fetus would have inherited the Rb susceptibility gene. Such a fetus almost certainly would develop retinoblastoma and be at much higher risk to have other malignancies, particularly osteosarcoma, in later years if the pregnancy reaches term. The cDNA probe could also allow one to identify some of the estimated 15% of individuals who have unilateral sporadic Rb but who unfortunately have the hereditary form of the disease.

REFERENCES AND NOTES

1. D. E. Comings, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3324 (1973); A. K. Knudson, Jr., *Semin. Oncol.* **5**, 57 (1978); L. C. Strong, V. M. Riccardi, R. E. Ferrell, R. S. Sparkes, *Science* **213**, 1501 (1981); W. F. Benedict *et al.*, *ibid.* **219**, 973 (1983); R. Godbout, T. P. Dryja, J. Squire, B. L. Gallie, R. A. Phillips, *Nature (London)* **304**, 451 (1983).
2. W. K. Cavenee *et al.*, *Nature (London)* **305**, 779 (1983).
3. T. P. Dryja *et al.*, *N. Engl. J. Med.* **310**, 550 (1984).
4. A. L. Murphree and W. F. Benedict, *Science* **223**, 1028 (1984).
5. A. G. Knudson, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 820 (1971).
6. J. J. Yunis and N. Ramsay, *Am. J. Dis. Child.* **132**, 161 (1978).
7. G. Balaban, F. Gilbert, W. Nichols, A. T. Meadows, J. Shields, *Cancer Genet. Cytogenet.* **6**, 213 (1982); G. Balaban-Malenbaum *et al.*, *ibid.* **3**, 243 (1981); W. F. Benedict, A. Banerjee, C. Mark, A. L. Murphree, *ibid.* **10**, 311 (1983).
8. D. H. Abramson, R. M. Ellsworth, F. D. Kitchin, G. Tung, *Ophthalmology* **91**, 1351 (1984).
9. M. F. Hansen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6216 (1985); T. P. Dryja *et al.*, *Am. J. Hum. Genet.* **38**, 59 (1986).
10. T. Motegi, M. Komatsu, Y. Nakazato, M. Ohuchi, K. Minoda, *Hum. Genet.* **60**, 193 (1982); P. Ward *et al.*, *J. Med. Genet.* **21**, 92 (1984).
11. M. Miyaki, N. Akamatsu, T. Ono, M. S. Sasaki, *Cancer Lett.* **18**, 137 (1983).
12. R. S. Sparkes *et al.*, *Hum. Genet.* **68**, 258 (1984).
13. M. Lalande *et al.*, *Cancer Genet. Cytogenet.* **13**, 283 (1984).
14. W. Benedict *et al.*, *Cancer Res.*, in press.
15. Ø. Fodstad *et al.*, *Int. J. Cancer* **38**, 33 (1986).
16. S. H. Friend *et al.*, *Nature (London)* **323**, 643 (1986).
17. W. H. Lee *et al.*, *Science* **235**, 1394 (1987).
18. T. Maniatis, E. F. Fritsch, J. Sambrook, Eds., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
19. A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983).
20. P. A. Krieg and D. A. Melton, *Nucleic Acids Res.* **12**, 7057 (1984).
21. Y. K. T. Fung, G. M. Shackleford, A. M. C. Brown, G. S. Sanders, H. E. Varmus, *Mol. Cell Biol.* **5**, 3337 (1985).

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Genetic Analysis of the Human Malaria Parasite *Plasmodium falciparum*

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Malaria parasites are haploid for most of their life cycle, with zygote formation and meiosis occurring during the mosquito phase of development. The parasites can be analyzed genetically by transmitting mixtures of cloned parasites through mosquitoes to permit cross-fertilization of gametes to occur. A cross was made between two clones of *Plasmodium falciparum* differing in enzymes, drug sensitivity, antigens, and chromosome patterns. Parasites showing recombination between the parent clone markers were detected at a high frequency. Novel forms of certain chromosomes, detected by pulsed-field gradient gel electrophoresis, were produced readily, showing that extensive rearrangements occur in the parasite genome after cross-fertilization. Since patients are frequently infected with mixtures of genetically distinct parasites, mosquito transmission is likely to provide the principal mechanisms for generating parasites with novel genotypes.

NEW METHODS ARE NEEDED FOR treating and controlling *Plasmodium falciparum*, the most pathogenic species of malaria parasite infecting humans. Control by chemotherapy has been complicated by the emergence of drug-resistant forms (*1*). Several antigens have been identified as candidates for vaccines but may exhibit considerable diversity in the parasite population (*2*). There is thus a risk that if vaccines based on such antigens are put into widespread use, new or alternative forms of the parasite will be selected and replace previously existing forms.

Few studies have been made on the basic genetics of malaria parasites by conventional strain hybridization techniques. This is mainly because the organisms undergo a complex life cycle in which asexual multiplication and gametocyte development occur in the vertebrate host, while fertilization between gametes takes place in the mosquito vector. Crossing experiments have been possible to date only with species that infect rodents (*P. yoelii* and *P. chabaudi*) (*3*), because the complete life cycle of these parasites can be maintained in the laboratory more easily than most other malaria species. Crosses between rodent parasites have been made by feeding mosquitoes on mixtures of two cloned lines to allow cross-fertilization to occur and then infecting rodents with the resulting sporozoites. The blood forms developing in these animals are then examined for the presence of forms exhibiting recombination between parent clone markers. These studies show that (i) the parasites appear to undergo a normal Mendelian pattern of inheritance of genetically determined characters; (ii) the blood forms are haploid, meiosis probably occurring during early division of the zygote (*4*); (iii) resistance to drugs such as pyrimethamine and chloroquine is due to gene mutations (*5*); and (iv) recombination between genes determining characters such as enzymes, antigens, and drug sensitivity occurs readily after cross-fertilization between clones (*3*).

The asexual erythrocytic forms (*6*) and gametocytes (*7*) of *P. falciparum* can be maintained in culture in vitro. Cultured gametocytes are infective to mosquitoes (*7*), and the resulting sporozoites infective to primates (*8*). Blood forms of *P. falciparum* can be cloned by limiting dilution (*9*) or micromanipulation (*10*), so that genetically pure lines can be obtained. Here we report successful crossing of two cloned lines of *P.*

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Table 1. Characteristics of parent clones. Pyrimethamine response was tested by the method of Thaithong and Beale (*13*), ADA typing was done by agarose gel electrophoresis (*14*), and antigen typing was done by immunofluorescence (*15*).

Characters	3D7	HB3
Origin	Netherlands	Honduras
Pyrimethamine response	Sensitive	Resistant
ADA form	ADA-1	ADA-2
Antigen type		
195-kD		
Mab 7.3	−	+
Mab 9.2	+	−
40-kD		
Mab 12.3	+	−

falciparum by infecting a chimpanzee with sporozoites from mosquitoes fed on a mixture of gametocytes of each clone. We show that genetic recombination occurs at a high frequency, resulting in the production of parasites with new genotypes. The term "genetic recombination" is used here to denote the appearance of forms exhibiting nonparental combinations of characters after the cross, which may be due to random assortment of chromosomes during meiosis or to physical exchange of genetic markers on homologous chromosomes.

The clones used as parent lines for the cross were 3D7, which was derived from isolate NF54 from the Netherlands (*11*) by limiting dilution, and HB3, derived from isolate H1 from Honduras by microscopic selection (*12*). The clones differed from one another in pyrimethamine sensitivity (*13*), in electrophoretic forms of the enzyme adenosine deaminase (ADA) (*14*), and in epitopes of two blood-form antigens, one of 195 kD having epitopes recognized by two monoclonal antibodies (Mabs) denoted 7.3 and 9.2, and one of 40 kD recognized by a Mab denoted 12.3 (*15*) (Table 1). They differed additionally in patterns of hybridization of a repetitive DNA probe (*16*), in certain proteins detected by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (*17*), and in chromosome sizes as revealed by pulsed-field gradient (PFG) gel electrophoresis (*18–20*).

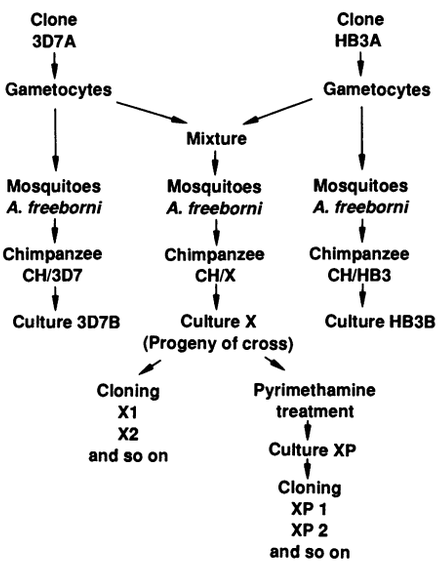

Fig. 1. Procedure used for crossing *P. falciparum* clones 3D7A and HB3A.

Cultures of each clone, denoted 3D7A and HB3A, were established in flasks, in conditions permitting development of gametocytes infective to mosquitoes (*21*) (Fig. 1). Fifteen days after initiating the cultures, when mature gametocytes were present, each culture was centrifuged (500*g* for 5 minutes), and the pellet of parasitized erythrocytes was resuspended in human serum at 50% hematocrit, at 37°C. Equal quantities of 3D7A and HB3A were mixed together and placed in a mosquito membrane-feeding apparatus. Approximately 1500 *Anopheles freeborni* were fed on the mixture. As controls, equivalent numbers of *A. freeborni* were fed on gametocytes of 3D7A and HB3A separately. After 7 days, 15 mosquitoes of each group were examined for oocyst development. In the mosquitoes fed on the mixture, an average of 17 oocysts per midgut was found; in 3D7A and HB3A, averages of 29 and 37 oocysts, respectively, per midgut were seen.

Sixteen days after the mosquitoes were fed on gametocytes, sporozoites were detected in their salivary glands. Sporozoites from each batch of mosquitoes were then used to infect splenectomized chimpanzees. One animal, denoted CH/X, received sporozoites from mosquitoes fed on the gametocyte mixture. Two additional animals, denoted CH/3D7 and CH/HB3, received sporozoites from mosquitoes fed on each respective parent line. Each chimpanzee received sporozoites from (i) the bite of infected mosquitoes and (ii) preparations of sporozoites isolated from mosquitoes and injected intravenously by syringe. For (i), approximately 500 mosquitoes of each batch were permitted to feed on each chimpanzee, which had been lightly anesthetized with ketamine. For (ii), sporozoites were extracted from mosquitoes by the method of Ozaki *et al.* (*22*) and suspended in Grace's insect medium (Gibco) containing 10% human serum, type AB. For chimpanzees CH/X and CH/3D7, the inocula each contained approximately 4×10^6 sporozoites, and for CH/HB3, approximately 2×10^6 sporozoites.

Parasites appeared in the blood of each chimpanzee on day 10 after sporozoite infection. Cultures of parasites from each animal were then established in human erythrocytes in flasks (*21*) and denoted X, 3D7B, and HB3B, respectively. The presence of recombinant forms among the progeny of the cross was tested in two ways: (i) by treating the uncloned progeny (X) with pyrimethamine and examining surviving parasites for enzyme and antigen markers, and (ii) by isolating clones from the progeny and characterizing them for parent line markers.

Table 2. Characters of parent clones and uncloned progeny of cross before and after pyrimethamine treatment. For methods of characterization, see (*13–15*).

Pyrimethamine treatment	ADA	195-kD antigen		40-kD antigen
		Mab 7.3	Mab 9.2	Mab 12.3
	Parasites 3D7B			
Untreated	1	−	+	+
Treated		−	−	−
	Parasites HB3B			
Untreated	2	+	−	−
Treated	2	+	−	−
	Parasites X			
Untreated	1, 2	+	+	+
Treated	1, 2	+	+	+

Cultures of the uncloned progeny X were first treated with $10^{-6}M$ pyrimethamine for 3 days; this treatment was shown to kill 3D7B, but not HB3B parasites. X parasites were found to survive pyrimethamine treatment, and these were used to establish new cultures denoted XP. X and XP, together with 3D7B and HB3B, were examined for ADA type and antigen markers (Fig. 2 and Table 2). In XP as well as X, both ADA-1 and -2 and antigens recognized by all three Mabs 7.3, 9.2, and 12.3 were present. Recombinant-type parasites, characterized by pyrimethamine resistance, ADA-1, and antigens positive for antibodies 9.2 and 12.3 were thus present among the uncloned progeny; these enzyme and antigen characters were not present in HB3B parasites that had survived pyrimethamine treatment, nor were they present in the cultures of 3D7B

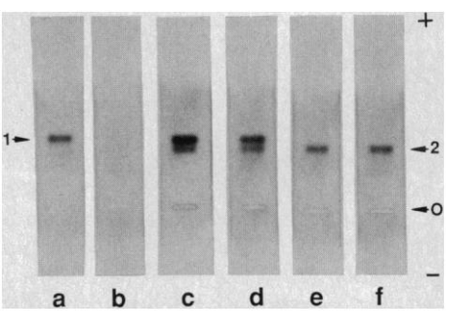

Fig. 2. Electrophoretic forms of adenosine deaminase (ADA) in parents and uncloned progeny of cross. Numerals 1 and 2 indicate positions of ADA-1 and ADA-2, respectively. O indicates origin; (a) 3D7B, (b) 3D7B pyrimethamine treated, (c) X, (d) pyrimethamine-treated X, (e) HB3B, (f) pyrimethamine-treated HB3B.

that had been drug-treated and in which some debris of dead parasites could be detected (Table 2).

Recombination between the antigenic markers was shown by immunofluorescence assays (IFAs) on the uncloned progeny. Preparations of X and XP containing schizonts were made on multispot slides on days 11 and 36 after cultures were established from the chimpanzee. The slides were incubated with either a mixture of Mabs 7.3 (isotype IgG2a) and 12.3 (IgG1), or a mixture of Mabs 7.3 (IgG2a) and 9.2 (IgG1). Each preparation was then stained with a mixture of two fluorescent reagents: a fluorescein-conjugated antibody that recognizes IgG2a, and a rhodamine-conjugated antibody that recognizes IgG1. The preparations were then examined by ultraviolet microscopy.

Preparations incubated with Mabs 7.3 and 12.3 showed three types of fluorescent organisms: those that stained only with fluorescein (7.3+, 12.3−); those that stained only with rhodamine (7.3−, 12.3+); and those that stained with both reagents (7.3+, 12.3+) (Fig. 3). Control preparations of each parent line stained by the same procedure showed only fluorescein-stained forms in HB3A and B, and only rhodamine-stained forms in 3D7A and B. The 7.3+, 12.3+ forms seen in X and XP were thus recombinants for the two antigens involved. Counts of the proportions of each type of fluorescent parasite in these preparations are shown in Table 3. Preparations of X and XP that were incubated with mixed Mabs 7.3 and 9.2 showed only two types of parasite, those that stained only with fluorescein and those that stained only with rhodamine. Thus, no recombination between these markers was detected.

Table 3. Changes in proportion of antigenically distinct parasites during cultures of untreated (X) and pyrimethamine-treated (XP) progeny of cross.

Parasites	Day of culture	Number of parasites counted	HB3-type 7.3+, 12.3− (%)	3D7-type 7.3−, 12.3+ (%)	Recombinant 7.3+, 12.3+ (%)
X	11	305	35.7	29.8	34.5
	36	168	81.5	10.8	7.7
XP	11	220	32.7	28.7	38.6
	36	215	83.3	6.0	10.7

Clones were established from X and XP on day 32 after cultures were set up from the chimpanzee. Each culture was diluted into aliquots of 0.1 ml, each containing an estimated 0.5 parasite, which were placed in wells of microtiter plates. After 14 days of culture, parasites were seen in 13 out of 40 wells set up from X, and in 19 out of 40 wells set up from XP. Each positive culture, denoted X1, X2, XP1, XP2, and so on, was grown in flasks to provide quantities sufficient for genetic characterization. Fourteen cultures were examined for pyrimethamine response, ADA type, and antigens (Table 4), and for chromosomes by PFG gel electrophoresis (*23*) (Figs. 4 and 5).

In cloning by dilution, it is expected that a minority of cultures will be initiated from more than one parasitized cell, or from cells containing more than one parasite. On the basis of an average of 0.5 parasite per well, it can be predicted from the Poisson distribution that approximately 77% of the positive cultures will be derived from single parasites, and the remainder from two or more parasites. Since the blood forms of malaria parasites are haploid, one can presume that cultures in which two forms of a given gene product are present are parasite mixtures. Cultures X1 and X9, which exhibited two forms of ADA, were thus most probably not clones. The remaining 12 cultures appeared to be pure clones, because they were characterized by only single forms of ADA, antigens, and other proteins detected by 2D-PAGE (*17*).

Five progeny clones (X4, XP1, XP6, XP7, and XP9) had pyrimethamine-response, enzyme, and antigen characteristics that were

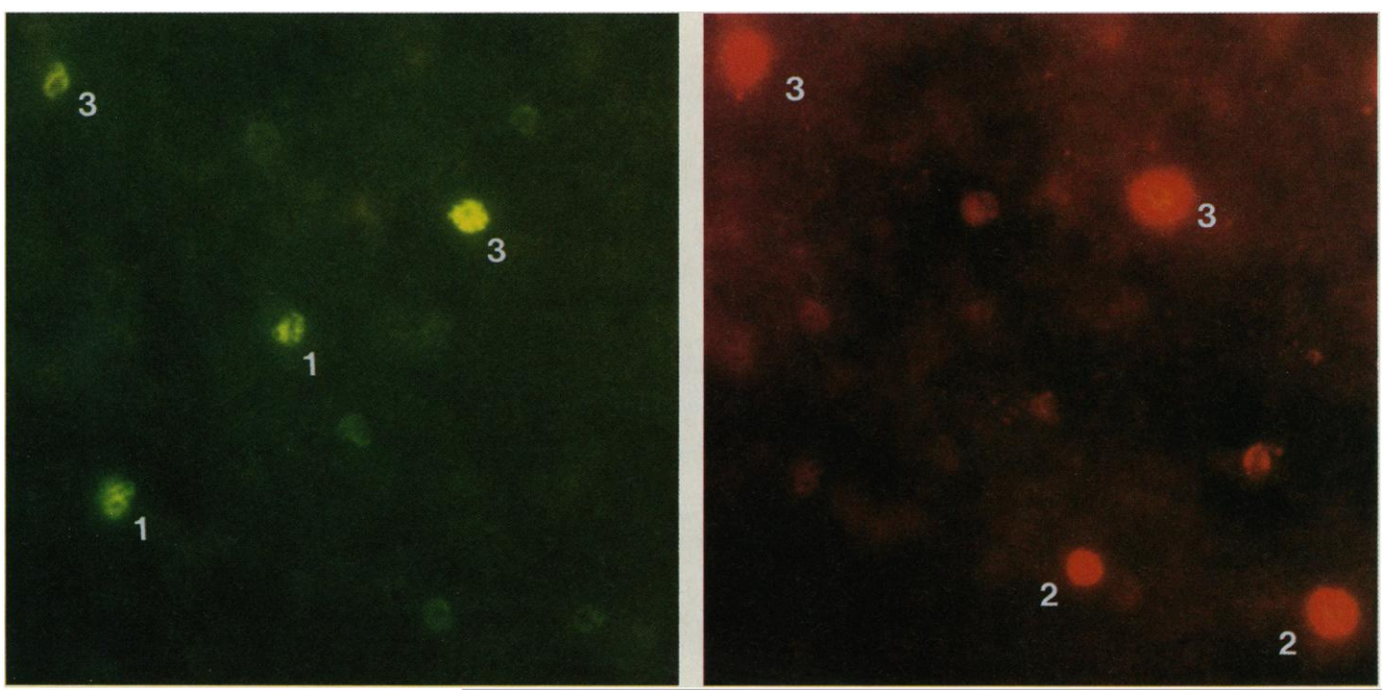

Fig. 3. Ultraviolet (UV) microscopy of uncloned progeny of cross incubated with Mabs 7.3 and 12.3, and stained with fluorescent reagents specific for each Mab. Pictures of the same microscope field show (**left**) fluorescein-stained schizonts (7.3+) and (**right**) rhodamine-stained schizonts (12.3+). Parasites numbered (1) are stained with fluorescein only (7.3+), those numbered (2) with rhodamine only (12.3+), and those numbered (3) with both reagents (7.3+, 12.3+).

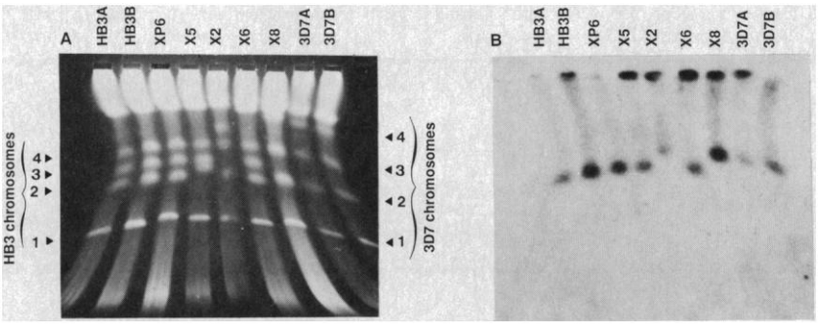

Fig. 4. Chromosomes of parents and five progeny clones visualized by PFG gel electrophoresis. Chromosome numbering is according to that of Kemp *et al.* (*19*). Sizing studies of these chromosomes with bacteriophage concatemers have shown that they range in size from 800 kb for chromosome 1 to 1500 kb for chromosome 4. (**A**) Ethidium bromide–stained gel. (**B**) Autoradiogram after hybridization of CSP gene probe (*26*) to Southern blot of gel. After hybridization, the blot was washed at moderate stringency [0.2× standard saline citrate (SSC) and 0.1% SDS, 50°C, 1 hour]. Note hybridization of probe to chromosome 3.

the same as HB3, and one (X8) had the same characteristics as 3D7. The other clones were recombinant for at least two parent-line markers. Thus X2 and X6 showed recombination between the two antigen markers, 7.3+, 12.3+ and 7.3−, 12.3−, respectively. Clones X5, XP2, XP4, and XP5 showed HB3-type drug-response and antigen characters, and 3D7-type ADA. The patterns of recombination and segregation of these characters were consistent with the view that *P. falciparum*, like the rodent malaria species, is haploid in the blood stages. With the exceptions of clones X1 and X9, only single forms of ADA (types 1 or 2) and of the 195-kD antigen (7.3+, 9.2− or 7.3−, 9.2+) were seen. This result establishes that the variant forms of ADA and of the 195-kD antigen are determined by allelic variation of each respective gene. There is evidence that the gene for the 195-kD antigen is present in the genome as only a single copy (*24*), which reinforces the view that polymorphism of this antigen is due to allelic variation.

The PFG gel technique distinguished clearly four chromosomal DNA bands in each clone, which we consider are equivalent to bands 1 to 4 described by Kemp *et al.* (*19*) (Figs. 4 and 5). In the top region of the gel, the "compression region," individual chromosomes could not be distinguished; although by using different electrophoretic conditions, at least ten additional chromosomes could be resolved (*25*).

Chromosomes 1 and 2 of the parent clones 3D7 and HB3 migrated to similar positions on the gel. Chromosomes 3 and 4, however, were larger in 3D7 than in HB3. The chromosome patterns of each parent were the same in 3D7A and B and in HB3A and B, respectively, indicating that their genomes had not undergone any major changes after mosquito transmission. Progeny clone X8 possessed a chromosome pattern similar to 3D7, and clones X4, X6,

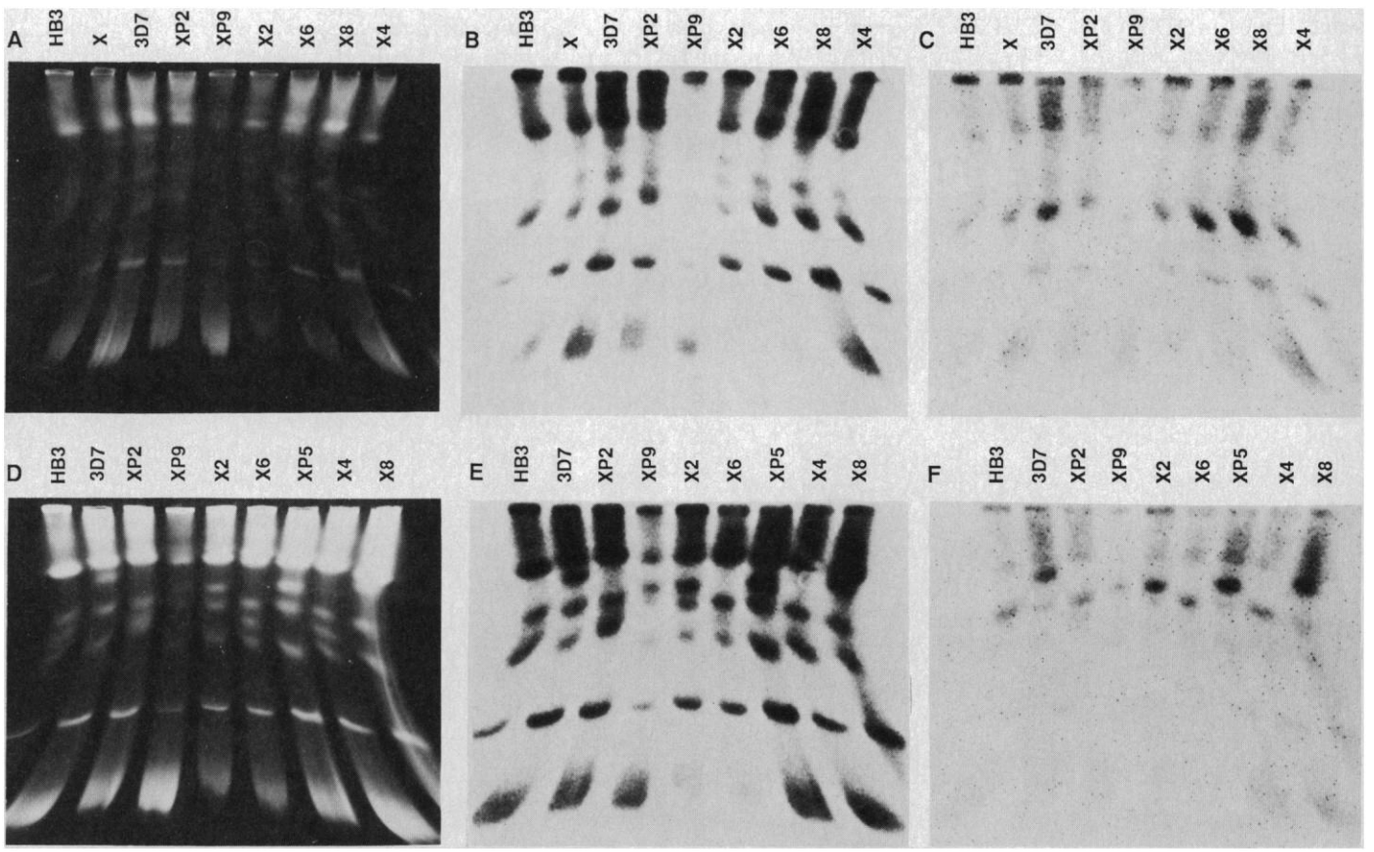

Fig. 5. (**A** and **D**) Chromosomes of parent and progeny clones. Ethidium bromide–stained PFG gels. (**B**, **C**, **E**, and **F**) Autoradiograms after hybridizations of total chromosome probes to Southern blots of gels. After hybridization the blots were washed at moderate stringency (0.2× SSC and 0.1% SDS, 50°C, 1 hour) and autoradiographed (B and E). Blots were then washed at high stringency (0.1× SSC and 0.1% SDS, 65°C, 1 hour) and autoradiographed again (C and F). Autoradiograms (B) and (C) are of blots of gel (A) hybridized with chromosome 2 probe. Autoradiograms (E) and (F) are of blots of gel (D) hybridized with chromosome 4 probe.

Table 4. Characteristics of parent clones and cultures of progeny. For methods of characterization, see (*13–15*). The pyrimethamine response is shown as S (sensitive) or R (resistant).

Clones	Pyrimethamine response	ADA	195-kD antigen		40-kD antigen
			Mab 7.3	Mab 9.2	Mab 12.3
Parents					
3D7A	S	1	−	+	+
3D7B	S	1	−	+	+
HB3A	R	2	+	−	−
HB3B	R	2	+	−	−
Progeny					
X1	R	1, 2	+	−	−
X2	R	1	+	−	+
X4	R	2	+	−	−
X5	R	1	+	−	−
X6	R	2	−	+	−
X8	S	1	−	+	+
X9	R	1, 2	+	−	−
XP1	R	2	+	−	−
XP2	R	1	+	−	−
XP4	R	1	+	−	−
XP5	R	1	+	−	−
XP6	R	2	+	−	−
XP7	R	2	+	−	−
XP9	R	2	+	−	−

XP1, and XP6 were similar to HB3. In the other clones, however, nonparental-type chromosomes were observed. In X5, XP2, and XP4, chromosome 2 appeared larger than in either parent, whereas in XP9 it was slightly smaller. Chromosome 4 in clones X2, XP5, XP7, and XP9 appeared intermediate in size between that of either parent. The chromosome patterns of parent and progeny clones were examined at intervals during 3 months' culture and appeared unchanged during this period.

To investigate the relation between chromosomes of each clone, we used three chromosome-specific probes in hybridizations with Southern blots of PFG gels. Chromosome 3 specificity was identified with a pUC8 clone containing the gene for the circumsporozoite antigen (CSP) of *P. falciparum* (*26*) (Fig. 4). Probes for chromosomes 2 and 4 were prepared by excising the respective bands from a preparative gel of X2 and extracting the DNA (*27*). The total DNA was then nick-translated and hybridized to PFG blots (Fig. 5). Autoradiography after moderately stringent washes showed hybridization of both probes to all chromosomes, presumably because of cross-hybridization of repetitive DNA or A-T rich sequences throughout the genome. Highly stringent washes removed most of the cross-hybridization, and each probe then showed a high degree of specificity for the homologous chromosome. The results of the hybridization experiments show that despite changes in size the chromosomes have retained their identity among the progeny clones. The relative order of chromosomes 1 to 4 is the same in all clones that have been examined.

Chromosome polymorphism in different isolates of *P. falciparum* has been reported previously. Corcoran *et al.* (*20*) showed that a small-sized chromosome 2 in two *P. falciparum* lines was correlated with the absence of the gene for a blood-form antigen from this chromosome. Pologe and Ravetch (*28*) showed that a reduction in the size of chromosome 2 in certain parasite lines was due to deletions involving segments of a knob-associated histidine-rich protein gene. It is not known whether the size variations seen in chromosomes 3 and 4 in 3D7 and HB3 involve coding or noncoding DNA, and the genetic mechanisms involved in generating the novel-sized chromosomes among the cross progeny are not understood. Unequal crossing-over events, reciprocal translocations, or gene conversion during pairing at meiosis could have produced chromosomes of new size.

The proportion of nonparental forms in the progeny of the cross is higher than expected. Whereas 6 of the 12 progeny clones examined had parent-type enzyme, drug sensitivity, and antigen markers, two of these (XP7 and XP9) were characterized by nonparental chromosome patterns. Assuming that gametes undergo random fertilization, it would be expected that half the progeny would result in parent-type zygotes, and half in hybrids. At least 50% of the progeny clones, therefore, would be predicted to be parental types. The reasons for the excessive numbers of recombinants are not understood. It is possible that cross-fertilization between the clones was favored in the mosquito host, even though self-fertilization could undoubtedly occur as shown by the parent line controls. There could also be selection in favor of certain genotypes during development of the progeny in mosquitoes, in the chimpanzee host, or during culture. When uncloned *P. falciparum* isolates containing several genetically distinct parasites are kept in culture for prolonged periods, variations in the proportions of each form are seen (*17*). In the present study, parasites characterized by HB3-type antigens appeared to outgrow other forms between days 11 and 36 of culture (Table 3). The X and XP clones, isolated on day 32, reflect the preponderance of these types of parasites (Table 4). An excess in the number of pyrimethamine-resistant forms was also apparent, even among the X clones that had not been drug-selected. Had cloning been carried out earlier during the culture, a greater proportion of parasites having 3D7-type antigens would probably have been isolated.

This work has shown that two genetically distinct clones of *P. falciparum* can readily undergo mating during mosquito transmission. It is not known whether the recombination between the genetic markers was due to simple reassortment of unlinked genes on separate chromosomes or whether crossing-over between genes on the same chromosome occurred. However, the chromosomal size changes suggest that rearrangements of the genetic material occur frequently in *Plasmodium*. In natural populations of *P. falciparum*, considerable diversity occurs in antigens and drug sensitivity, and patients are frequently infected with mixtures of genetically distinct parasites (*29*). This study has shown that mosquito transmission of such mixtures provides an efficient means of generating parasites with novel genotypes, a finding that has important implications for measures designed to control the parasite by chemotherapy or vaccination.

REFERENCES AND NOTES

1. H. C. Spencer, *Trans. R. Soc. Trop. Med. Hyg.* **79**, 748 (1985).
2. World Health Organization, *Bull. WHO* **62**, 715 (1984).
3. D. Walliker, *Adv. Parasitol.* **22**, 217 (1983).
4. R. E. Sinden and R. H. Hartley, *J. Protozool.* **32**, 742 (1985).
5. V. E. Rosario, *Nature (London)* **261**, 585 (1976).
6. W. Trager and J. B. Jensen, *Science* **193**, 673 (1976); J. D. Haynes, C. L. Diggs, F. A. Hines, R. E. Desjardins, *Nature (London)* **263**, 767 (1976).
7. T. Ifediba and J. P. Vanderberg, *Nature (London)* **294**, 364 (1981); T. Ponnudurai *et al.*, *Trans. R. Soc. Trop. Med. Hyg.* **76**, 242 (1982); I. A. Quakyi, L. Koontz, D. Seeley, R. W. Gwadz, *Abstracts Seventh International Congress of Protozoology,* Nairobi, Kenya, June 1985, p. 67.
8. C. C. Campbell, W. E. Collins, P. Nguyen-Dinh, A. Barber, J. R. Broderson, *Science* **217**, 1048 (1982).
9. V. Rosario, *ibid.* **212**, 1037 (1981).

10. W. Trager *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6527 (1981).
11. Isolate NF54 was derived from a patient living near Schipol Airport, Amsterdam, who had never left the Netherlands [see T. Ponnudurai, A. D. E. M. Leeuwenberg, J. H. E. Th. Meuwissen, *Trop. Geogr. Med.* **33**, 50 (1981)]. Clone 3D7 was derived from NF54 by limiting dilution (*9*). NF54 was cultured in a flask (*20*) to a parasitemia of approximately 0.5%. The culture was then shaken gently for 48 hours at 37°C so that most parasitized erythrocytes (about 90%) contained only single parasites. Dilutions were made until there was an average of 0.5 parasite per 0.1 ml of culture. Portions (0.1 ml) were placed in individual wells of 96-well microtiter plates and maintained in an incubator (5% CO_2, 5% O_2, 90% N_2). Medium was changed every 48 hours, and new erythrocytes, in a volume equal to that in each well, were added every 6 days. Eight out of 20 wells showed parasites after 14 days. The contents of one well, denoted 3D7, were grown in a flask and recloned by the same procedure. Two out of 20 wells contained parasites after 11 days. The contents of one well were taken for use as the 3D7 clone.
12. Clone HB3 was derived from isolate H1 from Honduras by W. Trager by microscopic selection.
13. Pyrimethamine response was determined by the method of S. Thaithong and G. H. Beale [*Trans. R. Soc. Trop. Med. Hyg.* **75**, 271 (1981)]. Parasites were cultured in microtiter plates and exposed to pyrimethamine at doses of $10^{-9}M$ to $10^{-5}M$ for 72 hours with daily changes of medium with drug. Giemsa-stained blood smears were examined to determine the minimum inhibiting concentration (MIC) of drug that killed all, or nearly all, the parasites. The MIC for 3D7 was $10^{-7}M$; for the purpose of this work, parasites with this MIC are classified as pyrimethamine-sensitive. For HB3, the MIC was $10^{-5}M$, and these parasites are classified as resistant.
14. Parasites were examined for electrophoretic forms of ADA by the method of A. Sanderson, D. Walliker, and J.-F. Molez [*Trans. R. Soc. Trop. Med. Hyg.* **75**, 263 (1981)], with the use of agarose gel electrophoresis (Corning) instead of starch gel electrophoresis; the banding patterns of ADA variants are similar in the two systems. Parasites were freed from host erythrocytes by lysis in saponin, concentrated by centrifugation, and disrupted by freezing and thawing prior to electrophoresis (for 25 minutes). The electrophoresis buffer was 0.05*M* sodium barbital, *p*H 8.6.
15. R. Hall *et al.*, *Mol. Biochem. Parasitol.* **7**, 247 (1983); J. S. McBride, C. I. Newbold, R. Anand, *J. Exp. Med.* **161**, 160 (1985); J. S. McBride, P. D. Welsby, D. Walliker, *Trans. R. Soc. Trop. Med. Hyg.* **78**, 32 (1984); J. S. McBride and C. Wilson, personal communication. The 195-kD and 40-kD antigens are associated with late-stage trophozoites and schizonts. Variant forms of these antigens were identified by indirect IFA, as described by J. S. McBride, D. Walliker, and G. Morgan [*Science* **217**, 254 (1982)]. Cultures containing schizonts were washed three times in RPMI 1640 medium without serum, and pipetted onto wells of multispot microscope slides so that each well contained approximately 10^4 schizonts. The preparations were dried, fixed in acetone, and stained with Mabs 7.3, 9.2, or 12.3, and then incubated with fluorescein isothiocyanate–conjugated antiserum to mouse immunoglobulin G (Sigma). Preparations were examined by ultraviolet microscopy. Wells contained either brightly fluorescing organisms that were scored +, or no fluorescing organisms that were scored −.
16. P. Oquendo *et al.*, *Mol. Biochem. Parasitol.* **18**, 189 (1986).
17. B. Fenton, A. Walker, D. Walliker, *ibid.* **16**, 173 (1985); B. Fenton, unpublished observations.
18. D. C. Schwartz and C. R. Cantor, *Cell* **37**, 67 (1984); L. H. T. Van der Ploeg *et al.*, *Science* **229**, 658 (1985).
19. D. J. Kemp *et al.*, *Nature (London)* **315**, 347 (1985).
20. L. M. Corcoran, K. P. Forsyth, A. E. Bianco, G. V. Brown, D. J. Kemp, *Cell* **44**, 87 (1986).
21. Cultures of *P. falciparum* were maintained in flasks by using modifications of the methods of Trager and Jensen and of Haynes *et al.* [see (*6*)]. For routine maintenance, cultures were kept in fresh human erythrocytes, type O, at 5% hematocrit in RPMI 1640 medium containing 10% human serum, with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Medium was changed daily, and fresh erythrocytes were added twice weekly. For gametocytes, cultures were established at a parasitemia of approximately 0.3% at 6% hematocrit. When the parasitemia had attained 5%, about 4 days later, the hematocrit was lowered to 3%. Cultures were then maintained without addition of further erythrocytes, but with daily changes of medium until day 15, when mature gametocytes were present.
22. L. S. Ozaki, R. W. Gwadz, G. N. Godson, *J. Parasitol.* **70**, 831 (1984).
23. PFG gel electrophoresis was carried out according to the system of G. F. Carle and M. V. Olson [*Nucleic Acids Res.* **12**, 5647 (1984)]. Parasites were prepared in agarose blocks that were incubated in a mixture of tris, EDTA, Sarkosyl, and Proteinase K for 48 hours as described by Kemp *et al.* (*19*) before insertion into the gel. The gel was 1.5% agarose (Seakem ME) in 0.5× tris-borate-EDTA (TBE) buffer. Electrophoresis was carried out at 300 V for 18 hours at 14° to 16°C with a pulse time of 75 seconds.
24. K. Tanabe, M. Mackay, M. Goman, J. G. Scaife, *J. Mol. Biol.*, in press.
25. T. E. Wellems and C. L. Smith, unpublished observations.
26. J. B. Dame *et al.*, *Science* **225**, 593 (1984).
27. Chromosomal DNA was extracted from PFG gel by a variation of the procedure of B. Vogelstein and D. Gillespie [*Proc. Natl. Acad. Sci. U.S.A.* **76**, 615 (1979)]. Agarose slices were dissolved in four volumes (by weight) of a mixture of 6*M* $NaClO_4$, 10 m*M* tris acetate, 1 m*M* EDTA, *p*H 8.0 (PTE), at 37°C. The DNA in solution was sheared twice by passing it through a 30-gauge needle. A 25% suspension (25 μl) of borosilicate glass powder was mixed with the solution for 2 hours on a rocking platform. The glass powder with bound DNA was recovered by centrifugation. After washes with PTE, isopropyl alcohol, and 100% ethanol, the powder was dried briefly in the open tube. DNA was eluted from the glass into two 50-μl washes of 10 m*M* tris acetate and 1 m*M* EDTA, *p*H 8.0, which were combined and passed through a Sephadex G-50 column.
28. L. G. Pologe and J. V. Ravetch, *Nature (London)* **322**, 474 (1986).
29. S. Thaithong *et al.*, *Trans. R. Soc. Trop. Med. Hyg.* **78**, 242 (1984).
30. We thank F. E. Neva, L. H. Miller, and W. E. Collins for advice on the design of this project; T. Ponnudurai for providing isolate NF54; P. Nguyen-Dinh for making available clone HB3; J. S. McBride for monoclonal antibodies; N. Papadopoulos and R. Costello for assistance with enzyme electrophoresis; and J. Dvorak for photographic assistance. Supported by the Medical Research Council of Great Britain (D.W.), and the United Nations Development Programme/World Bank, WHO Special Programme for Research and Training in Tropical Diseases (I.A.Q.).

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A Promoter with an Internal Regulatory Domain Is Part of the Origin of Replication in BPV-1

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Extrachromosomal elements that are stably maintained at a constant copy number through cell doublings are a good model system for the study of the regulation of DNA replication in higher eukaryotes. Previous studies have defined both cis and trans functions required for the regulated plasmid replication of the bovine papilloma virus in stably transformed cells. Here, a sequence known to be a cis-dominant element of the replication origin of the plasmid is shown to contain a promoter for transcription. Both in vitro and in vivo assays have been used to define this promoter and show that a sequence located just 3′ to the transcriptional start site is required for activity. This DNA sequence element, which has been defined through deletions, coincides with a binding site for a cellular factor and is also required for a functional origin of replication. Possible models for how a transcription factor may play a role in the regulation of DNA replication are discussed.

THE MECHANISMS BY WHICH THE eukaryotic DNA replication process is regulated are unknown (*1*). In particular, the structural features of a functional origin of DNA replication in higher eukaryotes and the nature of the factors that govern its activity have not been explored. However, the process of DNA replication is probably regulated at several different levels since it is a central part of the events in the cell cycle that commit the cell to divide. At present, virtually everything that is known about the process and the requirements for initiation of replication stems from studies of viral systems (*2*). Chromosomes from latent viruses that replicate as nuclear plasmids provide a model system in that their DNA synthesis must be regulated in order to maintain a constant copy number per nucleus. We are studying the regulation of bovine papilloma virus (BPV) DNA replication since this replicon appears to be intricately regulated to ensure orderly replication (*3, 4*). The viral components required for replication of BPV have been identified genetically (*5*). Two viral genes encoded in part by a single open reading frame (ORF) are required in the replication events (*6*). The R gene, which is encoded from the 3′ part of the E1 ORF acts in a positive way on replication, while the M gene, which is encoded by the 5′ part of the E1 ORF, serves as a negative regulator of DNA replication (*4, 6, 7*). It has been suggested that the activity of the M gene is what ensures the regulated and stable copy number of the DNA in transformed cells (*4, 7*).

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