Nucleic Acids Research, 1994, Vol. 22, No. 15 3099–3103

Chromatin structure determines the sites of chromosome breakages in *Plasmodium falciparum*

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Received April 25, 1994; Revised and Accepted June 20, 1994

ABSTRACT

Spontaneous chromosome breakages are frequently observed in the human malaria parasite *Plasmodium falciparum* and are responsible for the generation of novel phenotypes, which may contribute to the pathogenicity and virulence of this protozoan parasite. The identification of a hot spot of chromosome breakage within the coding region of the *KAHRP* gene revealed that these events do not occur randomly but follow a regular pattern with a periodicity of 155 bp. This phasing corresponds to the average repeat unit of *P.falciparum* nucleosomes. Furthermore, breakage events preferentially occur within the linker regions of nucleosomes, as demonstrated by mapping endonuclease hypersensitive sites of chromatin. These data suggest that, in *P.falciparum*, the chromatin structure is involved in the molecular process of chromosome breakage, a mechanism that may be common in other eukaryotes.

INTRODUCTION

Malaria has a world-wide prevalence of more than 400 million cases of which more than half suffer from malaria tropica, the most severe form of human malaria. Malaria tropica is an infectious disease that is caused by the protozoan parasite *Plasmodium falciparum*. This parasite is transmitted to humans by the bite of an infected female anopheline mosquito. Within the human host, *P.falciparum* invades hepatocytes and erythrocytes, in which parasite development and asexual multiplication occurs.

During development in the human host, the parasite is constantly challenged by the host's immune system. To evade this challenge the parasite has developed means to change its antigen repertoire at a frequency of 2 % per generation (1).

The molecular mechanisms of antigenic variation in *P.falciparum* are still obscure. However, it has been observed that the *P.falciparum* genome is highly polymorphic and subject to frequent DNA rearrangements (2, 3, 4, 5). In other human parasites, such as Trypanosomes, DNA rearrangements are responsible for creating novel phenotypes and thus, are indicative of a mechanism generating antigenic variation (6).

By cloning the entire *P.falciparum* chromosome 2, it has been demonstrated that DNA rearrangements preferentially occur at the ends of chromosomes, thereby defining the subtelomeric regions as polymorphic (7). The polymorphism often results from chromosomal truncations, internal double strand breakages that are healed by the *de novo* addition of *P.falciparum* telomere repeat sequences (4, 5). These events occur spontaneously during mitotic propagation.

So far, chromosomal breakages have been reported for the *P.falciparum* chromosomes 1, 2, 8, 9 and 10 (4, 5, 8, 9, 10, 11). However, considering the polymorphic nature of the entire *P.falciparum* genome it is likely that all 14 *P.falciparum* chromosomes are subject to these events.

Several chromosomal breakage sites have been mapped to genes encoding antigens, such as *KAHRP* on chromosome 2, *RESA* on chromosome 1, *HRPII* on chromosome 8, and *Pf11-1* on chromosome 10 (4, 5, 8, 9, 11). Since the chromosomal breakages disrupt the coding region of these genes, gene expression is abrogated. The result are mutant parasites with altered antigenic phenotypes.

The best studied example is the *KAHRP* gene. The *KAHRP* gene product is a component of the knob structure on the surface of parasitized erythrocytes that are responsible for cytoadherence (12, 13). Chromosomal breakage events disrupting the *KAHRP* gene result in parasite mutants that are knobless and cytoadherent-deficient (14, 15, 16). These mutant parasites possess a growth advantage in cell culture, but are cleared by the host's spleen *in vivo* (14).

Here we have analyzed the mechanism of chromosome breakage and healing in *P.falciparum* at a molecular level. Systematic screening for chromosomal breakpoints has revealed that the *KAHRP* gene is a hot spot for chromosome breakage and healing events. The breakpoints identified are clustered within the *KAHRP* transcribed region. The distance between individual breakpoints is phased with a periodicity identical to *P.falciparum* nucleosomes. Furthermore, the nucleosome organization is correlated with the chromosome breakage sites. Breakpoints preferentially map to nucleosome linker regions. These data

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suggest an involvement of the chromatin structure in determining the sites of chromosome breakage and healing in *P.falciparum.*

MATERIALS AND METHODS

P.falciparum strains and culture conditions

The *P.falciparum* strains 7G8 and HB2 used in this study were aclonal and displayed chromosome 2 polymorhism as a result of prolonged *in vitro* cultivation (7). The parasite strain Dd2 revealed a clonal knobless phenotype due to a chromosomal truncation that extends into the *KAHRP* gene (7). *P.falciparum* intra-erythrocytic stages were cultured as described (17).

DNA preparation and PCR amplification

The preparation of genomic *P.falciparum* DNA has been described (18). 200 ng of purified genomic *P.falciparum* DNA from the strains indicated was used for PCR amplification. The conditions for PCR amplifications are: 35 cycles for 1 min at 94°C, 2 min 50°C, 3 min at 72°C, using a Perkin Elmer Cetus DNA Thermal Cycler. These conditions favor the amplification of fragments shorter than 1000 bp. The oligonucleotides used are:

A, CGAATATACAAATTTCTAAGGGTGC;
B, GTTTTATTCGAAATGGTAGGCCTC;
C, AAAAGTTGTACATCTTAATTTATAT;
D, CATATATTTTTGAATGTAATGTG;
E, ATAGGATATAGGTACATTTTC;
F, ACCTATATCCTATTTTTAATAAAATGATACAAT;
G, AAACTGCATGTAGTGTAGTAA;
H, TTTGTTTTTATTTCACTTAATTG;
I, TTTAGGTGTAAAAAATAAC;
J, GTTATTTTTTACACCTAAA;
K, GGAAACGGGATCCGGTGACTCCTTCG;
L, GTGTTCATGTTGCTTTTGTGC;
M, CTAATCCTCCTAGTAATGAACC;
N, TACCATCGACAACATTTTCCT;
O, GGAAGTAATGGTATGCAAAA;
P, GGATCAAAAGCTCATGAAAAA;
Q, GGATGCTGTGGTTAAATAATC;
R, TATACAGAAAAGAGTTATTCT;
S, ATGATATTATTCTTTAACACCTAG;
telomere primer, $(TAAACCC)_4$

Preparation of *P.falciparum* nuclei

P.falciparum nuclei from the FCR3 strain were prepared as follows. All steps were carried out on ice. The content of twenty 10 cm petri dishes was collected at a parasitemia of 10 % and washed once in ice cold Trager's buffer (57 mM NaCl, 58 mM KCl, 1 mM NaH_2PO_4, 7 mM K_2HPO_4, 11 mM $NaHCO_3$, 14 mM glucose). Erythrocytes were lysed by the addition of an equal volume of 0.1 % saponin, followed by three washes in E buffer (250 mM sucrose, 20 mM PIPES pH 6.3, 0.5 mM $CaCl_2$, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml antipain, 0.1 mM benzamidine, 0.1 mM sodium metabisulfite). The free parasites were then resuspended in 3 ml of E buffer and subjected to N_2 cavitation at 1050 psi for 30 min (Parr Instruments). Nuclei were collected (10000 rpm, 10 min, Sorvall SM24 rotor), washed three times in E buffer and resupended in S buffer (1.0 M sorbitol, 20 mM Tris/HCl pH 7.4, 0.5 mM $CaCl_2$, protease inhibitors as indicated above) for storage at −80°C.

Micrococcal nuclease digestion

$(0.5-1)\times10^9$ nuclei were subjected to limited digestion with 3 units/ml of micrococcal nuclease (purchased from Pharmacia) as described (19). At the time points indicated aliquots were withdrawn. The reaction was terminated by the addition of stop buffer to a final concentration of 1 % SDS, 0.8 M NaCl. The DNA was purified by phenol/chloroform extraction and ethanol precipitated. The purified DNA was then cleaved by the restriction endonuclease *BamHI*, size fractionated by gel electrophoresis and transferred to nitrocellulose. Probe I corresponds to position 703 bp to 764 bp of the published *KAHRP* sequence, probe II 763 bp to 826 bp (20).

RESULTS

We have recently shown that *P.falciparum* lines are unstable and, though initially clonal, contain various subpopulations after prolonged cultivation (7). The structural basis of this polymorphism frequently are chromosomal truncations that result from double-stranded breakages followed by the *de novo* addition of telomere repeat sequences. Genomic DNAs from the aclonal

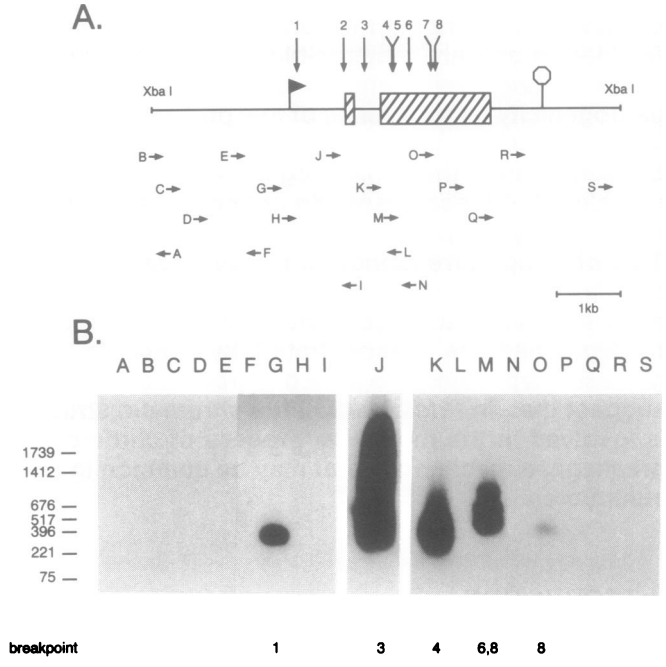

Figure 1. Identification of chromosome 2 breakpoints within the *P.falciparum KAHRP* gene. (**A**) Schematic drawing of the *KAHRP* gene locus. The coding region is indicated by rectangles, the transcription start site by an arrow and the termination site by a stop sign. 200 ng of genomic DNAs from the parasite lines 7G8, HB2 and Dd2 was subjected to PCR amplification using a series of *KAHRP* specific primers and an oligonuclotide to the telomere repeat sequences. The position and orientation of the *KAHRP* specific primers, designated as A to S, are indicated. Breakpoints are designated 1 to 8 and indicated by arrows (see Table 1). (**B**) PCR products derived from the indicated primers were analyzed by gel electrophoresis and hybridized with a probe spanning the entire *KAHRP* locus. The breakpoints 4, 5 and 6 were observed in the parasite strains 7G8 and HB2, breakpoint 1 in 7G8, breakpoint 3 in Dd2, breakpoint 2 in D3 (ref. 4), breakpoint 5 in FVO^- (ref. 5), and breakpoint 7 in FCC1 (ref. 9). The upward smear seen in several lanes is indicative of PCR products with variable numbers of telomere repeat sequences. The smear is particularly profound in lane J, since this experiment was conducted with genomic DNA from the clonal parasite strain Dd2. Dd2 is a clonal population in regard to breakpoint 3, as demonstrated by Southern analysis (ref. 7). Of the three hybridization signals seen in lane M, the upper signal revealed breakpoint 8, whereas the two lower signals, distinguished by the number of telomere repeat sequences, correspond to breakpoint 6. A DNA size standard is indicated in base pairs.

P.falciparum lines, 7G8 and HB2, were screened for subpopulations that reveal chromosome 2 breakage and healing events within a 7 kb region flanking the *KAHRP* gene. In addition, DNA from the clonal knobless parasite strain Dd2 was examined.

Novel breakpoints were identified using a PCR-based approach. A series of *KAHRP* specific oligonucleotides were used as the first primer and an oligonucleotide specific for *P.falciparum* telomere sequences was used as the second primer (Fig. 1A). *KAHRP* primers to both strands of the gene were utilized since chromosomal breakage events in *P.falciparum* can be preceded by DNA inversions (8). PCR products were size-fractionated and analyzed by hybridization using probes to the entire *KAHRP* locus and to the *P.falciparum* telomere repeats (Fig. 1B). Specific fragments were then cloned in *E.coli* and multiple subclones were sequenced (Table 1). All of them revealed novel breakpoints within the *KAHRP* gene. The breakpoints 4, 6 and 8 were present in the parasite strains 7G8 and HB2. Breakpoint 1 was found in 7G8, and breakpoint 3 was observed in Dd2. None of the breakage events identified were preceded by other rearrangements such as inversions.

The breakpoints cluster within the transcribed region of the *KAHRP* gene (Fig. 1A). No breakpoints were detected in the upstream or downstream untranscribed regions. This suggests that transcription through this region of DNA may contribute to the pathway of chromosome breakage and healing.

A compilation of all *KAHRP* breakpoints known to date revealed no striking homologies, with the exception of a conserved CA dinucleotide that immediately precedes the breakage site (Table 1).

To determine if the sites of chromosome breakage and healing are non-randomly distributed, the distance between individual breakpoints was calculated. A regular pattern emerged, with a phasing of 155 ± 6 base pairs (Fig. 2). This phasing is reminiscent of the average nucleosomal repeat unit, thus suggesting an involvement of the chromatin structure in

Table 1. Compilation of sequences at breakpoints of the chromosome 2 *KAHRP* gene.

Breakpoint No.	Sequence at Breakpoint
1	T A A A A C A T A T **C A** T C A T A
2	A T A T A T T T T A **C A** A A A T T
3	A T A T T T A T T T **C A** T A T A T
4	C C A A G C A C C A **C A** G G T T C
5	A C C A C A G G T T **C A** C C A A C
6	G A A A A T G G T C **C A** A A T A T
7	T T T A A T G G T G **C T** C C T G G
8	G C A G G C T A T T **C A** G C T C C

Breakpoints are indicated by arrow heads. The conserved CA dinucleotide is indicated in bold letters. The exact positions of the breakpoints are indicated in figure 4. Breakpoints 1, 3, 4, 6, 8 this study; breakpoints 2 and 5 ref. 4 and 5; breakpoint 7 ref. 9.

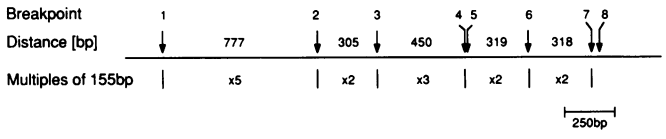

Figure 2. Phasing of breakpoints in *P.falciparum*. The positions of the *KAHRP* breakpoints are indicated by arrows. The distance between breakpoints is indicated in base pairs and expressed in multiples of 155 bp, the nucleosome repeat length.

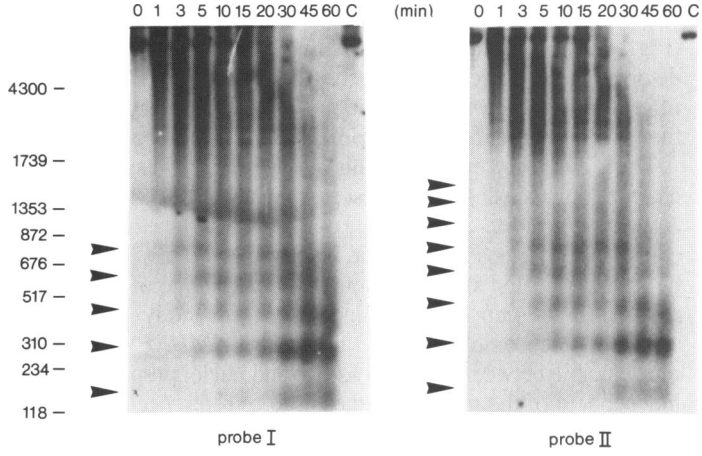

Figure 3. Determination of micrococcal nuclease hypersensitive sites along the *KAHRP* gene. *P.falciparum* nuclei prepared from the schizont stage were subjected to limited digestion with micrococcal nuclease. At the time points indicated in minutes aliquots were withdrawn. The genomic DNA was extracted and digested to completion with *BamHI*. The DNAs were size-fractionated and examined by Southern analysis using the probes I and II that flank the *BamHI* site of the *KAHRP* gene. Specific hybridization signals are indicated by arrowheads. The analysis of these data are summarized in figure 4.

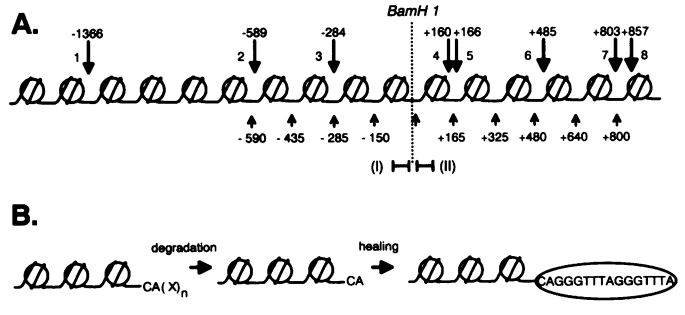

Figure 4. **(A)** Model of chromosome breakage and healing in *P.falciparum*. The primary chromatin structure of the *KAHRP* gene as determined in figure 3 is shown, with circles symbolizing nucleosome core particles. The positions of breakpoints are indicated by arrows above the array of nucleosomes, the positions of micrococcal nuclease hypersensitive sites below. All sites are given in reference to the *KAHRP BamHI* restriction site. The probes used in figure 3 are indicated. **(B)** Two step process of chromosome healing. The terminal DNA $(X)_n$ is degraded until a CA dinucleotide is reached, then telomere repeat sequences are added.

determining the sites of chromosome breakage and healing in *P.falciparum*.

To examine this possibility, the chromatin structure surrounding the breakpoints was characterized. Intact *P.falciparum* nuclei were prepared from a synchronized erythrocytic culture of FCR3 schizont stage parasites. The parasite strain FCR3 contains a functional *KAHRP* gene. The purified schizont nuclei were then subjected to limited digestion with micrococcal nuclease, an enzyme that preferentially cleaves within the linker region of nucleosomes. The DNA was then purified and digested with the restriction endonuclease *BamHI*. This enzyme cleaves the *KAHRP* gene once. Upon size-fractionation and Southern transfer of the *P.falciparum* genomic DNA, the nitrocellulose filters were sequentially hybridized with probes flanking the *BamHI* site of the *KAHRP* gene.

Short overlapping oligonucleotides of about 60 bp were used as probes. These oligonucleotides abut the *BamHI* restriction site (Fig. 4). These indirect end-labelling experiments allow one to determine the nucleosome positions along the *KAHRP* gene in reference to the *BamHI* restriction site by measuring the size of the DNA fragments that hybridize to the probes used.

The indirect end-labelling experiments revealed the characteristic phasing of nucleosomes on both sides of the *KAHRP BamHI* restriction site (Fig. 3). Probe I indicates up to six consecutive nucleosomes, probe II up to eight. Thus, the position of at least the first six nucleosomes on either side of the *BamHI* restriction site can be determined from this study (Fig 3).

The nucleosome phasing along the *KAHRP* gene was reproducible between different assays and nuclei preparation, and independent of the restriction sites used as reference points (data not shown).

The average distance between nucleosomes was found to be 156 ± 5 bp (Fig. 3). The length of the DNA fragment associated with a histone octamer in a core particle is 148 ± 5 bp, as determined by prolonged digestion of *P.falciparum* chromatin with micrococcal nuclease (data not shown). The nucleosome linker region is 8 ± 3 bp in length.

When the nucleosome positions along the *KAHRP* gene were compared with the breakpoints, a correlation emerged. Breakpoints map to nucleosome linker regions, with the single exception of breakpoint 8 (Fig. 4A). This suggests that the nucleosomal organization is involved in determining the site of chromosome breakage in *P.falciparum*.

DISCUSSION

The structural basis for the polymorphism associated with *P.falciparum* chromosomes are terminal truncations caused by mitotic, chromosomal breakage and healing events (4, 5, 7, 8). The *KAHRP* gene maps 90 kb distal from a chromosome 2 telomeric end and is subject to these chromosomal truncations (4, 5, 7).

The identification of eight independent breakpoints within the *KAHRP* gene suggests that this region of DNA represents a hot spot for chromosome breakages in *P.falciparum*. The breakpoints were non-randomly distributed and revealed a periodicity of 155 bp. This periodicity was found to be identical to the phasing of *P.falciparum* nucleosomes. Limited digestion of *P.falciparum* chromatin with micrococcal nuclease demonstrated a nucleosomal phasing of 156 ± 5 bp in this pathogen (Fig. 3).

This repeat unit consists of the internucleosomal linker of 8 ± 3 bp and the core DNA fragment of 148 ± 5 bp, as determined by subjecting *P.falciparum* chromatin to prolonged digestion with micrococcal nuclease (data not shown). The size of the DNA fragment in a nucleosome core particle conforms, within the margins of error, to the canonical value of 146 bp (21).

Preliminary data suggest that the protein composition of *P.falciparum* chromatin is similar to other eukaryotes and contains the histones H1, H3, H2A, H2B and H4 (22). The *P.falciparum* histone H2A has recently been cloned (23). Thus, the *P.falciparum* histone constitution conforms to other eukaryotes.

Interestingly, the nucleosomal organization along the *KAHRP* gene and the sites of chromosome breakage are closely correlated. This observation suggest a model for chromosome breakage and healing in *P.falciparum*. The data suggest that double-stranded breakages of *P.falciparum* chromosomes preferentially occur within nucleosome linker regions. This model is further supported by the clustering of breakpoints 4 and 5 within the same nucleosome linker.

However, only some linker regions seem to be suitable sites for chromosome breakages since the breakpoints identified are not equally distributed along the 7 kb *KAHRP* gene locus but instead cluster within 2 kb of coding sequence. Thus, the internucleosomal linkers of this region are obviously predisposed to chromosome breakages. One possible model could involve an unspecific endonucleolytical activity that requires opening of chromatin and unpacking of the DNA prior to inducing double-stranded breaks.

As has been demonstrated in other systems, transcriptional elongation results in disruption of the nucleosome structure (24, 25, 26). Specifically, transcribed genes are partially depleted of the histone H1, which extends beyond the core particle along the DNA into the internucleosomal linker (27, 28). Despite the depletion of H1, transcribed domains still retain a nucleosomal conformation (29, 30). This was confirmed for the *KAHRP* gene. During the transcriptionally active ring stage, the *KAHRP* gene is still assembled into nucleosomes as demonstrated by subjecting ring stage nuclei to limited digestion with micrococcal nuclease (data not shown). However, the indirect end-labelling experiment revealed only the average nucleosome distribution. It is likely that, at any given time during the ring stage, only a subset of the *KAHRP* genes investigated are transcribed, and thus would reveal transcriptionally induced changes of the nucleosome structure.

Recent data indicate that the transcribing RNA polymerase progressively translocates nucleosome core particles (24, 25, 26, 31). Our findings suggest that during this process, the DNA is inaccessible to DNA breakage events since the breakpoints are phased and not randomly distributed.

Thus, chromosome breakages in *P.falciparum* may be initiated by an endonucleolytic activity that cleaves double-stranded DNA within H1 depleted nucleosome linker regions. Following a breakage event, the DNA is then degraded until the first accessible CA dinucleotide within a linker region is encountered (Fig. 4B). Alternatively, the endnucleolytical activity could possess a cleavage preference 3′ to a CA dinucleotide. The CA dinucleotide is not only conserved among all known *KAHRP* breakpoints but is also found at the breakage sites of other *P.falciparum* genes (5, 8, 9, 11).

While it is likely that transcriptional activity is involved in predisposing nucleosome linker regions to chromosome breakages, other mechanisms can not be excluded. Alternatively, the presence or absence of a CA dinucleotide could determine if a linker region is prone to chromosome breakages. However,

CA dinucleotides are also found in nucleosomal linker regions that are apparently not prone to breakages. This is true for internucleosomal linkers of transcribed as well as non-transcribed regions of the *KAHRP* gene. However, there is an accumulation of CA dinucleotides within the *KAHRP* coding region, in which CA dinucleotides are about 7-fold more abundant than elsewhere within the locus. Thus, the increased number of CA dinucleotides could also account for the clustering of breakpoints within the *KAHRP* coding region.

Since *P.falciparum* telomere repeat units are composed of GGGTTTA or GGGTTCA, it is conceivable that a CA dinucleotide is a necessary and perhaps sufficient signal for the addition of telomere repeat sequences by the *P.falciparum* telomerase (32). This model is consistent with the observation that the telomerases of other eukaryotes possess a 3′ to 5′ exonuclease activity and are capable of elongating non-telomeric primers (33, 34, 35).

Chromosome breakage and healing events are also observed in other eukaryotes. A well characterized example is a terminal truncation of the human chromosome 16 that is associated with α-thalassaemia (36). It is tempting to speculate that, in other eukaryotes, the sites of mitotic chromosome breakages also map to nucleosome linker regions. In addition to determining the sites of mitotic DNA breakages, the chromatin structure is also involved in determining the position of transient meiosis induced double-strand breakages as has recently been observed in yeast (37).

ACKNOWLEDGEMENTS

We thank J.Hoheisel and A.J.Lustig for critically reading the manuscript, G.Lanzer for discussion, and M.Samuels and C.Ralphe for technical assistance. This work was supported by the World Health Organization and grants from the US army and the German Bundesministerium für Forschung und Technologie.

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