Chromosomes and Sexual Development of Rodent Malaria Parasites

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Key words: *Plasmodium* - rodent malarial parasites - sexual development - chromosomes - gene expression

The unicellular eukaryotic parasites which are members of the genus *Plasmodium*, have a complex life cycle, in which several rounds of asexual multiplication alternates with a sexual phase. During the sexual development male and female gametocytes are formed in the blood of the vertebrate host, which are the precursors of the gametes. The haploid gametes are formed in the midgut of the mosquito host. Fertilization results in the production of diploid zygotes which develop into motile ookinetes. Although the sexual development is well studied at the cellular level and the light- and electron microscopic morphology of the different developmental stages have been described, nothing is yet known about the molecular basis of the differentiation of asexual blood stage parasites into male and female sexual cells. Fertilization and the formation of the diploid zygote followed by meiosis, appears to resemble these processes in other eukaryotes. However, the genetic and environmental factors which underlie differentiation of male and female gametocytes/gametes are unknown and apparently different from sexual differentiation in other (closely related) eukaryotes. We studied the chromosomal organization of rodent parasites and expression of genes which are transcribed early during sexual development to get more insight into the genetic factors which direct the sexual differentiation in *Plasmodium*.

MATERIALS AND METHODS

Methods to study chromosomal structure have been published in detail elsewhere (Ponzi et al. 1990, Janse et al. 1992). Methods for the study of gene expression have been described in Paton et al. 1993). Parasites, culture techniques for the asexual and sexual stages of *P. berghei* and purification and collection of the developmental stages have been described in Janse et al. (1984, 1985, 1989).

RESULTS AND DISCUSSION

GENOME AND CHROMOSOME STRUCTURE

The genome of the four rodent parasites *P. berghei, P. yoelii, P. chabaudi* and *P. vinckei* comprise fourteen chromosomes, ranging in size between 0.5 and 3.5 Mb. Both intra- and interspecific size differences exists between homologous chromosomes of these species. Size differences of up to 0.5 Mb have been found between several homologous chromosomes of different strains of one species.

We have studied in detail size polymorphisms in *P. berghei* chromosomes, which arose in cloned lines during asexual multiplication in the blood of the vertebrate host. The following processes appeared to be responsible for changes in the size of chromosomes: Deletion and acquisition of subtelomeric repeat-sequences, chromosome duplication, chromosome breakage followed by healing by telomere addition, chromosome translocation and possibly mitotic recombination between non-homologous chromosomes (Janse 1993). Most size polymorphisms in *P. berghei* could be explained by variation in numbers of subtelomeric repeated sequences. The subtelomeric regions of several chromosomes contain different numbers of a 2.3 kb repeat. Changes in the number of this repeat unit occur frequently and apparently do not affect the viability of the parasites (Ponzi et al. 1990).

In *P. berghei* chromosomal rearrangements appear to affect mainly the subtelomeric regions of

The work in the three laboratories was financied by the EEC in the framework of the Program 'Science and Technology for Development' (TS3*-CT92-0116).

chromosomes, while the internal regions appear to be less prone to large scale rearrangements. On the other hand we have found that exchange of genes between non-homologous chromosomes occurs as a result of chromosome translocations (Janse et al. 1992). Moreover, evidence has been presented for the occurence of mitotic recombination between non-homologous chromosomes resulting in the exchange of DNA-sequences (Pace et al. 1990). If large scale rearrangements which affect the location of genes in the genome, occur frequently one could question whether the different chromosomes of malarial parasites could be defined as conserved entities containing groups of closely linked genes, comparable to chromosomes of higher eukaryotes. To answer this question we compared the location and linkage of 50 genes on the polymorphic chromosomes of the four rodent parasites. It appeared that the location and linkage of genes on homologous chromosomes was quite conserved despite extensive intra- and interspecific size polymorhisms of chromosomes (manuscript in preparation). This result indicates that large scale rearrangements involving inter-chromosomal translocations of genes does not significantly contribute to size polymorphisms in *Plasmodium* species which infect rodents. Parasites with internal, large scale rearrangements apparently have a selective disadvantage in nature.

The results of these studies on chromosomes of rodent parasites show that chromosomes of *Plasmodium* closely resemble chromosomes of other eukaryotes. Chromosomes are comprised of closely linked genes and size polymorphisms are mainly due to variation in non-coding subtelomeric repeats. Both the function of these repeats and the function of variation of the number of repeats is unknown. Parasites with large scale rearrangements which affect expression and location of genes most likely arise as a result of aberrant processes during DNA replication and recombination. These parasites are often found in *in vitro* cultures or after artificial selection procedures and are less frequently observed in natural isolates. These aberrant processses are not unique for *Plasmodium* but occur in the DNA of many other eukaryotes.

CHROMOSOME STRUCTURE AND SEXUAL DEVELOPMENT

Comparison of linkage between genes in closely and distantly related species might be useful to establish common and conserved linkage groups and in addition may provide information about the evolution of genomes. The presence of conserved linkage groups of genes in distantly related species might be an indication of the existence of functional relationships between linked genes. For example coordination of timing of expression of a set of genes might require linkage. In many lower eukaryotes groups of genes which are involved in sexual determination are closely linked in the genome. Interestingly, in the rodent malaria parasites several genes which are expressed during early sexual development (C-type rRNA, PbS21, -tubulin) are located on chromosome 5 in the rodent malaria parasites (manuscript in preparation).

The capacity to produce sexual cells can easily be lost by the rodent parasites during prolonged periods of asexual multiplication. The loss is irreversible and is most likely due to mutations in or deletions of genes which are necessary for the development of the sexual cells. We studied the karyotype, chromosome size polymorphism and large scale deletions in detail in a number of cloned lines of *P. berghei* which lost the capacity to produce gametocytes (Janse et al. 1991). We found evidence that deletions in the subtelomeric region of chromosome 5 coincided with the loss of gametocyte production.

Moreover, in two non-producer lines of *P. berghei*, expression of a gene which is located on chromosome 5, is lost as a result of rearrangement. This rearrangement appears to be located at the same site of the gene in both lines (Ponzi et al. this issue). The function of this gene is yet unknown but studies are underway to characterize the gene and the DNA-rearrangement in more detail.

Characterization and localization of more genes involved in sexual development is necessary to establish whether chromosome 5 might play an crucial role in the sexual cycle. If indeed genes involved in sexual development appear to be clustered in the genome of *Plasmodium* , the study of the region of clustered genes might provide information about genes which determine the initial steps in the sexual differentiation of *Plasmodium.*

GENE EXPRESSION AND SEXUAL DEVELOPMENT

Two genes which are expressed in sexual stages and which are located on chromosome 5 are under study.

We are investigating the control of expression of the gene encoding the ookinete specific protein of *P. berghei* designated Pbs21 (Paton et al. 1993). Northern analysis using stage specific RNA preparations from pure cultured sexual forms demonstrated that production of the gene transcript initiated in gametocytes yet translation is evident only after fertilisation and the transcript is considered to be translationally repressed. Furthermore we have now evidence that in later ookinete stages (14-20 hours after fertilisation) the ~1500 nt transcript is processed and reduced in size by some 50-100 nt. Mapping studies are therefore in hand to demonstrate both the start point of transcription and the site of processing in the mRNA. Preliminary evidence indicates that the start site of transcription lies ~350 nt upstream of the translational start site and that a small subset of poly(A) addition sites are used, clustered within 20 nt. The cDNA sequence reported by Paton et al. (1993) employs one of this subset. An additional 500bp of upstream sequence and 300bp of downstream sequence have been cloned and characterised. This will include the transcriptional initiation and termination sites and the developmentally regulated promoter region. Structural analysis failed to reveal conserved motifs or higher order structures in the untranslated regions. Future research will pinpoint the transcriptional start sites and site(s) of processing of the transcript. No introns have yet been detected in the gene.

The nuclear rRNA genes are expressed in a stage specific fashion. The 4 rRNA units (AD) of *P. berghei* are divided into two groups, A-type (A and B units) expressed predominantly in asexual blood stage parasites and C-type (C and D units), expressed in the insect stages. We have developed probes which allow the demonstration of transcriptional activity of the two classes of rRNA gene in the highly developed *P. berghei*-rodent model available in the laboratory (manuscript in preparation). The probes detect the external transcribed spacer (ETS) of the two types of rRNA unit and demonstrate not only transcription but also the degree of conservation between the genes comprising the two types of rDNA unit. An A gene ETS PCR probe recognises both the A and B units and the C unit ETS probe recognises the C and D units, demonstrating the extent of the sequence identity. Interestingly the A gene ETS probe also hybridises to the homologues in all other rodent malaria species whereas the C probe only recognises units from *P. berghei*. Thus the ETS region appears to diverge at different rates (manuscript in preparation).

Using the C gene probe we have demonstrated a biphasic mode of C-type unit transcription. There is an initial pulse of transcription at 20 hours after erythrocyte invasion. This pulse occurs to a different extent in different strains of *P. berghei* including clones which cannot produce gametocytes. Given the different kinetics of gametocyte formation in *P. berghei* (30 hours) and *P. falciparum* (8 days), it is possible that this transcriptional pulse in *P. berghei* is the equivalent to the published transcriptional event seen in mature gametocytes of *P. falciparum*. The significance of this event is the subject of continuing investigation. A second C-type transcriptional event occurs between 14 and 20 hours after fertilisation in ookinetes which is apparently the onset of continuous C-type transcription. This is the first demonstration of the precise timing of the transcriptional switch in the stage specific rRNA genes in *Plasmodium*. C-type transcription continues throughout parasite development within the mosquito but demonstrates a temporal pattern of precursor formation. Currently this is interpreted as differential expression of the C and D units indicating that they generate specific precursors distinguishable by their size.

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