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Antifolate Drug Selection Results in Duplication and Rearrangement of Chromosome 7 in *Plasmodium chabaudi*

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We selected lines of *Plasmodium chabaudi* that are resistant to high levels of the antifolate drug pyrimethamine and have shown that rearrangement and duplication of a portion of chromosome 7 has occurred in the resistant lines. This chromosomal duplication results in an increase in the chromosome number from 14 to 15; two derived chromosomes (450 kilobases and 1.1 megabases) were smaller than the original chromosome 7 (1.3 megabases), so that essentially only a 200-kilobase region was duplicated. This region contained the *DHFR-TS* gene and the closely linked *Hsp70* gene. We have macrorestriction mapped chromosome 7 from the pyrimethamine-susceptible line (DS) and also the duplicated chromosome 7s in the resistant line. From these maps, we have proposed a process for the karyotype changes. Sequencing of the *DHFR* gene from the parent and derived chromosomes showed that there were no mutations in the coding sequence. As a result of the duplication of the *DHFR-TS* gene, there is at least a twofold increase in expression of the *DHFR-TS* gene, and this may explain the ability of the pyrimethamine-resistant lines to grow in increased amounts of the drug.

The development of drug resistance in malaria parasites has caused a crisis in the selection of suitable drugs for treatment and prevention of this disease. The antifolate drug pyrimethamine is an effective antimalarial agent. However, resistance develops rapidly, thus reducing its usefulness. Consequently, pyrimethamine and other antifolate drugs are usually used in combination with potentiating drugs, such as sulfonamide, to guard against this problem. Pyrimethamine binds to the enzyme dihydrofolate reductase (DHFR) (11), which exists as a bifunctional enzyme with thymidylate synthase (TS) in *Plasmodium chabaudi*, *P. falciparum*, and other protozoans (12, 13; see below). DHFR and TS catalyze sequential reactions in the synthesis of dTTP, and consequently, pyrimethamine inhibits the growth of the parasites by disrupting replication of the DNA and reducing the supply of essential folate cofactors.

The rapid emergence of pyrimethamine-resistant (Pyr^r) *P. falciparum* strains in the field suggested that a single mutation may be responsible, and genetic crosses with Pyr^r and Pyr^s lines of *P. chabaudi* (32, 34) have shown a pattern of inheritance typical of a single gene. Further work on other Pyr^r clones of *P. chabaudi* (29) has shown a decrease in inhibitor binding to DHFR, indicating that changes to the DHFR enzyme may account for the observed resistance.

Analysis of the DHFR enzyme and gene in *P. falciparum* lines has indicated that the predominant mechanism of resistance to pyrimethamine consists of mutations within the *DHFR* gene that alter the binding affinity of the drug to the enzyme (5, 10, 19, 21). The DHFR activity of the Pyr^s *P. falciparum* clone 3D7 has been compared with that of the Pyr^r clones HB3 and 7G8, and a decreased affinity of pyrimethamine to a structurally altered enzyme in the resistant clones can explain the level of resistance observed (19). Comparison of the *DHFR* sequences of 3D7 with those of 7G8 and HB3 has implicated a single amino acid change that confers resistance (10, 21). The progeny of a genetic cross between 3D7 and HB3 (33) has been analyzed, and a restriction fragment length polymorphism has been identified that segregates with Pyr^r (21). This polymorphism is tightly linked to the *DHFR-TS* gene. The same amino acid that correlates with Pyr^r in HB3 has been shown to be present in the *DHFR* genes of all Pyr^r lines derived from the field that have been examined so far (10, 21). The only example of overexpression of the DHFR enzyme has been the 5- to 10-fold increase in expression of the enzyme in a Pyr^r clone of *P. falciparum* selected by in vitro drug pressure (16). However, there were clearly structural changes in the DHFR enzyme that also altered its activity, and also there was an apparent increase in the *DHFR* gene copy number. This cloned line was obtained by mutagenesis and selection after sequential increases in drug concentration, and it seems likely that a number of events have combined to produce the observed drug resistance phenotype.

In the work described in this report, we have derived lines of the mouse malaria parasite *P. chabaudi* that grow in increased amounts of pyrimethamine, to find whether changes in the *DHFR* gene are involved and to use these lines as a model system with which to study drug resistance in malaria parasites. We show that pyrimethamine drug pressure has selected for duplication of the *DHFR-TS* gene. This was achieved not by tandem duplication but, surprisingly, by duplication and rearrangement of part of chromosome 7. The *DHFR* gene from the Pyr^s parent and both genes from the Pyr^r derived lines were identical in sequence. Thus, the duplication itself (and not a mutation), which resulted in increased expression of the DHFR-TS transcript and presumably the enzyme, has allowed the parasite to grow in an increased amount of pyrimethamine.

MATERIALS AND METHODS

Parasites, DNA, and RNA. The DS cloned line of *P. chabaudi* subsp. *adami* was obtained from David Walliker, Department of Genetics, University of Edinburgh, Edinburgh, Scotland.

The pyrimethamine-resistant line Pr4 was derived from *P. chabaudi* DS by four sequential passages in the BALB/c strain of mice. Parasites (10^7 per mouse) were injected intravenously, and the mice were treated intraperitoneally

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with 0.05 mg of pyrimethamine per kg daily for the next 4 days. Parasitemias were monitored, blood was harvested between 40 and 80% parasitemia, and the line Pr1 was produced from these parasites. Pr2, Pr3, and Pr4 were produced in the same way, except that the amount of pyrimethamine administered was doubled at each step. Pr4 was cloned by diluting the parasites and injecting one parasite each into 16 BALB/c mice; of these, 7 developed parasitemias. One of these lines was recloned by limiting dilution such that the probability of a parasite in the inoculum was 0.3; it was injected into 16 mice. Two of the mice developed parasitemias (none of eight mice injected with half of the above dose developed a parasitemia). Pr4-c was developed from this second cloning. DNA and RNA were produced as previously described (8).

Cloning and sequencing. The *P. chabaudi DHFR-TS* gene was isolated from *Rsa*I-cut *DS* genomic DNA by ligating *Eco*RI linkers on the DNA fragments and cloning in λgt10. The gene was isolated by probing the library generated with a portion of the *P. falciparum DHFR-TS* gene. The 2.2-kilobase (kb) fragment isolated contained most of the *DHFR-TS* gene except for the last 45 base pairs of the *TS* gene. The 3′ end of the gene was isolated by obtaining two oligonucleotides facing in different directions and amplifying the *Sau*3A-cut and ligated DNA by the technique of inverted polymerase chain reaction (31). The *DHFR-TS* gene was sequenced on both strands by the dideoxy-chain termination method (25) after fragments had been subcloned into M13mp18 and M13mp19. When suitable restriction fragments were not available, oligonucleotide primers were used to complete the sequence.

Polymerase chain reaction. Oligonucleotide primers from the 5′ (GCTAGTAACAATTGTGTAGTGCTTATATATA TACAC) and 3′ (CCTGTTCTATCATCTTGTTTATTTCCA TGC) ends of the *DHFR* gene were used to amplify the intervening region by using the polymerase chain reaction as described previously (24). The amplified fragment was cloned into M13mp18 and sequenced. At least four independent clones were sequenced for DS, Pr1, Pr3, Pr4, and Pr4-c from two different polymerase chain reactions.

PFG electrophoresis and chromosome mapping. Pulsed-field gradient gel (PFG) electrophoresis (26) was performed in a contour-clamped homogeneous electric field apparatus (6). Running conditions were as described previously (9). Chromosome mapping was performed as described previously (9). Sizes were determined by using chromosomes from *Saccharomyces cerevisiae* YNN295 (6).Genomic DNAs from the different *P. chabaudi* lines were digested with *Eco*RI, fractionated on 0.7% agarose gels, and transferred to Hybond-N membranes. Total RNA was fractionated on 1.5% agarose gels in E buffer (36 mM Na_2HPO_4, 4 mM NaH_2PO_4)–6% formaldehyde and transferred to Hybond-N membranes. All filters were hybridized in 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate [pH 7])–1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin)–0.1% sodium dodecyl sulfate–500 mg of salmon sperm DNA at 65°C.

RESULTS

Isolation and sequence analysis of the *DHFR-TS* gene of *P. chabaudi*. To understand the molecular basis of pyrimethamine resistance in *P. chabaudi*, we isolated the *DHFR-TS* gene by homology with the *P. falciparum* gene that has previously been analyzed (3, 10, 21). The *TS* gene is more conserved than the *DHFR* gene, and so a fragment containing all of the TS gene of *P. falciparum* was used to isolate a 2.2-kb *Rsa*I fragment from *P. chabaudi* genomic DNA. Sequencing of this DNA fragment revealed a high degree of homology with the *P. falciparum DHFR-TS* gene, and comparisons indicated that the *P. chabaudi Rsa*I fragment contained 296 base pairs 5′ to the first methionine codon and encoded almost the entire protein except for the last 15 amino acids. To isolate the rest of the *P. chabaudi DHFR-TS* gene, we designed two oligonucleotide primers from the 3′ end of the TS portion and amplified the remaining portion of the gene by the inverted polymerase chain reaction (31).

The complete sequence of the *P. chabaudi DHFR-TS* gene with the predicted amino acid sequence is shown in Fig. 1. The initiator methionine is located at position 1, and there is a continuous open reading frame to a stop codon at position 1652 containing no apparent introns. The *P. falciparum DHFR-TS* gene also does not contain any introns. Comparison of the *P. chabaudi* and *P. falciparum DHFR-TS* gene sequences indicates 78% homology at the DNA level and 72% homology at the protein level.

Comparison of the *P. chabaudi* and *P. falciparum* DHFR-TS protein sequences shows that, as expected, the TS portion (90%) of the bifunctional enzyme is much more conserved than either DHFR (63%) or the join region (38%) of the molecules (Fig. 2). TS is highly conserved throughout evolution (30); however, DHFR shows much less conservation at the amino acid level, although the amino acids involved in the active site of the enzyme are highly conserved.

Comparison of the *P. chabaudi* DHFR-TS protein with the *P. falciparum* enzyme (Fig. 2) and other species suggests that the DHFR domain consists of 222 amino acids at the amino terminus and the TS region is 287 amino acids at the carboxy terminus. The two enzymatic domains are separated by a join region of 74 amino acids that is much shorter in *P. chabaudi* than the corresponding region in the *P. falciparum* enzyme (100 amino acids). This region is also not homologous to the equivalent region of the *Leishmania tropica* DHFR-TS bifunctional enzyme (1), either in size or in sequence, and suggests that the only function of this region is to bring the catalytic sites of both enzymes into close proximity.

Selection of pyrimethamine-resistant *P. chabaudi*. To understand the possible role of changes within the *DHFR-TS* gene in the development of resistance to antifolate drugs, we subjected the DS cloned line of *P. chabaudi* to increasing doses of pyrimethamine. Each passage involved a twofold increase in the level of drug used, from 0.05 to 0.2 mg/kg, and the parasite lines Pr1, Pr2, Pr3, and Pr4 were obtained from each of these steps. The *P. chabaudi* line Pr4 was cloned in mice twice (see Materials and Methods), so that we could be confident of the clonality of this parasite line, which we have called Pr4-c.

The *DHFR-TS* gene is duplicated by selection with pyrimethamine. To test whether selection with pyrimethamine induced changes in the *DHFR-TS* gene copy number, we used Southern blot analysis to probe genomic DNAs of the different *P. chabaudi* lines (Fig. 3). The 2.2-kb *Rsa*I fragment, containing almost the entire *DHFR-TS* gene, was hybridized to *Eco*RI digests of genomic DNAs from the parent cloned line DS and also the pyrimethamine-selected lines Pr1 and Pr4 and the cloned line Pr4-c. The *DHFR-TS* probe hybridized to an 8.2-kb *Eco*RI fragment in all cases. However, densitometry readings of the band indicated that

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 M  E  D  I  S  E  I  F  D  I  Y  A  I  C  A  C  C  K  V  L  N  S  N  E  K  A  G  C  F  S  N  K  T  F  K  G  L  G  N  E
ATGGAAGATATCTCTGAAATATTCGATATATATGCAATTTGTGCATGTTGTAAAGTTCTGAACAGTAATGAAAAGGCTGGCTGTTTTAGTAATAAGACTTTTAAGGGACTTGGGAATGAA 120

 G  G  L  P  W  K  C  N  S  V  D  M  K  H  F  S  S  V  T  S  Y  V  N  E  T  N  Y  M  R  L  K  W  K  R  D  R  Y  M  E  K
GGGGGGTTACCTTGGAAATGTAATTCAGTAGATATGAAACATTTTAGCTCTGTAACATCCTATGTTAATGAAACTAACTATATGAGATTAAAATGGAAAAGGGATAGATATATGGAAAAA 240

 N  N  V  K  L  N  T  D  G  I  P  S  V  D  K  L  Q  N  I  V  V  M  G  K  A  S  W  E  S  I  P  S  K  F  K  P  L  Q  N  R
AATAATGTAAAATTAAATACTGATGGAATACCCTCCGTTGATAAGTTACAAAATATTGTAGTAATGGGAAAAGCAAGTTGGGAAAGCATCCCCTCAAAATTTAAGCCATTACAAAATCGA 360

 I  N  I  I  L  S  R  T  L  K  K  E  D  L  A  K  E  Y  N  N  V  I  I  I  N  S  V  D  D  L  F  P  I  L  K  C  I  K  Y  Y
ATAAATATTATATTGTCTAGAACATTGAAAAAAGAAGATCTTGCAAAAGAATATAATAATGTTATTATAATTAATAGTGTGGATGATTTATTTCCTATTTTAAAATGCATAAAATATTAT 480

 K  C  F  I  I  G  G  A  S  V  Y  K  E  F  L  D  R  N  L  I  K  K  I  Y  F  T  R  I  N  N  A  Y  T  C  D  V  L  F  P  D
AAATGTTTTATTATAGGAGGTGCATCTGTTTATAAAGAGTTTTTAGATCGTAATTTAATAAAAAAAATATATTTTACAAGAATAAATAATGCTTATACTTGTGATGTTTTATTTCCAGAT 500

 I  N  E  D  L  F  K  I  T  S  I  S  D  V  Y  S  S  N  N  T  T  L  D  F  V  I  Y  S  K  T  K  E  I  H  E  E  I  N  P  N
ATCAATGAAGATTTATTTAAAATAACATCAATTAGTGATGTATATAGTAGTAATAACACGACTTTAGATTTTGTAATTTATAGTAAGACAAAAGAAATACATGAAGAAATTAATCCCAAC 620

 D  E  L  F  N  N  T  F  L  G  V  C  D  E  K  N  T  N  F  D  D  E  D  D  Y  T  Y  F  S  F  N  K  H  K  D  N  I  K  K  N
GATGAACTATTTAATAACACATTTTTAGGTGTGTGTGATGAAAAAAATACAAATTTTGATGATGAAGATGATTATACATATTTCAGTTTTAATAAACATAAAGATAATATTAAAAAAAAT 740

 S  E  H  A  H  H  F  K  I  Y  N  S  I  K  Y  K  H  H  P  E  Y  Q  Y  L  N  I  I  Y  D  I  I  M  H  G  N  K  Q  D  D  R
TCGGAGCATGCTCATCATTTTAAAATATATAATAGTATAAAATATAAACATCATCCTGAATATCAATATTTAAATATTATATATGATATAATAATGCATGGAAATAAACAAGATGATAGA 860

 T  G  V  G  V  L  S  K  F  G  Y  M  M  K  F  N  L  S  E  Y  F  P  L  L  T  T  K  K  L  F  V  R  G  I  I  E  E  L  L  W
ACAGGTGTTGGTGTATTAAGTAAATTTGGATATATGATGAAATTTAATTTAAGTGAATATTTTCCATTATTAACAACAAAAAAATTATTTGTAAGAGGTATCATTGAAGAGTTGTTATGG 980

 F  I  R  G  E  T  N  G  N  T  L  L  E  K  N  V  R  I  W  E  A  N  G  T  R  E  F  L  D  N  R  K  L  F  H  R  E  V  N  D
TTTATAAGGGGAGAAACAAATGGAAATACATTATTAGAAAAAAATGTAAGAATATGGGAAGCTAATGGGACAAGAGAGTTTTTAGATAATAGGAAATTATTTCATAGAGAAGTTAATGAC 1100

 L  G  P  I  Y  G  F  Q  W  R  H  F  G  A  E  Y  T  D  M  H  A  D  Y  K  D  K  G  V  D  Q  L  K  N  I  I  N  L  I  K  N
TTAGGTCCAATTTACGGATTTCAATGGAGGCACTTTGGTGCTGAATATACAGATATGCATGCTGATTATAAAGATAAAGGTGTTGATCAACTAAAAAATATTATAAATTTAATTAAAAAC 1220

 D  P  T  C  R  R  I  I  L  C  A  W  N  V  K  D  L  D  Q  M  A  L  P  P  C  H  I  L  C  Q  F  Y  V  F  D  G  K  L  S  C
GATCCGACATGTAGACGAATTATTTTATGTGCATGGAATGTAAAAGATTTAGATCAAATGGCATTACCTCCTTGTCATATTTTATGTCAATTTTATGTTTTTGATGGAAAATTATCATGT 1340

 I  M  Y  Q  R  S  C  D  L  G  L  G  V  P  F  N  I  A  S  Y  S  I  F  T  Y  M  I  A  Q  V  C  N  L  Q  P  A  E  F  I  H
ATTATGTATCAAAGATCTTGTGATTTAGGTCTTGGTGTTCCATTCAATATTGCTTCCTATTCTATATTTACATATATGATAGCACAAGTATGTAATTTACAACCAGCTGAATTTATACAT 1460

 V  L  G  N  A  H  V  Y  N  N  H  V  E  S  L  K  V  Q  L  N  R  T  P  Y  P  F  P  T  L  K  L  N  P  E  I  K  N  I  E  D
GTATTGGGAAATGCTCATGTTTATAATAATCATGTTGAAAGCTTAAAAGTTCAGTTAAATAGAACACCCTATCCTTTCCCTACTCTTAAATTAAATCCGGAAATTAAAAATATCGAGGAT 1580

 F  T  I  S  D  F  T  V  Q  N  Y  V  H  H  D  K  I  S  M  D  M  A  A  *
TTTACAATTTCAGATTTTACTGTACAAAATTATGTCCATCACGATAAAATCAGTATGGATATGGCAGCTTAA                                                1652
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the *P. chabaudi* DS *DHFR-TS* gene. The nucleotides are numbered positively from the ATG of the start codon toward the 3′ end.

Pr4 and Pr4-c contained twice as many copies of the *DHFR-TS* gene than did DS or Pr1. The same filter was hybridized to a known single-copy gene (Ag352 [V. Marshall, unpublished data]), and this probe hybridized equally to all of the tracks. Therefore, not only the full *DHFR-TS* gene but also the regions around the gene have been duplicated in Pr4 and Pr4-c as the restriction sites are conserved around the duplicated copy (data not shown). Hybridization of the *DHFR-TS* probe to genomic DNA from the Pr3 line showed no increase in the copy number of the gene (data not shown); therefore, the duplication event(s) occurred during the pyrimethamine selection between the Pr3 and Pr4 passages.

We hybridized the *Hsp70* gene (27, 28) to the same filter initially to show that equal amounts of DNA were loaded in all of the tracks but surprisingly, we found that this gene was also duplicated in Pr4 and Pr4-c (Fig. 3). Hence, we concluded that duplication of the *DHRF-TS* gene also involved the *Hsp70* gene and that the two genes are likely to be linked (see below). Also, as for the *DHFR-TS* gene, the restriction sites surrounding the duplicated *Hsp70* copy are retained and the duplication event includes sequences outside the 3.1-kb *Eco*RI fragment.

Duplication of the *DHFR-TS* and *Hsp70* genes involves changes in karyotype. To understand the duplication event(s) at the chromosomal level, we separated chromosomes by PFG electrophoresis and hybridized the *DHFR-TS* and *Hsp70* probes separately to the same filter (Fig. 4). The *DHFR-TS* and *Hsp70* genes are both present on chromosome 7 in the parent DS cloned line (28). The chromosomal patterns of DS and Pr1 are identical; however, Pr4 and Pr4-c both have two hybridizing bands that are smaller than the original parent chromosome 7. As the chromosomal pattern in Pr4 is identical to its cloned derivative Pr4-c, both bands that hybridize to DHFR-TS and Hsp70 are present in all parasites and do not represent two events maintained as a mixed population. Therefore, duplication of the *DHFR-TS* and *Hsp70* genes involves rearrangement of the parent chromosome to create the two smaller chromosomes in Pr4 and Pr4-c. We have designated these two chromosomes Pr4/ch7 and Pr4/ch7s, the latter being the smaller.

Amplification of the *DHFR-TS* gene in *L. tropica* on extrachromosomal circular molecules has been described (14), and to determine whether such a mechanism occurred in Pr4 and Pr4-c, we constructed physical maps of the parent chromosome 7 from DS (DS/ch7) and the two hybridizing bands in Pr4. Figure 5 shows the macrorestriction maps that were generated by using four restriction enzymes (*Bss*HII, *Bgl*I, *Sma*I, and *Eag*I) which cut *P. chabaudi* infrequently and the markers *DHFR-TS*, *Hsp-70*, and the telomeric repeat (23). Chromosome 7 from DS is approximately 1.3 Mb in length, and the DHFR-TS gene is located on a 180-kb fragment in the left end of the chromosome. The *Hsp70* gene is present on a 50-kb *Bgl*I fragment, also toward the left end of the chromosome but to the right of the *DHFR-TS* gene.

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                10         20         30         40         50         60         70         80         90        100
P.c .MEDISEIFD IYAICACCKV LNSNE..KAG CFSNKTFKGL GNEGGLPWKC NSVDMKHFSS VTSYVNETNY MRLKWKRDRY MEKNNVKLNT DGIPSVDKLQ
     **  *  ** **********    **  *    * * ** ** ** * *****  * *** *   ** **** *   ** **  *   *  *  *     *  ***
P.f MMEQVSHVFD IYAICACCKV ESKNEGKKNE VFNNYTFRGL GNKGVLPWKC ISLDMKYFCA VTTYVNESKY EKLKYKRCKY LNKETVD.NV NDMPNSKKLQ

               110        120        130        140        150        160        170        180        190        200
P.c NIVVMGKASW ESIPSKFKPL QNRINIILSR TLKKEDLAKE .YNNVIIINS VDDLFPIL.K CIKYYKCFII GGASVYKEFL DRNLIKKIYF TRINNAYTCD
    * ****  ** **** *****  **** **** ******      *    ***  * **   * *    ******* **  ** ***    ******* ****  * **
P.f NVVVMGRTSW ESIPKKFKPL SNRINVILSR TLKKEDFDED VY....IINK VEDLIVLLGK .LNYYKCFII GGSVVYQEFL EKKLIKKIYF TRINSTYECD

                                DHFR ⟷ JOIN
               210        220        230        240        250        260        270        280        290        300
P.c VLFPDINEDL FKITSISDVY SSNNTTLDFV IYSKT..KEI HEE..IN... .....P..ND ELFNNTFLGV CDEKN.TNF. .......... ......DDED
    * ** ***    * * ****  ********  ** **  *   *  *          *  **      *      * *  * *                        **
P.f VFFPEINENE YQIISVSDVY TSNNTTLDFI IYKKTNNKML NEQNCIKGEE KNNDMPLKND D..KDT.... CHMKKLTEFY KNVDKYKINY ENDDDDEEED

                                JOIN ⟷ TS
               310        320        330        340        350        360        370        380        390        400
P.c DYTYFSFNKH KDNIKKNSEH AHHFKIYNSI KYKHHPEYQY LNIIYDIIMH GNKQDDRTGV GVLSKFGYMM KFNLSEYFPL LTTKKLFVRG IIEELLWFIR
    *  ** ***  *    *** *    * ****  *** ****** ******* *  **** ***** ******** * ** ** **** ******* ** **********
P.f DFVYFNFNKE KEEKNKNSIH PNDFQIYNSL KYKYHPEYQY LNIIYDIMMN GNKQSDRTGV GVLSKFGYIM KFDLSQYFPL LTTKKLFLRG IIEELLWFIR

               410        420        430        440        450        460        470        480        490        500
P.c GETNGNTLLE KNVRIWEANG TREFLDNRKL FHREVNDLGP IYGFQWRHFG AEYTDMHADY KDKGVDQLKN IINLIKNDPT CRRIILCAWN VKDLDQMALP
    *********  ********** ********** ********** ********** **** *  *   ******** ********** *** ***** **********
P.f GETNGNTLLN KNVRIWEANG TREFLDNRKL FHREVNDLGP IYGFQWRHFG AEYTNMYDNY ENKGVDQLKN IINLIKNDPT SRRILLCAWN VKDLDQMALP

               510        520        530        540        550        560        570        580        590        600
P.c PCHILCQFYV FDGKLSCIMY QRSCDLGLGV PFNIASYSIF TYMIAQVCNL QPAEFIHVLG NAHVYNNHVE SLKVQLNRTP YPFPTLKLNP EIKNIEDFTI
    ****** *** ********** ********** ********** * ******** *** ****** ********  *** **** * **********  *********
P.f PCHILCLFYV FDGKLSCIMY QRSCDLGLGV PFNIASYSIF THMIAQVCNL QPAQFIHVLG NAHVYNNHID SLKIQLNRIP YPFPTLKLNP DIKNIEDFTI

               610        620
P.c SDFTVQNYVH HDKISMDMAA
    ****  **** * ********
P.f SDFTIRNYVH HEKISMDMAA
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FIG. 2. Comparison of the *P. chabaudi* and *P. falciparum* DHFR-TS protein sequences. P.c is the *P. chabaudi* sequence, and P.f is the *P. falciparum* sequence. The proteins are numbered from the initiator methionine. Dots representing spaces have been inserted to optimize homology.

The small chromosome in Pr4 (Pr4/ch7s) could be easily purified from the other chromosomes and restriction mapped separately by using the markers *DHFR-TS*, *Hsp70*, and the telomere. The telomere always hybridized to two bands when this chromosome was cut with any of the four restriction enzymes, as would be expected for a linear chromosome. Detailed restriction mapping revealed that Pr4/ch7s behaved as a linear chromosome of approximately 450 kb (Fig. 5). The *DHFR-TS* gene was located on the same 180-kb *Bgl*I-*Sma*I fragment as in DS chromosome 7. The *Hsp70* gene was also present on a *Bgl*I fragment of the same size as that found in DS chromosome 7, but it is located at the right end of the chromosome, just beside the telomere. The restriction map of this small chromosome is identical to the left 450 kb of the parent chromosome.

A restriction map of the larger hybridizing band showed that it behaved as a linear chromosome of approximately 1.1 Mb. Mapping of the telomeric ends of the chromosome was difficult because it was not possible to separate it from chromosomes 5 and 6. To circumvent this problem, we cut out agarose plugs from PFG electrophoresis-separated chromosomes of Pr4 that contained chromosomes 5, 6, and Pr4/ch7. The corresponding plugs from DS chromosomes contained only chromosomes 5 and 6. The telomeric fragments of chromosome Pr4/ch7 were then identified by comparison with identical digests of the DS chromosomes 5 and 6. The *DHFR-TS* gene is located on a 50-kb telomeric fragment at the left end of the chromosome, but the rest of the chromosome is identical in its restriction map to the parent chromosome 7. Therefore, the chromosome Pr4/ch7 differs from DS/ch7 only at the left end, which lacks approx-

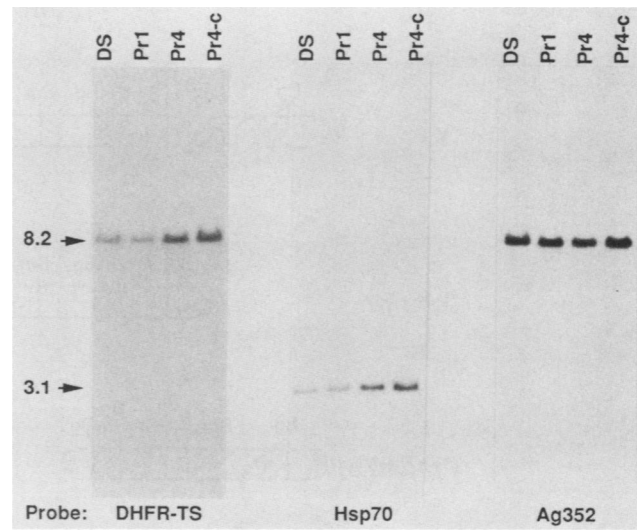

FIG. 3. Determination of the copy number of the *DHFR-TS* and *Hsp70* genes in the *P. chabaudi* lines. Chromosomal DNAs from the isolates DS, Pr1, Pr4, and Pr4-c were digested with *Eco*RI, fractionated on 0.7% agarose gels, blotted onto a Hybond-N filter, and hybridized with the indicated probes (DHFR-TS, Hsp70, and Ag352). Each probe was sequentially hybridized to the same filter after washing the filter with NaOH.

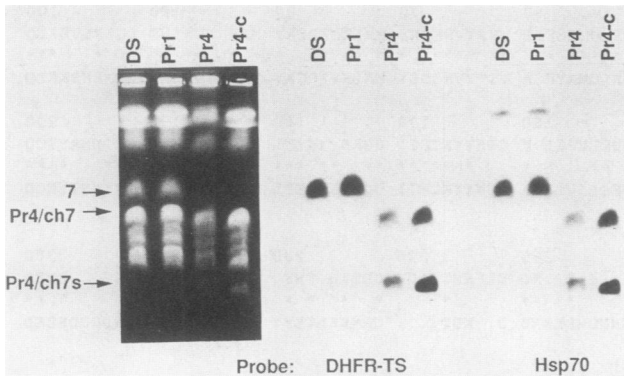

FIG. 4. PFG electrophoresis of chromosomes from DS, Pr1, Pr4, and Pr4-c. Intact chromosomes were separated by PFG electrophoresis, blotted onto Hybond-N, and hybridized with the indicated probes (DHFR-TS and Hsp70). The probes were hybridized sequentially to the same filter after removal of the remaining signal with NaOH. Chromosomes in DS have been numbered sequentially from the smallest to the largest.

imately 200 kb of the parent chromosome. Consequently, the *DHFR-TS* and *Hsp70* genes are duplicated in the *P. chabaudi* pyrimethamine-resistant lines Pr4 and Pr4-c because the two chromosomes (Pr4/ch7 and Pr4/ch7s) share the same region encoding both closely linked genes.

The sequence of the *DHFR* gene is the same in DS, Pr1, Pr4, and Pr4-c. The mechanism of Pyr^r in *P. falciparum* field isolates is clearly a result of mutations in the *DHFR* gene (10, 21) that alter the binding affinity of the drug to the enzyme (5). It is possible that in the *P. chabaudi* Pyr^r lines we have generated, changes in the sequence of the *DHFR* gene were selected. To test this possibility, we sequenced the *DHFR* gene from DS, Pr1, Pr3, Pr4, and Pr4-c by amplifying the gene from genomic DNA via the polymerase chain reaction (24). The duplicated *DHFR* genes located on the different forms of chromosomes 7 from Pr4 and Pr4-c were sequenced by amplifying the gene from the individual chromosomes that had been purified from PFG electrophoresis gels. All of the *DHFR* genes sequenced were identical to that of DS (Fig. 1). Therefore, no changes to the *DHFR* gene have been selected by growth in the presence of pyrimethamine.

The pyrimethamine-resistant lines overexpress the *DHFR-TS* and *Hsp70* transcript. Duplication of the *DHFR-TS* gene would presumably result in an increase in expression of the gene, and this could explain the ability of the Pr4 *P. chabaudi* line to grow in increased amounts of pyrimethamine. We isolated total RNA from DS and Pr4 trophozoites, fractionated equal amounts on agarose gels, transferred them to nylon filters, and hybridized them with the *DHFR-TS* gene probe (Fig. 6). Hybridization of the *Rsa*I fragment containing almost the entire *DHFR-TS* gene revealed that the gene is expressed at least at a twofold higher level in Pr4 than in DS, presumably as a result of the duplication of the portion of the chromosome containing the gene. Therefore, duplication of the chromosome 7 portion containing the *DHFR-TS* gene has resulted in a twofold overexpression of the gene.

DISCUSSION

Mechanism of resistance to pyrimethamine. We have selected lines of *P. chabaudi* that are resistant to pyrimethamine and shown that this selection has altered the karyotype of the parasite and duplicated the *DHFR-TS* gene, thus allowing at least a twofold overexpression of the transcript and presumably the enzyme. No mutations within the coding region of the *DHFR-TS* gene have been identified that would confer the drug resistance phenotype observed. Therefore, it appears that the pyrimethamine resistance of the *P. chabaudi* line Pr4 can be explained by at least a twofold increase in expression of the *DHFR-TS* transcript and presumably the enzyme. The *P. chabaudi* lines Pr1 and Pr3 have a single-copy *DHFR-TS* gene and yet grow in an increased amount of pyrimethamine; however, it is possible that a mutation(s) in other genes has occurred that allows these parasites to grow in these concentrations of the drug.

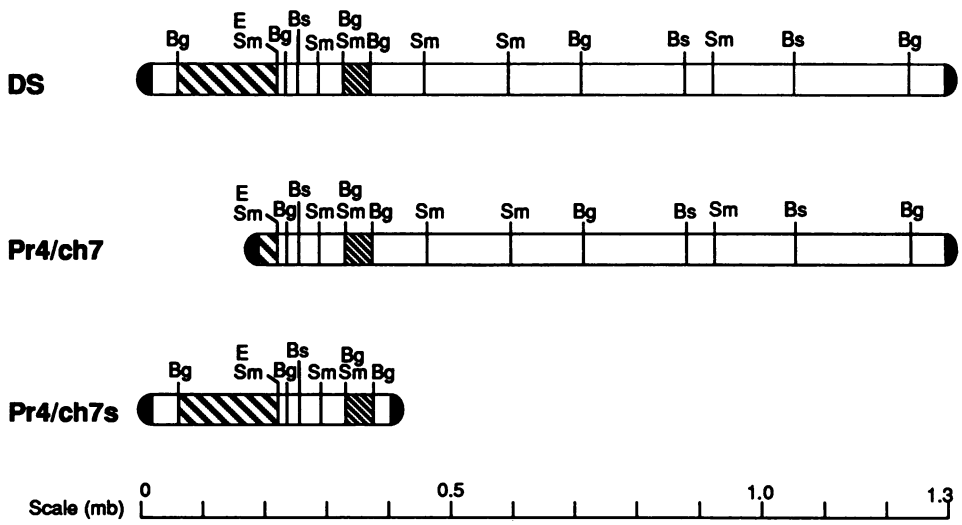

FIG. 5. Macrorestriction maps of chromosome 7 from DS, Pr4, and Pr4-c. Restriction digests of whole chromosomes were fractionated by PFG electrophoresis and probed with various cloned probes (DHFR-TS, Hsp70, and telomere). The sizes were calculated by using *S. cerevisiae* chromosomes as markers (see Materials and Methods) and used to construct these maps. Abbreviations: Bs, *Bss*HI; Bg, *Bgl*I; Sm, *Sma*I; E, *Eag*I. The shaded regions indicate restriction fragments which hybridize to the probes: ◖, telomere; ▨, DHFR-TS; ▧, Hsp-70.

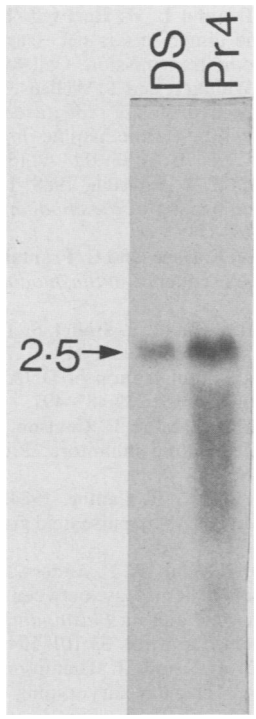

FIG. 6. The DHFR-TS transcript is expressed at a higher level in Pr4 than in DS. Total RNA (20 μg) was fractionated on a 1.5% agarose gel and transferred to a nylon filter (ethidium bromide staining of the gel indicated that there were equal amounts of the rRNA bands in the two tracks). The RNAs from DS and Pr4 were probed with the *Rsa*I fragment that includes almost the entire *DHFR-TS* gene.

Nevertheless, it is clear that the final selection passage to obtain Pr4 has resulted in duplication of *DHFR-TS* and an increase in the level of the transcript as a result of the chromosomal changes observed.

What mechanism has produced the changes in karyotype? The most likely mechanism that would result in the changes of karyotype due to drug selection observed in Pr4 is an initial step of nondisjunction. The accuracy of disjunction of chromosomes in yeast cells has been measured (7) and is very high, approximately one error in 10^5 mitotic divisions. We have applied selective pressure to increase the copy number of the *DHFR-TS* gene by the use of pyrimethamine. Consequently, there is a strong pressure to select any daughter cells after mitosis that have undergone a nondisjunction event involving chromosome 7, which encodes the *DHFR-TS* gene. It is likely that at least a twofold increase in expression of the DHFR-TS enzyme would allow the *P. chabaudi* Pr4 line to grow in the small amounts of pyrimethamine that we have used.

Duplication of chromosome 7 by nondisjunction would presumably be lethal for the daughter cell that has lost a chromosome, but it would leave the second daughter cell with a double complement of the full-length chromosome 7. Random nondisjunction can lead to instability of the karyotype of a cell, possibly by overproduction of gene products essential for correct maintenance of the cell cycle (15, 20). Our findings suggest that the full-length duplicated chromosome 7 is unstable and that, consequently, further secondary rearrangements have deleted the unwanted regions of chromosome 7.

A single centromere on each chromosome is essential for correct segregation to the daughter progeny during mitosis, and so both rearranged chromosomes 7 in the Pyr^r Pr4 parasite line must contain a centromere. We conclude that the centromere of chromosome 7 is localized in the region common to both of the chromosomes (Fig. 5).

The two rearranged chromosomes in the Pyr^r Pr4 parasite line could be derived, after a nondisjunction event, either by deletion of the regions between the telomere or by chromosome breakage and rehealing of the ends. Deletion events would maintain the original telomeres on the chromosomes. Hybridization of a telomere probe to digests of the purified chromosomes (Pr4/ch7 and Pr4/ch7s) with *Eco*RI and *Pvu*II shows different patterns from that observed in chromosome 7 in DS (data not shown). This suggests that chromosome breakage and rehealing of the telomeres has occurred by either de novo synthesis at the ends or repair by homologous recombination with the ends of other chromosomes (17). Telomere healing by de novo synthesis at the ends in *Schizosaccharomyces pombe* (18) and *P. falciparum* (4, 22) has recently been reported, and subtelomeric deletions are common in both in vitro cultured (9) and field isolates (2) of *P. falciparum*.

Regardless of the exact mechanism that has generated the rearranged chromosomes, it is clear that both chromosomes together include all of the original chromosome 7 with a 200-kb portion duplicated. These results are the first example of an increase in chromosome number in *P. chabaudi* and *P. falciparum* as a result of drug selection. The duplication of the *DHFR-TS* gene presumably confers the observed drug resistance. Generation of a small chromosome with a selectable marker such as *DHFR-TS* suggests the possibility that it may be used as a vector for transfection of the parasite. The centromere has also been defined to a small region of that chromosome, and it may be possible to use this information to clone this important chromosomal region.

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LITERATURE CITED

1. **Beverley, S. M., T. E. Ellenberger, and J. S. Cordingly.** 1986. Primary structure of the gene encoding the bifunctional dihydrofolate reductase-thymidylate synthase of *Leishmania major*. Proc. Natl. Acad. Sci. USA **83**:2584–2588.
2. **Biggs, B., D. J. Kemp, and G. V. Brown.** 1989. Subtelomeric chromosome deletions in field isolates of *Plasmodium falciparum* and their relationship to loss of cytoadherence in vitro. Proc. Natl. Acad. Sci. USA **86**:2428–2432.
3. **Bzik, D. J., W.-B. Li, T. Horii, and J. Inselburg.** 1987. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc. Natl. Acad. Sci. USA **84**:8360–8364.
4. **Cappai, R., M.-R. van Schravendijk, R. F. Anders, M. G. Peterson, L. M. Thomas, A. F. Cowman, and D. J. Kemp.** 1989. Expression of the RESA gene in *Plasmodium falciparum* isolate FCR3 is prevented by a subtelomeric deletion. Mol. Cell. Biol. **9**:3584–3587.
5. **Chen, G.-X., C. Mueller, M. Wendlinger, and J. W. Zolg.** 1987. Kinetic and molecular properties of the dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistant clones of the human malaria parasite *Plasmodium falciparum*. Mol. Pharmacol. **31**:430–437.

6. **Chu, G., D. Vollrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour-clamped homogenous electric fields. Science **234**:1582–1585.
7. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature (London) **287**:504–509.
8. **Coppel, R. L., A. F. Cowman, K. R. Lingelbach, G. V. Brown, R. B. Saint, D. J. Kemp, and R. F. Anders.** 1983. Isolate-specific S-antigen of *Plasmodium falciparum* contains a repeat sequence of eleven amino acids. Nature (London) **306**:751–756.
9. **Corcoran, L. M., J. K. Thompson, D. Walliker, and D. J. Kemp.** 1988. Homologous recombination within sub-telomeric repeat sequences generates chromosome size polymorphisms in *Plasmodium falciparum*. Cell **53**:807–813.
10. **Cowman, A. F., M. J. Morry, B. A. Biggs, G. A. M. Cross, and S. J. Foote.** 1988. Identification of amino acids linked to pyrimethamine resistance in dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **85**:9109–9113.
11. **Ferone, R., J. J. Burchall, and G. H. Hitchings.** 1969. *Plasmodium berghei* dihydrofolate reductase. Isolation, properties and inhibition by antifolates. Mol. Pharmacol. **5**:49–59.
12. **Ferone, R., and S. Roland.** 1980. Dihydrofolate reductase: thymidylate synthase, a bifunctional polypeptide from *Crithidia fasciculata*. Proc. Natl. Acad. Sci. USA **77**:5802–5806.
13. **Garrett, C. E., J. A. Coderre, T. D. Meek, E. P. Garvey, D. M. Claman, S. M. Beverley, and D. V. Santi.** 1984. A bifunctional thymidylate synthetase-dihydrofolate reductase in protozoa. Mol. Biochem. Parasitol. **11**:257–265.
14. **Hightower, R. C., M. L. Wong, L. Ruiz-Perez, and D. V. Santi.** 1987. Electron microscopy of amplified DNA forms in antifolate-resistant *Leishmania*. J. Biol. Chem. **262**:14618–14624.
15. **Holliday, R.** 1989. Chromosome error propagation and cancer. Trends Genet. **5**:42–45.
16. **Inselburg, I., D. Bzik, and T. Horii** 1987. Pyrimethamine-resistant *Plasmodium falciparum*: overproduction of dihydrofolate reductase by a gene duplication. Mol. Biochem. Parasitol. **26**:121–134.
17. **Jager, D., and P. Phillipsen.** 1989. Stabilization of dicentric chromosomes in *Saccharomyces cerevisiae* by telomere addition to broken ends or by centromere deletion. EMBO J. **8**:247–254.
18. **Matsumoto, T., K. Fukui, O. Niwa, N. Sugawara, J. W. Szostak, and M. Yanagida.** 1987. Identification of healed terminal DNA fragments in linear minichromosomes of *Schizosaccharomyces pombe*. Mol. Cell. Biol. **7**:4424–4430.
19. **McCutchan, T. F., J. A. Welsh, J. B. Dame, I. A. Quakyi, P. M. Graves, J. C. Drake, and C. J. Allegra.** 1984. Mechanism of pyrimethamine resistance in recent isolates of *Plasmodium falciparum*. Antimicrob. Agents Chemother. **26**:656–659.
20. **Meeks-Wagner, D., and L. H. Hartwell.** 1986. Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission. Cell **44**:43–45.
21. **Peterson, D., D. Walliker, and T. Wellens.** 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc. Natl. Acad. Sci. USA **85**:9114–9118.
22. **Pologe, L. G., and J. V. Ravetch.** 1988. Large deletions result from breakage and healing of *Plasmodium falciparum* chromosomes. Cell **55**:869–874.
23. **Ponzi, M., T. Pace, E. Dore, and C. Frontali.** 1985. Identification of a telomeric DNA sequence in *Plasmodium berghei*. EMBO J. **4**:2991–2995.
24. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuichi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
26. **Schwartz, D. C., and C. R. Cantor.** 1984. Separation of yeast chromosome-sized DNAs by pulse field gradient gel electrophoresis. Cell **37**:67–75.
27. **Sheppard, M., D. J. Kemp, R. F. Anders, and A. M. Lew.** 1987. High level sequence homology between a *Plasmodium chabaudi* heat shock gene and its *Plasmodium falciparum* equivalent. Mol. Biochem. Parasitol. **33**:101–104.
28. **Sheppard, M., D. J. Kemp, J. Thompson, R. F. Anders, and A. M. Lew.** 1989. Molecular karyotyping of the rodent malaria *Plasmodium chabaudi*, *Plasmodium berghei* and *Plasmodium vinckei*. Mol. Biochem. Parasitol. **34**:45–52.
29. **Sirawaraporn, W., and Y. Yuthavong.** 1984. Kinetic and molecular properties of dihydrofolate reductase from pyrimethamine-resistant and pyrimethamine-sensitive *Plasmodium chabaudi*. Mol. Biochem. Parasitol. **10**:355–367.
30. **Takeishi, K., S. Kaneda, D. Ayusawa, K. Shimizu, O. Gotoh, and T. Seno.** 1985. Nucleotide sequence of a functional cDNA for human thymidylate synthase. Nucleic Acids Res. **13**:20.
31. **Triglia, T., M. G. Peterson, and D. J. Kemp.** 1988. A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. **16**:8186.
32. **Walliker, D., R. Carter, and A. Sanderson.** 1975. Genetic studies on *Plasmodium chabaudi*: recombination between enzyme markers. Parasitology **70**:19–24.
33. **Walliker, D., I. A. Quakyi, T. E. Wellems, T. F. McCuthchan, A. Szarfman, W. T. London, L. M. Corcoran, T. R. Burkot, and R. Carter.** 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science **236**:1661–1666.
34. **Walliker, D., A. Sanderson, M. Yoeli, and B. J. Hargreaves.** 1976. A genetic investigation of virulence in a rodent malaria parasite. Parasitology **72**:183–194.