karyotypes, repetitive DNA sequences, kinetoplast DNA sequences, and restriction enzyme fragment size polymorphisms as revealed by conserved sequence probes.

It is concluded that *T. congolense* is comprised of genomically diverse trypanosomes that probably do not have the capacity to participate in the conventional exchange of genetic material. Such trypanosomes could constitute distinct species or subspecies.

Points arising during discussion

It was pointed out that the Kilifi isolates were identical to the *T. congolense* isolates from Matuga at the Kenya Coast described by Dr. Gashumba. However, the isoenzyme profiles of these isolates were different from those of the savannah- and forest-type isolates described by Christine Young. Furthermore, the *T. congolense* isolates of the forest type did not occur in East Africa.

The number and structure of chromosomes in *T.b. brucei* nuclei: an electron microscopical approach

G. Steinert, F. Luhahi Lumba and M. Steinert

Although trypanosome nuclei have a structure very similar to that of higher eukaryotic cells, their mitosis proceeds differently. The nuclear envelope persists throughout the cell division cycle and the chromosomes, which never condense, cannot be visualized by classical cytological methods. Therefore, the questions of the size and number of chromosomes in trypanosomes have been approached by completely different methods, namely pulse field gradient (PFG) gel electrophoresis, which allows the separation of chromosomes-sized DNA. It has been demonstrated in this way that the trypanosome genome includes a number of large and medium sized chromosomes in addition to about 100 minichromosomes. To date, only VSG (variant surface glycoprotein) genes have been localized on these minichromosomes. How these chromosomes segregate at mitosis is not known, but, if they are typically eukaryotic, each of them should have two telomeres, one centromere and several replication initiation sites. Only one of these essential features, the pair of telomeres, has been clearly demonstrated to be present in these small trypanosome chromosomes. The present investigation was aimed at finding out whether they have the centromere.

Previous electron microscopic studies in several trypanosome species have shown that mitosis involves the formation of an intranuclear spindle and that 'dense plaques' seem to be associated with spindle microtubules. These plaques are thought to represent the kinetochores which characterize the centromere in mitotic chromosomes. Solari (1980) demonstrated by serial sectioning and three-dimensional reconstruction that there are 10 such plaques in *T. cruzi*. The same ultrastructural approach was used in the present investigation to find out how many dense plaques are present in the mitotic nucleus of the bloodstream form of *T.b. brucei* (EATRO 1125, AnTar 1) and whether this number may be correlated to the data obtained by PFG gel electrophoresis.

The spindle appears to be very similar to the one described by Solari in *T. cruzi*, although more elongated and less regular in shape, and mitosis proceeds in *T.b. brucei* in very much the same way as in the American trypanosome. Dense plaques have been easily identified within the spindle, especially in longitudinal section where they appear as characteristic symmetrical and laminated structures, composed of two thick central bodies separated by a clear zone from the two outer elements. They seem to be randomly distributed in the centre of the metaphasic nucleus. Microtubules either run tangentially to the plaques or seem to be attached to the outer elements. Each plaque is associated with several mictotubules. Chromatin appears to be closely associated with the plaques. At anaphase, the plaques split into half plaques which segregate toward the poles of the elongating nucleus. New microtubules appear between these half plaques and continue to grow until complete separation of the daughter nuclei. Only 9 or 10 dense plaques have been counted in metaphasic nuclei which have been

completely cut in serial sections. Since the probability of counting the same body twice in serial sections is lower than that of missing one, we propose that *T.b. brucei* has 10 dense plaques (or, more precisely, kinetochore pairs). The number of normal centromeric chromosomes should thus be 10 (i.e. 5 pairs) per diploid nucleus. We assume that these 10 chromosomes are the few 'large' chromosomes that are reportedly retained in the slots of PFG electrophoresis gels. This conclusion raises the question of the segregation mechanism used by the numerous chromosomes of the lower size classes in which we have not observed the dense plaques and which thus appear to lack a normal centromere.

Points arising during discussion

There was a question as to whether there may have occurred degradation of material and if this could have led to the observation of only a small number of kinetochores (dense plaques). The response was that a constant number of kinetochores was observed on several serial sections made from different trypanosomes. Thus, if there was any degradation, it was minimal and could not have affected the number of kinetochores observed.

It was observed that no *in situ* hybridizations with purified minichromosomal DNA had been done. However, even if they had been done, the hybridizations might not have been specific to particular kinetochore structures because all trypanosome chromosomes have common repetitive sequences.

The serial sections examined were believed to be from trypanosomes at metaphase and not those at anaphase. The dense plaques observed were similar to those seen in *Plasmodium* during mitosis. The speaker explained that attempts to block mitosis in trypanosomes, using colchicine, had not been successful.

Structure of the *T. brucei* genome and an analysis of recombinational events

M.G.-S. Lee, C. Shea and L.H.T. Van der Ploeg

The parasitic protozoans *T. brucei* (Van der Ploeg et al. 1984a), *P. falciparum* (Van der Ploeg et al. 1985) and several *Leishmania* species (Giannini et al. 1986) have chromosome banding patterns (karyotypes) that vary among different geographic isolates. The rearrangement events underlying the chomosome length variability may be of advantage to the parasites because it can contribute to the regulation of differential gene expression. However, different recombinational mechanisms appear to be involved among these species (Langsley et al. 1986). We studied chromosome structure in *T. brucei* and analysed recombinational events in antigenic switches in order to understand mechanisms that contribute to alterations in the karyotypes.

Chromosomes in *T. brucei* appear mitotically stable (Van der Ploeg et al. 1984b). Chromosomes have CCCTAA telomere repeats which are recombinogenic (Van der Ploeg et al. 1984c). These repeats were found at internal positions in the chromosomes, sometimes in association with VSG genes (Van der Ploeg et al. 1986). We tried to identify recombinogenic sequences in a 430 kb chromosome, which varies in size in five different antigenic variants. One hundred randomly isolated clones derived from a 430 kb chromosome all contained repeats. Twenty were analysed in detail and were shown to contain repetitive elements that were shared in groups among different chromosomes and were polymorphic when compared between different geographic isolates. However, this analysis did not allow us to identify specific sequences, other than telomere repeats, that were involved in recombinational events in the variants we studied. In order to gain insight into specific sequences that contribute to recombinational events, we analysed recombinations that occur during antigenic switches in: (A) the alterations in the molecular karyotypes in a series of antigenic switches (Shea et al. 1986) and (B) the events at independent duplicative transpositions of a single VSG gene (Lee & Van der Ploeg 1986). We showed that the switch-off of the 1.8 VSG expression site can occur by different reciprocal recombinations. These result in translocation of the 1.8 expression site from a 430 kb chromosome to a larger chromosome and lead to the appearance of new chromosomes of 140 kb in variant 118b' and 350 kb in variant MiTat 1.2000. The appearance of a single variant (117 or 118) in a parasitaemia resulted from many independent duplication transpositions of these VSG genes. We showed that the duplicative transpositions of these VSG genes occur intra-chromosomally (Van der Ploeg et al. 1984b) between sequences that are homologous at the 5' ends of the transposed segment and expression site (Lee & Van der Ploeg 1986). However, sequences other than the previously reported 70 bp repeats can be involved (Lee & Van der Ploeg 1986). The presence of extra 70 bp repeats in the basic copy and expression-linked copy of one variant indicated that the 70 bp repeats may determine a region involved in the initiation of recombination. We hope that these data will aid in our understanding of mitotic and meiotic recombination in these parasitic protozoans.

Points arising during discussion

It was noted that the expression site for the 118 VSG gene was different when this gene was re-expressed by individual trypanosome clones in a relapse population. Similar observations have been made by other investigators with the AnTat 1.1 VSG gene. These findings indicate the existence of several independent VSG gene expression sites in trypanosomes. From the data obtained so far, it is not possible to present a scheme of events which may have led to the 113 VSG gene occupying different expression sites in different trypanosome clones.

Several recombinant clones made from the 430 kb chromosome were found to contain repetitive sequences which were co-expressed with VSG genes in the bloodstream forms, but not in procyclic forms. The whole chromosome appears to be transcriptionally active in bloodstream-form trypanosomes and the transcripts produced are discrete in size. The size of these transcripts varies from 1 to 4 kb, as determined by northern blot analyses.

Session 4a: GENETIC EXCHANGE IN TRYPANOSOMES--EXPERIMENTAL STUDIES

Experimental approach to produce hybrid trypanosomes in *Glossina*

L. Jenni

Tait (1983) has reviewed the evidence provided by various experimental approaches which have recently been applied to the long-standing question of kinetoplastid sexuality. Increasing evidence for genetic exchange emerged from analyses of the distribution of enzyme variants in African trypanosome stocks (*T. (T.) brucei* and *T. (N.) congolense*) isolated from different areas in Africa during the past 10 years (Gibson et al. 1980; Richner & Jenni 1986; Tait 1980; Young & Godfrey 1983). These authors found many enzymes which showed single and multiple-banded patterns after electrophoresis, depending on the trypanosome population analysed. Organisms showing one band were assumed to be homozygous at the respective locus, whereas those showing multiple bands were assumed to be heterozygous. By applying the Hardy-Weinberg law, Tait (1980) found that the frequencies of the various forms of each enzyme in the parasite populations provided good evidence that mating between trypanosomes was taking place.

The data obtained by Culwick et al. (1951) and Letch (1984) indicated that sexual processes of *T. (T.) brucei* probably occur during cyclical development in the tsetse fly vector. Based on these data, a series of mixed transmission experiments was carried out in Basel using cloned and well characterized trypanosome populations which differed from each other in several aspects (Jenni et al. 1984). The prerequisites for these simultaneous transmission studies were that both parental clones had: (1) to be homozygous single banded for distict isoenzymes with different electrophoretic mobilities, (2) to be transmissible with comparable speed and mature infection rates through the same tsetse species, (3) to show distinct DNA hybridization patterns with specific DNA probes, (4) to belong to distinct serodemes and (5) to have differential drug sensitivities.

In all mixed transmission experiments the same two clones were used. One parental clone was obtained from a game animal in the Serengeti, East Africa (STIB 247-L) and the other was derived from a human in Ivory Coast (STIB 386AA).

In one series of experiments, cloned progeny trypanosome populations showed 'hybrid' properties for most of the above markers. The parental and progeny clones (labelled STIB 723C) have been analysed extensively in collaboration with several research groups and the results have been published (Jenni et al. 1986). The four enzyme phenotypes of the stocks derived from the mixed transmissions were found to differ from those of either parental clone. They also appeared to be heterozygous for the variant alleles, while the parental clones were homozygous. Comparable results were obtained with DNA probes showing different hybridization patterns between the parental clones, thus demonstrating that the 'offspring' were hybrids not only at the level of gene products but also at the level of the genes examined.

In addition, analysis of the inheritance of size variation of the intermediate size class of chromosomes, separated by PFG, and restriction site and length polymorphisms of kinetoplast DNA showed an uniparental inheritance (Sternberg et al. in press). These experiments have clearly demonstrated that gene exchange can occur between African trypanosomes of the subgenus *T.* (*T. brucei*) and that it most probably occurs at specific stages during cyclical development in the fly.

In extending the analysis of hybrid formation, the antigenic relationship between metacyclic trypanosomes of the parent clones and those of the hybrid clones has been investigated by indirect immunofluorescence, neutralization assays and immunization experiments in collaboration with ILRAD. When antibodies specific for parental metacyclic VATs were tested in a double indirect fluorescent antibody test (IFA) on metacyclics from saliva after a mixed infection, they revealed some forms that expressed parental epitopes. In another experiment, immunization of mice with the hybrid forms by infection and treatment protected them against challenge by hybrid forms and by one of the parents (STIB 386), but not by the other parent (STIB 247). This suggested that almost all 386 epitopes were present in the hybrid, but many of the 247 epitopes must be lacking.

The results point to a recombination of metacyclic variable antigen types (mVATs) between both parental clones. They thus provide additional phenotypic evidence for the occurrence of hybridization during cyclical development in the fly.

Points arising during discussion

The presence of two parental epitopes in the hybrid does not constitute an argument for the diploid nature of the metacyclic form, since their allelic nature is not known. Also it is not known whether both parental VSGs are present on the hybrid metacyclics or one recombinant VSG. In other protozoa such as *Paramecium*, two alleles are often co-expressed. Also it has to be shown whether all hybrids possess the same VAT repertoire. From the mixed transmission experiment, only three clones were obtained with the same isoenzyme and DNA-hybridization patterns. These might therefore be derived from the same initial hybrid. In the system used, there might be preferential selection for some types of hybrids and other types might be lost. In another laboratory, under different conditions, two additional, different hybrid clones were obtained from the same parents.

After four to five consecutive transmissions through the fly, the hybrids were still stable, suggesting that the recombined alleles did not segregate.

The frequency of hybrid formation during cyclical transmission

J. Schweizer

The frequency of hybrid formation between two *T. (T.) brucei* clones during cyclical transmission through *Glossina morsitans centralis* has been analyzed. In two independent experiments, teneral *G.m. centralis* were infected with an equal mixture of two parental clones which showed different homozygous patterns for isocitrate dehydrogenase (ICD; E.C. 1.1.1.42) and alkaline phosphatase (AP; E.C. 3.1.3.1). From 23 positive flies, trypanosomes were cyclically transmitted to mice and the subsequent bloodstream form populations were characterized by isoenzyme electrophoresis for ICD and AP.

Initial results obtained in collaboration with A. Tait indicate that hybrid formation with heterozygous isoenzyme patterns occurred in at least 12 of the 23 tsetse. There is further evidence that the extrusion of hybrid parasites in the saliva of a single fly is not necessarily continuous, but can alternate with the occurrence of either or both of the homozygous parental clones in the course of time. In general, the frequency of hybrid occurrence increased with the time after fly infection.

Points arising during discussion

Although the data presented only showed flies that transmitted one of the parental types plus hybrids, a single fly has been observed to transmit the two parental types and a hybrid at different times. It was pointed out that one might have a selective outgrowth of the hybrids in the fly. The number of potential hybrid-producing flies (12/23) might also be higher, since many flies died early.

Mixing of the parental forms in the animal (by syringe, bites with two flies, or mixing of saliva before injection) did not result in hybrid formation.

It was not possible to answer the question whether all hybrid progeny came from a single initial event or from multiple, continuously occuring events.

Isoenzymatic analysis of trypanosome populations and their progeny after mixed cyclical transmission

B. Betschart

Lysates of parent clones I (386 AA) and II (247-L), as well as those obtained after cyclic transmission through *G.M. centralis*, were analysed for their isoenzyme patterns using isoelectric focusing (IEF) on ultrathin polyacrylamide gels. The isoenzyme patterns of phosphoglucomutase (PGM) and isocitrate dehydrogenase (ICD) were followed in order to screen trypanosome populations for hybrid patterns after mixed cyclical transmission (Jenni et al. 1986).

Isoenzyme patterns are widely used to study the taxonomic relationships between closely related organisms. Isoenzymes, defined as multiple forms of a given enzyme, are conventionally separated either on starch gel or cellulose acetate. Taxonomic characterization as well as genetic studies of trypanosomes of the subgenus *Trypanozoon* have mainly been made by electrophoretic separation on starch gels (Gibson et al. 1980; Tait 1983). The introduction of IEF for separating proteins necessitates a critical evaluation of the results obtained by both techniques. The IEF method not only offers many technical advantages, but also gives an excellent separation of different isoenzymes, which cannot be obtained with the conventional techniques. The isolates tested so far showed similar patterns to those obtained after electrophoresis on starch gels, justifying the use of an alternate technique.

The analysis of isoenzyme patterns is normally carried out on lysates from bloodstream forms. This necessitates the infection of a rather large number of experimental animals so that sufficient numbers of trypanosomes can be obtained for isoenzyme analysis. To circumvent this problem, we initiated the use of procyclic forms, which can be grown in large quantities without major problems. Bloodstream forms were passaged into tsetse, and 10 days later the midgut procyclics were put into cultures. Most of the enzyme patterns tested can be directly compared with those of the bloodstream forms. Some differences were detected with PGM and ICD. Whereas the lysates from procyclic forms of 247-L showed one band of PGM like the bloodstream forms, the lysates from 386 AA had two distinct bands, which were also found in the hybrid 723 CA. Cloned 386 AA bloodstream forms showed only one band, and the hybrid had the corresponding bands of the two parental clones. This can only be interpreted as an expression of a further isoenzyme during differentiation from bloodstream to the insect stage. ICD lysates showed a reduction of bands in the procyclic forms compared with the bloodstream forms. This also is taken as an indication of a differentiation process, where bloodstream forms contain soluble forms of ICD which are membrane bound in the procyclic forms.

These dynamic changes indicate the difficulty of making a simple interpretation of isoenzymatic patterns in trypanosomes. A careful analysis of the subunit structure of the individual isoenzymes would most certainly help to elucidate their relationship to the genomic organization. Whereas the more classical techniques of isoenzyme analysis are very helpful in taxonomic classification, their usefulness for detailed genetic analysis must be evaluated.

Points arising during discussion

Considerable discussion ensued on the qualitative and quantitative differences seen in IEF gels of procyclic and bloodstream-form isoenzymes from parental and hybrid organisms. Although these differences could be interpreted as expression of another isoenzyme between different developmental stages, other reasons which could account for this finding included: (1) the amount of enzyme loaded, (2) different protein turnover rates, (3) different enzyme activities, (4) different association constants between homo- and hetero-meric enzyme subunits, (5) differential post-translational modification and (6) influence of sample handling, namely the use of ethylene diamine tetra-acetic acid (EDTA) and DTT known to activate trypanosome lysosomal proteases.

Nuclear DNA content during cyclic transmission of *T. brucei*

F. Zampetti-Bosseler

The relative nuclear DNA content of *T. brucei* was measured using quantitative fluorescence cytophotometry of individual Feulgen-pararosaniline stained cells. These analyses were performed on trypanosomes (provided by D. Le Ray and D. Aerts of the Institute of Tropical Medicine, Antwerp, Belgium, and by L. Jenni, D. Richner and J. Schweizer of the Swiss Tropical Institute, Basel) during their developmental cycle in the mammalian host (bloodstream forms) and in the tsetse fly (procyclic, proventricular, epimastigote, premetacyclic and metacyclic forms). Cells of different developmental stages were fixed on the same slide and were processed in the same conditions. All measurements were done on the same day. Fluorescence was measured 7 seconds after illumination, with background fluorescence subtracted.

On the basis of DNA microfluorometric measurements, we propose that mature metacyclic *T. brucei* have a haploid amount of DNA, compared to that of all other developmental stages in the mammal and insect, and that premetacyclic trypanosomes are probably those which undergo meiotic division. It can be inferred that a sexual cycle exists in *T. brucei*, with trypanosome meiosis and gametogenesis occurring in the salivary glands of the vector and syngamy taking place in the mammalian host.

Points arising during discussion

The accuracy of the method used seemed to be confirmed by the observation of 30% less DNA content in *T.b. gambiense* than in *T.b. brucei* as already described by others. This decrease may be accounted for by the lower number of minichromosomes observed in *T.b. gambiense* and some VSG gene depletion.

A 2-fold difference in DNA content may be easily observed because of artifacts related to the technique used for hydrolysing the samples. An example is human neurone cells which were incorrectly described as nearly tetraploid. These artifacts may be overcome with good reagents and experimental conditions.

The necessity of including reliable controls in the experiments was discussed. In the results presented the bloodstream forms were used as controls since they are thought to be diploid. Moreover, many nuclei from different clones at different stages were measured at the same time, and these showed similar distribution for comparable stages. Some of the material prepared was reanalyzed 1 month later; all the samples showed a decrease in the fluorescence measured, but with the same relative intensity. It was mentioned that the bloodstream forms might not be the best control as they are dividing and therefore do not comprise a narrow, evenly distributed population.

The results presented seem to indicate a haploid stage and thus some kind of meiosis but, from a geneticist's point of view, segregation has to be observed before being certain of meiosis. It

was pointed out that in a classical miotic process a tetraploid stage should be observed. Some cells showed a tetraploid DNA content with the technique used, but were found only in salivary forms and were described as duplicating epimastigotes. Some salivary forms were observed with two nuclei; preliminary measurements suggested that each nucleus had a haploid DNA content, so these may be premetacyclics.

Analysis by flow cytometry of DNA synthesis during the life cycle of *T. brucei*

S.Z. Shapiro

DNA content at different stages in the life cycle of the haemoprotozoan parasite *T. brucei* has been analysed with a fluorescence-activated cell sorter (FACS) using chromomycin A_3 to stain DNA. It was observed that the long slender bloodstream forms and procyclic culture forms (analogous to the tsetse fly midgut stage) are dividing cell populations with cells in G1, S, G2 and mitosis. Short stumpy bloodstream-form and metacyclic-form populations are composed of non-dividing parasites stablized at G1 and G0 of the cell cycle. Haploids, possible sexual forms, were not detected.

When transferred to a culture system which mimics the fly midgut, short stumpy bloodstream-form parasites were readily able to initiate DNA synthesis and differentiate into dividing procyclic culture forms. In earlier experiments where ethidium bromide had been used to quantitate DNA, stumpy-form parasites cultured for 3 to 6 hours in the procyclic culture system appeared to have half the G1 amount of DNA. Determination of DNA content in these parasites by the diphenylamine reaction showed no difference when compared to stumpy forms. It is suggested that this result may have been an artefact caused by the dye binding quantitatively differently to DNA in the organism at different stages in its life cycle.

Points arising during discussion

Signals of low fluorescence intensity were omitted in histograms from metacyclics, and therefore possibly from haploids, because too much debris was present. The purity of the population was also discussed, as the samples were prepared from salivary glands and a more homogenous population can be obtained from tsetse saliva. The morphology of the trypanosomes purified on di-ethyl aminoethyl (DEAE) columns corresponded to that of metacyclic forms. No clumping was observed. A 90° scatter signal could help in eliminating the contribution of debris.

Preliminary cytophotometric results on DNA complement size and ploidy of different stages of *T. congolense* and *T. brucei*

J.P. Overdulve with R.F. Kooy (Utrecht), M. Van der Ploeg (Leiden), H. Hirumi, S.K. Moloo and V. Nantulya (ILRAD)
(C.J. Janse assisted in measuring the *T. brucei* metacyclics)

Cell by cell microfluorometry of Feulgen-stained protozoan parasites has successfully been applied recently to determine (1) the genome sizes of different sporozoan parasites, including *Plasmodium*; (2) the ploidy of successive stages--hence the position of meiosis--in the life cycle of the coccidian parasite *Isospora (Toxoplasma) gondii* (Cornelissen et al. 1984a); (3) time and rate of DNA synthesis during sexual and intra-erythrocytic asexual reproduction of the rodent malaria parasite *P. berghei* and during gametocytogenesis of the human malaria parasite *P. falciparum* (Janse et al. 1986a; 1986b; submitted-a). We are now using the same methods and equipment (see also Cornelissen et al. 1984b) to measure the DNA contents of different stages of different isolates of *Trypanosoma congolense* and *T. brucei*. In addition, flow cytometry with a FACS analyser is being applied after staining the parasites with Hoechst 33258 and 33342 (Janse et al. submitted-b).

Apart from adequate staining conditions and quality of the dye itself, three factors are of crucial importance in fluorometry after Feulgen staining. First, there should be adequate hydrolysis, i.e. at room temperature in 5N HCL instead of at 60°C in 1N HCL, to ensure that no DNA is broken down during treatment. Second, the pre-exposure of stained cells to green light should be reduced or, whenever possible, avoided since the rate of fading is maximal during the first seconds of exposure. Cells should be stained under 'green light-free' (± 450 - 150 nm) conditions and afterwards should be kept in the dark until the time of measuring and they should preferably be searched for and focussed with long-wavelength light. Third, reliable references should be hydrolysed, stained and measured along with the stages that are investigated in order to enable adequate corrections, e.g. for fluctuations in lamp intensity. The reference should consist of uniform cells with uniform compactness of DNA, all non-DNA-replicating (g1) and non-DNA-amplifying--i.e. producing narrow symmetrical DNA histograms--and having a genome of about the same size as the cells to be measured. In previous investigations (see above) intro-erythrocytic ring forms of *P. berghei*, preferably from a synchronized infection, appeared to fulfil these requirements excellently, especially so since their (haploid) genome is small (25 Mbp) and since they do not synthesize DNA at all during at least the first 15 hours of development.

So far, we have measured bloodstream forms of (1) two *T. congolense* isolates, (2) mature metacyclic forms from culture (after passage through DE52) of another *T. congolense* isolate (all three isolates from East Africa), (3) bloodstream forms, both from culture (Baltz-medium at 37°C) and from mouse blood, of a *T. brucei* isolate from Uganda and (4) metacyclics from tsetse salivary probes of *T. brucei* isolate STIB 247 from Tanzania, a clone of which was used as a parent by Jenni et al. (1986). Histograms of Feulgen- and of Hoechst-stained bloodstream forms of both species

showed a large peak of cells in G1, a minor proportion of cells in G2 with twice the amount of DNA as the G1 peak, and the remaining cells with DNA values in between (S-phase). In *T. brucei* bloodstream forms, the kinetoplast was found to divide after onset but before completion of nuclear DNA replication, hence during nuclear S-phase. Metacyclic forms of both species produced symmetrical single peaks. Among *T. congolense* metacyclics from culture, a few cells in S-phase and in G2 were also present, but not among *T. brucei* metacyclic forms from tsetse salivary probes. Absolute values, in 10^{15}g $\pm$ s.e.m., are 102 $\pm$ 1.2 (n=95) and 104 $\pm$ 0.9 (n=128) for *T. congolense* bloodstream forms (G1-peak); 98 $\pm$ 1.0 (n=162) for *T. congolense* metacyclics; 114 $\pm$ 0.7 (n = 226) for *T. brucei* bloodstream forms in culture; 119 $\pm$ 3.1 (n=24) for *T. brucei* bloodstream forms in mouse blood; and 121 $\pm$ 2.8 (n=43) for *T. brucei* metacyclics.

It may be concluded:

1. There is a small but significant difference in genome size between the two species.
2. No significant differences were found between different isolates of either species.
3. In both species, metacyclic and bloodstream forms have the same ploidy, hence metacyclics must also be considered diploid.
4. In neither species were metacyclic of bloodstream forms found with significantly less than the diploid value or significantly more than twice the diploid value.

Points arising during discussion

Overdulve's finding of a normal DNA content in metacyclic forms is in direct conflict with that of the Brussels group. One other group independently corroborated Overdulve's results. They used *T. congolesne* with adsorption spectrophotometry. According to those involved, the timing of fluorescence measurement did not seem to be the reason for the differences observed. Since different material was tested in all groups concerned, it was agreed that exchange of material could establish whether the differences were due to biological or technical causes.

Session 4b: GENETIC EXCHANGE IN TRYPANOSOMES--EXPERIMENTAL STUDIES

Trypanosome hybrids generated in tsetse flies by nuclear fusion

P. Paindavoine, F. Zampetti-Bosseler, E. Pays,
J. Schweizer, M. Guyaux, L. Jenni and M. Steinert

Genetic exchange may occur between two particular *T. brucei* clones simultaneously transmitted by the same tsetse fly (Jenni et al. 1986). We report here that this exchange takes place in the fly through nuclear fusion. The resulting hybrids appear to be subtetraploid, some DNA sequences from one of the parental stocks being lost before enough cloned hybrid trypanosomes could be harvested for DNA analysis. A further reduction of the DNA content of these hybrids occurs gradually upon growth and yields near diploid DNA values in a major part of the population. This mode of hybrid generation is different from the fusion of haploid gametes, which is thought to occur normally upon inoculation of metacyclic trypanosomes in their mammalian host (Zampetti-Bosseler et al. 1986). In this process the subtetraploid hybrids appear to result from meiosis in the fly, generating subdiploid metacyclic forms which then fusion in mammalian blood.

Points arising during discussion

It was noted that, while cells with an increased DNA content were observed, tetraploidy has not been proven. The speaker was asked whether parasexual models could be invoked to explain his observations. He said that he had, in fact, considered 18 possible models but had presented only the 2 which he considered most straight forward.

The speaker pointed out that he did not see any indication of segregation in Southern blot analyses of DNA from the hybrids studied.

The suggestion was made that if pairing preferentially occurs between hybrid members of homologous sets, this might explain the failure to observe the formation of homozygotes by DNA loss. This failure was mentioned in the presentation as evidence against the former model.

It was pointed out that if one of the populations was heterozygous for a null allele of malic $enzyme_A$, then these results would become consistent with the proposed meiosis followed by segregation.

The suggestion was made that variation in times of harvest of hybrid clones from mice by different investigators might account for the observed variations in DNA content. All the hybrids in this study were harvested on day 7 after infection. Dr. Wells observed that the DNA content of the three bloodstream-form clones was stable for over 160 days. He indicated that variation in the DNA content of cloned metacyclic hybrids was observed but could not be correlated with the presence or absence of any markers.

The contribution of telomere growth and loss to changes in DNA content was discussed. It was suggested that in order to unequivocally establish whether a 500 kb chromosome was actually lost from the hybrids' genomes, it should be possible to cut this band from a gel and use it as a probe to see if the sequences had been transferred to other chromosomes. This was accepted as a good suggestion but it was also pointed out that the likely presence of repetitive sequences common to many chromosomes may present difficulties in interpreting positive signals. Sequences unique to this chromosome would be required as probes.

Flow cytofluorometric (FCF) analysis of the DNA content of parental and hybrid trypanosomes

T.D. Prospero, J.M. Wells, and R.W.F. Le Page

The DNA content of several of the cloned trypanosome stocks isolated by Jenni et al (1986) was determined by use of the Ortho H50 Cytofluorograph. Staining was by a mithramycin-propipidium iodide mixture, which recognizes double-stranded DNA with high specificity. In addition, chemical measurements of DNA content were made using a micro-diphenylamine method.

When the parental and derivative hybrid clones were compared as bloodstream forms 23 to 40 days after their isolation from infected tsetse, all the hybrid clones were found to contain elevated levels of DNA. On average the hybrids had about 50% more DNA than the parental stocks. This value was stable, both in serial blood passage (for 160 days) and following transformation to procyclic organisms (for 360 days). One hybrid clone showed a small early fall in DNA content, from about 60 to 70% more than that of the parental clone to 50 to 60% more. Isolated, purified preparations of the nuclei of procyclic organisms increased the precision of measurement of the G1/G0 DNA content values. The micro-diphenylamine assays yielded average values for the hybrid organisms 33% greater than the mean value for the parental stocks (102 femtograms per nucleus of bloodstream forms), and these analyses indicated that our FCF measurements overestimated true DNA content by a constant relative amount, attributable to the dye-binding properties of trypanosome chromatin. In combination with our studies of the karyotypes of the trypanosome hybrids, we conclude that these organisms have stable DNA contents that are about 33% greater than those of the parental stocks, and that up to half of this increase could arise from the minichromosomes.

Analysis of restriction fragment length polymorphisms (RFLP) and minichromosomes in parental and hybrid trypanosomes

J.M. Wells, T.D. Prospero and R.W.F. Le Page

Probes detecting RFLPs in parental stocks have been used to study genetic exchange in hybrid trypanosomes (Jenni et al. 1986). Analysis of other cloned trypanosome isolates strongly suggests that these polymorphisms arise from allelic sequences in a diploid genome. Two probes detecting RFLPs which are homozygous and different in the parental stocks revealed heterozygous patterns in all hybrid clones analysed. RFLPs which are heterozygous in the parent stocks have shown segregation for the alleles in hybrid clones. These patterns of inheritance cannot distinguish between models for hybrid formation which involve meiosis or those which invoke loss of DNA from synkaryons.

The minichromosomes of parental and hybrid trypanosomes have been resolved by OFAGE using very short pulse times (3 seconds). The patterns detected in the parent stocks were distinctly different. All the hybrids differed from a deliberate mixture of the parental minichromosome arrays by the acquisition of two minichromosomes and the loss of three in the 50 to 100 Kb size range. The field inversion gel electrophoresis (FIGE) procedure permitted measurement of the total amounts of minichromosome DNA in the parental and hybrid stocks by densitometric scanning of the gels. The hybrids contained almost double the amount of minichromosome DNA detected in the parental stocks. The larger minichromosomes (100 to 150 Kb) of one of the hybrids examined were also distinguishable from the others by the patterns seen in the gel scans.

Points arising during discussion

It was asked why there was such a big differece in intensity of allelic marker fragments between parent trypanosomes and hybrids. This was because additional DNA in hybrid clones will dilute out some sequences in the genome, which was noticeable from the lower intensity of hybridization to most fragments. However, if the intensity of the allelic marker fragments in hybrids and parents is expressed as a ratio to a common band detected by the same probe at the same locus, the copy number of allelic marker fragments in hybrids is found to be about half that in the parent. This would be expected for a heterozygote which has inherited one homologous chromosome from each of two homozygous parents.

Since it was indicated that minichromosomes may contribute to DNA increases between parent and hybrid parasites, a question was raisee concerning the localization of the probes used to detect RFLPs. The speaker indicated that these single copy genes were located in large chromosomes.

Allelic segregation of enzyme markers and inheritance of intermediate chromosomes and kinetoplast DNA in trypanosome crosses

A. Tait and J. Sternberg

We fed a mixture of *T. brucei* clones STIB 386-AA and STIB 247-L to *G.m. morsitans* 'super flies'. Flies in which the trypanosomes developed cyclically to metacyclic forms were allowed to feed on mice at regular intervals and the resultant parasitaemias were scored for alkaline phosphatase and isocitrate dehydrogenase isoenzyme phenotype by starch gel electrophoresis. Hybrid formation was observed at high frequency. From one of the hybrid trypanosome-producing flies, we analysed the metacyclic population in detail by preparing metacyclic forms for DNA content measurement and by cloning metacyclics for marker analysis.

The metacyclic clones were scored for the inheritance of isoenzyme phenotype using isocitrate dehydrogenase, alkaline phosphatase, malic enzymes$_{A + B}$, phosphoglycerol mutase and tyrosyltyrosyl tyrosine peptidase. One metacyclic clone was identified as parental STIB 247-L, the other six were hybrids. All the enzymes except malic enzyme$_A$ showed inheritance consistent with meiosis followed by allelic segregation. This result was also obtained when the hybrid clones were screened with two DNA probes detecting single-copy RFLPs. The hybrid clones were shown to inherit kinetoplast DNA in a uniparental fashion, with either parent capable of contributing the kinetoplast. A complex mode of inheritance was observed for intermediate chromosomes resolved on pulse field gels.

Our measurements of trypanosome nuclear DNA content showed that the hybrid trypanosome clones varied in DNA content from the parental value up to approximately 1.6 x the parental value. We examined the metacyclic population from which the clones were derived and found the same range of DNA content. We suggest that such variation in DNA content is not related to ploidy, and therefore supports equally well models of genetic exchange involving meiosis and models involving fusion of diploid nuclei followed by rapid chromosomal loss or segregation.

Points arising during discussion

It was asked whether a DNA sequence that disappeared in the course of hybrid growth was in large or small chromosomes. The disappearing sequence was in a large chromosomes.

It was also asked whether all eight cloned hybrids were from the same fly. They were. Thus it was pointed out that the sequence could have been lost in a single gene conversion event.

The point was made that the cloning frequency of the hybrids was low. However, it was suggested this might have been due to the presence of many stumpy forms in the hybrid bloodstream parasite populations.

The question was raised whether the increase in the DNA content of hybrids might be a reflection of the presence of aberrant parasites which are often noticed following fly transmission. However, it was pointed out that comparison with fly-transmitted parental controls make this an unlikely explanation.

GENERAL DISCUSSION

General discussion focused on three questions that arose from differences in the day's presentations. A major discrepancy in the observations of different laboratories concerned the DNA content of metacyclic forms. Exchange of material may be the only way to resolve this question. Also, while most groups agreed that there could be an increased DNA content in hybrid trypanosomes, there was some disagreement about the observation of DNA loss during the growth of hybrids in the mammalian bloodstream. It was suggested that the different observations could reflect different selective environments, as it was recognized that mouse strains and tsetse flies may have differed and the cloning efficiencies of hybrids from different laboratories were very different. Lastly it was noted that the hybrids under study were the product of two parental strains which have been geographically isolated from each other for probably thousands of years. Consequently, aberrant recombinations might have occurred which may bear little relation to what happens in genetic exchange in a natural field situation.

Two models were presented in an attempt to reconcile all of the data. In one model there was meiotic division with subsequent fusion to form a diploid hybrid. In the other, model fusion of diploid parents with subsequent segregation and reduction of DNA content occurs. It was suggested that the observation of an increased DNA content in hybrids favoured the second model. However, it was also pointed out that the parasite appears to have difficulty with inheritance of its minichromosomes and perhaps the first applies, except that there is no segregation and reduction of minichromosomes, leading to an increased overall DNA content in the hybrids. The point was made that one should not necessarily equate DNA content with ploidy. Furthermore it was observed that the two models need not be regarded as exclusive since some other protozoa seem to utilize both types of genetic exchange at different times. Lastly, it was noted that the STIB 386/247-L cross may be one of the most intensively analysed single mating events in the history of genetics.

Session 5: OTHER ASPECTS OF GENETIC EXCHANGE

Towards a genetics system for *Crithidia fasciculata*

D.A. Hughes

The lack of defined mutants or a workable genetics system for the members of the trypanosomatid family essentially prohibits any functional genetic characterization of these parasites. A series of projects is reported that was designed with the goal of establishing a workable genetics system for the trypanosomatids using *Crithidia fasciculata* as a model.

It was found that drug-resistant mutants of *Crithidia* could be derived at a high frequency, although the mechanism of resistance in one case was an adaptive process involving a large percentage of the population. If this is a general phenomenon, drug resistance may not be a suitable marker for genetic analysis. An interesting physical phenomenon, involving the specific paired attachment by the flagella of a high percentage of cells in liquid culture, was observed in certain drug-resistant strains which may represent mating or a conjugation event. Also, plasmid DNAs were constructed for the development of a DNA-mediated transformation system for *Crithidia*. One such plasmid clearly entered the cell using polyethylene glycol and in most cases underwent a recombination event whereby it acquired a specific genomic fragment and was dramatically rearranged. Electroporation (the application of a high-voltage pulse) dramatically increased the association of DNAse-insensitive plasmid with living cells. This association was stable for at least 5 but less than 19 hours. This technique is thus potentially useful for the introduction of DNA into *Crithidia*.

Points arising during discussion

The question was asked whether cell fusion occurred during electroporation and/or polyethylene glycol (PEG) treatment. Fusion was observed in electroporation experiments carried out with low capacitance. These were not pursued because the membrane perturbation was considered insufficient for transformation studies. PEG treatment produced extensive clumping so that it was not possible to determine whether proper fusion had occurred.

Only 1% of drug-resistant *Crithidia* colonies obtained in a transformation experiment using the neo-gene contained sequences from the transforming plasmid. This indicated a high level of phenotypic expression of resistance that might not be a consequence of transformation. A plasmid recovered from a drug-resistant *Crithidia* colony did contain part of the neo-gene, but did not confer neomycin resistance in *E. coli*, and probably did not contain the whole gene.

The possibility was raised that the *Crithidia* sequence acquired by the transforming plamid might be a *Crithidia* replication origin and might be useful in transformation vector construction.

Ploidy of genetic loci of *Leishmania major* and attempts at DNA transformation

S. Beverley, J. Cordingley, D. Iovannisci and G.M. Kapler

The average genetic ploidy of *Leishmania* has been estimated to be approximately diploid, based upon studies of total DNA content, kinetic complexity and/or isoenzyme frequencies. We have examined the ploidy of the dihydrofolate reductase/thymidylate synthetase (DHFR/TS) locus and the chromosome bearing the mini-exon locus in *L. major*, which like the APRT locus and a dispersed beta-tubulin locus appear to be at least diploid. Our studies are based on molecular analysis of these loci in drug-resistant or spontaneous mutants.

The coding region of the DHFR/TS resides on a chromosome of about 500 to 600 kB, as revealed by pulsed field gradient electrophoresis. The gene is flanked on either side by short direct repeats, separated by about 30 kB. Methotrexate-resistant cells frequently amplify this locus into an extra-chromosomal circular DNA (Beverley et al. 1984) in which an apparently homologous recombination has occurred between the repetitive elements (Cordingley & Beverley in preparation). This process may occur in a conservative manner, in which no alterations of the wild-type locus are found, or in a deletional manner, leading to excision of the DHFR/TS gene from the chromosome. Analysis of the deletion lines reveals that both a wild-type chromosome and a DHFR/TS deleted chromosome are present, and clonal derivatives all exhibit equal levels of both chromosomes. This indicates that the DHFR/TS locus is diploid in this isolate of *L. major* (Beverley et al. in preparation).

During normal laboratory propagation of our wild-type *L. major*, the population was observed to exhibit reduced levels of chromosome 2 in pulsed-field gradient analysis. No alterations in apparent viability or other properties were observed in this population. The starting and clonal lines of the variant exhibitied identical reductions in the level of chromosome 2 to the uncloned population. Using isolated chromosome 2 as a hybridization probe, we found that the variant line exhibited reduced levels of chromosome 2 and a new larger chromosome which co-migrated with chromosome 3. Interestingly, the clonal lines of the variant exhibit some heterogeneity in the size of the larger chromosome identified by the chromosome-2 probe. No other alterations in the molecular karyotype have been observed. Using an oligonucleotide corresponding to the mini-exon sequence of *L. major* (kindly provided by J. Miller of Harvard Medical School), we showed that chromosome 2 contains the mini-exon locus: this probe yielded hybridization results identical to those with the total chromosome 2 probe. Our data suggest that the mini-exon chromosome must be diploid (at least), though the approximate ploidy remains to be quantitated. We also suggest that the change in size of chromosome 2 may be due to amplification of the mini-exon repeating unit on the chromosome, as other models, such as translocations or unequal crossing-over, predict additional chromosomal modifications which are not observed. Further testing of this proposal is in progress.

The extra-chromosomal circular DNAs of methotrexate-resistant *Leishmania* are reminiscent in many ways of bacterial drug-resistance plasmids, and we have sought to use this property to develop a system of DNA transformation of *Leishmania*. We have employed methodology derived from transfections of cultured mammalian cells. If one transfects a DHFR gene into a wild-type cell and then applies methotrexate (MTX) drug pressure, a large proportion of the MTX-resistant colonies exhibit high levels of the transfected gene. Using total DNA from the amplified *Leishmania* lines, we attempted to introduce the DNA by numerous and diverse protocols followed by selection as described above, so far without unambiguous success. An unanticipated problem was that, unlike mammalian cells in which different lines exhibit very different patterns of sequence amplification, repeated elements in *Leishmania* appear frequently to direct and canalize the pattern of amplification into identical structures, making it difficult to distinguish the pattern of transfected DNA from amplification of the endogenous DHFR. We are now pursuing a systematic approach to this problem, ultimately leading to the introduction of a dominant selectable marker into the extra-chromosomal circular DNA.

Small nucleic acids in *Leishmania*

K. Stuart

We have examined the DNA karyotype of several species and subspecies of *Leishmania* to determine the utility of PFG in identifying and discriminating among *Leishmania*, to examine the heterogeneity of uncloned stocks, to invstigate the number and size of chromosomes in these stocks, and to determine the chromosomal location of various genes. During the course of these studies, we discovered in each of two isolates relatively small, multicopy nucleic acids which we have begun to characterize.

PFGE was found to be very useful in distinguishing among stocks of *Leishmania* but more limited in identifying species and subspecies, although this limitation was improved when gene localization using molecular probes was also used. Isolates were initially identified according to conventional criteria including isoenzyme, kDNA restriction, and monoclonal antibody analyses. Each isolate examined had a distinct karyotype. However, there was a direct correlation between karyotype and taxonomic position. The more closely related isolates had the most similar karyotypes while those most distantly related had the most different karyotypes.

At least 15 DNAs were resolved by PFG for each isolate, but comigrating DNAs were evident. Quantitative analyses allowed us to estimate that 26 to 33 distinct DNAs were actually resolved, which represent between one quarter and one half of the estimated nuclear DNA content. The rRNA, DHFR/TS, H region, and alpha tubulin genes were each located on single chromosomes. Beta tubulin genes were found on four chromosomes in *L. braziliensis braziliensis*, on three in *L. donovani chagasi* and *L. mexicana mexicana*, and on two in *L. mexicana amazonensis*.

The same genes were located on chromosomes with similar but not identical sizes among isolates, suggesting that these chromosomes are homologs with size differences of $\pm$ 50 kb. The basis for these chromosome size differences is not known, nor is it known if they reflect evolutionary or physiological processes. In addition, some chromosomes present in some isolates were absent in other isolates. Subclones from each of several isolates had karyotypes identical to those of the uncloned isolates. Thus, the cultured stocks were homogeneous and the DNA karyotype did not result from a mixture of subpopulations.

One isolate contained an approximately 6-kb multicopy RNA which was designated LR1. Polyethylene glycol-precipitated LR1 was recovered from culture supernatants, but the RNA did not enter agarose gels even when treated with EDTA. It did so when treated with SDS, suggesting that it exists in a complexed form, at least in the medium. LR1 was shown to be RNA by virture of its sensitivity to RNAse and NaOH but not DNAse and by its red-orange staining with acridine orange. It is single stranded, based on RNase sentitivity at different salt concentrations. It is not polyadenylated by the criterion of not binding to oligo d(T) cellulose.

The LR1 was reverse-transcribed using random primers, but this required a high-temperature pretreatment implying the presence of considerable secondary structure. LR1 cDNA probe hybridized to LR1, but did not appear to hybridize to chromosomal DNA. Our working hypothesis is that LR1 is a virus and we are now testing its transmissibility.

Another isolate contained a 250-kb multicopy DNA which was designated LD1. LD1 is DNA, based on its sensitivity to DNAse but not RNAse and its green/yellow staining with acridine orange. It is linear and double stranded, based on its mobility in PFG and cleavage by restriction endonucleases and since it can be nick-translated. Restriction fragments total about 100 kb, raising the possibility that LD1 may be dimeric. LD1 does not hybridize to DHFR/TS or H-region probes and nick-translated LD1 hybridizes only to itself and not other chromosomes. Thus, LD1 does not contain highly repeated sequences present in other *Leishmania* chromosomes. LD1 appears to be a multicopy chromosomal sequence or an extrachromosomal element such as a virus or plasmid.

Points arising during discussion

The location of the small nucleic acids could be determined by *in situ* hybridization and might help in determining whether they are components of viruses. Their location is not yet known.

Some investigators have inferred the presence of viruses in *Leishmania* by electron microscopy and detection of small nucleic acid molecules, while others have been unable to confirm their presence. The abundance of the DNA species described here is sensitive to growth conditions, being highest in stationary phase. This consideration may be important in attempts to identify comparable molecules in other systems.

The possibility was raised that changes in the size of the chromosome on which specific genes are located might be related to processes of gene amplification and loss. This was not investigated in the work described.

The question of whether the molecules described might belong to viruses could be approached by attempts to infect *Leishmania* strains lacking the molecules. For this purpose, cloned probe sequences to detect the infection would be desirable. Transfer to these strains might also be possible when optimum conditions for introduction of the nucleic acid molecules themselves have been determined.

Genes induced during meiosis in *Tetrahymena*

D. Martindale

Tetrahymena thermophila is a 'typical' ciliated protozoan and is easily grown and manipulated in the laboratory. Its sexual process (conjugation) is particularly amenable to study because of the ease with which it can be synchronously induced in over 90 % of a large cell population (Martindale et al. 1982). Large quantities of stage-specific material can be obtained, facilitating biochemical and molecular analyses of the process. We found that meiotic prophase is a particularly active time for the synthesis of stage-specific proteins (Martindale et al. 1985). Sequences representing genes induced during meiotic prophase were isolated through the construction of a cDNA library using meiotic prophase polyA+ RNA (Martindale & Bruns 1983). Eight genes have been examined. Three of these (cnj A, B and C) make transcripts that are found only during early conjugation (meiosis), with the transcripts peaking in abundance during mid-meiotic prophase. The physical arrangement of these genes in *Tetrahymena*'s two nuclei (macro- or somatic nucleus and micro- or germinal nucleus) has been examined (Martindale et al. 1986).

Reasoning that meiosis is a fundamental eukaryotic process and therefore the DNA sequence of some of the genes involved in the process might be conserved, cDNA-plasmids representing each of the eight genes were used as probes against the genomic DNA of other organisms. Two of the eight (pC7 = cnj C and pC8 = cup C) strongly cross-hybridized to different-sized restriction fragments from yeast DNA under stringent conditions; pC7 also hybridized to *Drosophila* DNA. The pC7 transcript is meiosis-specific, while the pC8 transcript is strongly induced during meiosis but present as well during starvation and vegetative growth.

Clones were isolated containing yeast and *Drosophila* DNA that cross-hybridized to the cDNA-plasmid pC7. *In-situ* hybridization to polytene chromosomes (with M. Goldberg, Cornell University) determined that a PC7-homologous region resided in the same band as zeste, an interesting gene that interacts with several loci when they are synapsed. This greatly facilitated the cloning of the zeste gene (Gunaratne et al. 1986), but the pC7-homologous region was found to be about 10 kb from zeste, in a region where no known gene exists. Yeast DNA carrying a pC7-homologus region was found to hybridize to two yeast polyA$^+$ RNAs, one of which is quickly induced in sporulation medium but which is not under mating-type control. The cross-hybridizing region of pC7 was localized to the 3' end of the cDNA and this region was sequenced. Sequencing the homologous region in yeast DNA has proven difficult, probably due to a long polyA track. This would indicate that 'conserved' behavior of pC7 is due to a cDNA artifact, even though pC7-homologous yeast DNA was found to cross-hybridize to pC7 only and not to any of the other cDNA plasmids (all of which should have a polyA run at one end of the cDNA insert). Further evidence that the conserved behavior of pC7 may be an artifact comes from the inability of pC7 to cross-hybridize to DNA from other ciliates (*T. pyriforms* and the hypotrichs *Stylonychia* and *Oxytricha*). The function of the

starvation-induced yeast gene will be investigated by directed mutagenesis in yeast.

The cDNA plasmid pC8 behaved as expected of a conserved gene when it was used to probe the genomic DNA of several organisms: it strongly hybridized to DNA from all ciliates examined (*T. pyriformis*, *Stylonychia* and *Oxytricha*) as well as yeast. Little or no hybridization to *Drosophila* was detected. The chromomal DNA of the hypotrichs is processed to gene-sized pieces in the macronucleus; pC8 (mRNA is 4200 bases) hybridized to a 4200 bp and a 4800 bp DNA fragment from *Stylonychia* and *Oxytricha* respectively. Yeast DNA homologous to pC8 has been isolated and hybridizes to both pC8 and *Tetrahymena* genomic DNA. Preliminary evidence suggests that the pC8-homologous yeast DNA is transcribed. Sequencing of this yeast DNA and pC8 has begun.

We reasoned that protozoan genes active in meiosis and conserved might be used as probes against *Trypanosoma* DNA and, if hybridization was seen, these genes might be used to determine at what stage in the trypanosome life cycle the genes are active. Hybridization against trypanosome DNA at high and low stringencies with cDNA-plasmid representing the eight *Tetrahymena* genes described above has been negative, even with pC8. A consistant background of hybridization (high molecular weight smear) was seen with all probes (including vector alone) against trypanosome DNA. This was not seen with any other genomic DNA and may be obscuring bands. However, a recent examination of the evolutionary diversity of eukaryotic small-subunit rRNA genes (Sogin et al. 1986) suggests that *Trypanosoma* diverged much earlier than the ciliates and, surprisingly, that the evolutionary distance between *Tetrahymena* and *Trypanosoma* is much greater than that between *Tetrahymena* and yeast.

Points arising during discussion

Another cDNA clone, pC5, contains an extremely abundant transcript that is induced upon starvation, anoxia or heat shock. Having been originally identified in starvation, it may not be correct to regard it as a heat-shock gene.

RNA joining and the construction vectors for DNA transformation

N. Agabian, W. Murphy, K. Perry, K. Watkins and S. Michaeli

Gene expression in trypanosomes involves a novel process of RNA joining where two unlinked genetic loci, one which encodes a spliced leader (SL)-RNA and the other a target gene transcript, are joined to produce a single mRNA. Since the discovery of chimeric mRNAs in trypanosomes, the resolution of the mechanism of RNA joining has become a crucial factor in understanding the regulation of gene activity in this system. Whether RNA joining occurs during transcription of the target gene or post transcriptionally via RNA splicing will influence strategies for construction and presentation of DNA constructs in developing DNA transformation protocols.

Our laboratory has recently demonostrated that RNA joining occurs via transRNA splicing. The SL-RNA, bearing a highly modified cap structure beginning with zone G, serves as a donor of the 35-nucleotide SL to the target mRNA. Specific intermediates in the reaction which are compatible only with a trans-splicing model have been identified and characterized. The mechanism appears similar to cisRNA-splicing in other eukaryotes except that the 'intron' sequences (3' 100 nucleotides of the SL-RNA and 5' untranslated sequences of pre-mRNA) are not joined before splicing. The discontinuous intron results in the formation of a 'y' rather than a lariat intermediate.

Since essentially all mRNAs in trypanosomatid organisms contain the SL-RNA at their 5'-termini, DNA vectors in gene transformation must be constructed in such a manner as to include appropriate splice recognition sequences so that the heterologous or exogenous DNA can receive a capped SL sequence. While there is no evidence that the SL is absolutely required for mRNA function, the ubiquity of this sequence would suggest that inclusion of appropriate splice recognition sites would be useful in ensuring gene expression.

Points arising during discussion

Assuming that the SL sequence and components of the splicing complex will be available within a trypanosome into which a transfection vector has been introduced, it should not be necessary to include the leader sequence within a transformation vector. Probable requirements are the gene to be expressed, appropirate signals to ensure transcription and sequences recognized by the splicing complex. The latter might incude sequences at the splicing intermediate branch point and structural elements involved in formation of the complex. Indeed, inclusion of the SL sequence within the same transcript as the gene might interfere with proper recognition by other components of the complex.

The idea of an extra-nuclear expression vector has the advantage that the complex process of SL addition might not be required, since transport into the cytoplasm may be one of the functions of this sequence. However, there is no reason to expect that transcription in the cytoplasm is possible with such a vector, and the SL addition also provides the mRNA cap which may be required for stability. The

speaker considered that a vector designed to operate within the nucleus was required.

Transcripts from VSG gene expression sites and those from the repeated tubulin genes are probably very long and poly-cistronic. Identification of the regulatory and splicing sequences for these genes has been difficult. Single-copy genes with well defined primary transcripts may provide a better means of identifying those sequences for inclusion in an expression vector.

The splicing complex particles have been isolated in the speaker's laboratory. The secondary structure of the SL-RNA within the splicing particle, predicted from computer analysis, contains an unpaired loop at the end of one arm of a Y-shaped molecule. This may be exposed in the particle which contains RNA species 1, 2 or 3 bases longer than the genuine 100 bp splicing product. These are due to cleavage of this loop which may be a nuclease-hypersensitive site within the particle. Otherwise the particle is stable.

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ACRONYMS

ADA	adenosine deaminase
AP	alkaline phosphatase
APRT	adenosine phosphoribosyl transferase
AnTar	Antwerp *Trypanozoon* antigen repertoire
bp	base pair
DEAE	di-ethyl aminoethyl
DHFR	dihydrofolate reductase
DHFR/TS	dihydrofolate reductase/thymidylate synthetase
DNA	desoxyribonucleic acid
DNAse	desoxyribonuclease
DTT	dithiothreitol
EATRO	East African Trypanosomeasis Research Organization (now Ugandan Trypanosomiasis Research Organization)
EDTA	ethylene diamine tetra-acetic acid
ELC	expression-linked copy
ESAG	expression-site associated gene
FACS	fluorescence-activated cell sorter
FCF	flow cytofluorometry
FIGE	field inversion gel electrophoresis
HRP	histidine rich protein
ICD	isocitrate dehydrogenase
IEF	isoelectric focusing
IFA	indirect fluorescent antibody
ILRAD	International Laboratory for Research on Animal Diseases
MTX	methotrexate
Mb	megabase pairs
OFAGE	orthogonal-field alternation gel electrophoresis
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PFG	pulse field gradient gel electrophoresis
poly A+	polyadenylated (RNA)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
s.e.m.	scanning electron micrograph
SL	spliced leader
VAT	variable antigen type
VSG	variable surface glycoprotein