sludges for elevated concentrations of toxic organic chemicals and to study their formation and degradation during wastewater treatment, sludge treatment, and sludge disposal.

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15. We thank the operators of the treatment plant who helped us during sample collection; the colleagues who sent us sludge samples; K. Grob and G. Grob for supplying gas chromatography capillaries; M. Ahel, H. Mönch, and M. Tschui for technical assistance; and D. H. Welti for NMR work. This project was supported by the COST 64b and 68 programs of the European Communities and by the Swiss National Science Foundation.

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Mung Bean Nuclease Cleaves *Plasmodium* Genomic DNA at Sites Before and After Genes

Abstract. *Mung bean nuclease was found to cut the genomic DNA of the malaria parasite* Plasmodium *at positions before and after genes but not within gene-coding regions. This cleavage, which had nearly the preciseness of a restriction nuclease, required controlled conditions in the presence of formamide. Southern blot analysis showed that the coding areas for* Plasmodium *actin, circumsporozoite protein, histidine-rich protein, ribosomal RNA's, and tubulin are each cleaved from genomic DNA to yield a single major band on an agarose gel. DNA sequence data on several clones of mung bean nuclease cleavage products containing the gene for the circumsporozoite protein of* Plasmodium falciparum *confirmed that cleavage sites are before and after genes. Recognition and cleavage of DNA did not seem to be related to any primary sequence but may be related to structural features of the DNA duplex that demarcate genes. Mung bean nuclease–cleaved DNA could be inserted directly into a λ expression vector, yielding a representative but small gene bank of intact gene fragments.*

The conformation of double-stranded DNA has been shown to be variable. In addition to the familiar β-form helix, DNA can contain bends and kinks as well as have a Z-DNA configuration. Distinguishing these features along the DNA molecule undoubtedly plays a large part in the regulation of gene expression. Using species of the malaria parasites, *Plasmodium*, we show here that mung bean nuclease recognizes and cleaves the genomic DNA at specific locations before and after genes. The cleavage is directed by the structure of naked DNA since the nuclease cuts both cloned and genomic DNA to yield the same products. Because the cleavage points are related to the beginning and ending of genes and not to a primary sequence, we propose that the nuclease is recognizing defined conformational structures that demarcate genes in the DNA of *Plasmodium* and of other organisms.

We cleaved genomic DNA with mung bean nuclease under a series of conditions and analyzed the products by the Southern blot techniques. Cloned genes isolated from *Plasmodium* and other organisms, or synthetic oligonucleotides, were used as probes. In each case, single major bands of *Plasmodium* DNA hybridized to each probe. Further, the bands were approximately the size needed to encode the corresponding gene. A number of genes were investigated in the same manner to see if the phenomenon was a general one or was specific to a single gene.

Four different DNA's were used as radioactively labeled probes for Southern blot analysis to investigate the results of mung bean cleavage of genomic DNA. Complementary DNA's (cDNA's) for chicken β-actin (*1*) and *Chlamydomonas* tubulin (*2*) were selected because they have been used to find corresponding genes in a broad range of organisms including parasitic protozoa (*3*). A series of different cleavage conditions was tried (*4*). Figure 1 shows an analysis of the reactions in 40 and 45 percent formamide. Southern blot analysis of *Plasmodium knowlesi* DNA after cleavage in 45 percent formamide shows a single predominant fragment at 1.6 kb hybridizing to the actin probe (Fig. 1A) and one at 2.6 kb hybridizing to the tubulin probe (Fig. 1B). These fragments are of adequate size to encode the corresponding genes. The 40 percent formamide reactions contain larger fragments that hybridize to the probes.

The area surrounding the histidine-rich protein (HRP) of *P. lophurae* (*5*) was investigated with a radioactively labeled oligonucleotide consisting of a sequence of five tandem histidine codon triplets as described (*6*). DNA from *P. lophurae* was made greater than 95 percent pure by passing it through a Hoechst dye CsCl gradient (*7*), and was then digested with mung bean nuclease in 30, 35, and 40 percent formamide. Analysis of the 30 and 35 percent reactions showed a band at 2.2 kb (Fig. 1C). This is the same size as the messenger RNA (mRNA) described (*6*) which codes for the HRP and hybridizes to the oligonucleotide probe. Again the mung bean nuclease seemed to excise a gene-sized fragment.

The gene for the circumsporozoite (CS) protein of *P. falciparum* was investigated by using as a probe the clone pmPf5, which contains the complete coding area of the gene (*8*). In Southern blots only one band at 2.3 kb was seen in the 35 percent reaction. One major band of 1.3 kb and one minor species of slightly larger size were seen in the 40 percent reaction.

In general, the number of bands hybridizing to a probe did not increase as the formamide in the reaction mix was increased. The results indicated that the region of identity between each probe and the *Plasmodium* DNA was not cleaved under any of a series of conditions that reduce the size of the gene-containing fragments from large to "gene sized." We assume that this means that the coding regions are not cleaved even though, in the case of probes from other systems, sequence identity may not extend over an entire *Plasmodium* gene.

To determine the site of mung bean nuclease cleavage we cloned and se-

quenced fragments generated by mung bean nuclease digestion. To do this we combined equal portions of DNA from the 35 and 40 percent formamide mung bean nuclease reactions shown in Fig. 1D and ligated them into the expression vector λgt11 (*9*). Two hundred thousand inserts containing clones were immunologically screened with monoclonal antibodies specific for the CS protein of *P. falciparum* (*8*). Seven of 35 positive clones were analyzed in detail. The fragments detected by Southern blot analysis in Fig. 1D were all found in this group. Three clones contained a 2.3-kb fragment (35 percent formamide reaction, Fig. 1D, lane 1). Three contained a 1.3-kb fragment and one contained a 1.35-kb fragment (40 percent formamide reaction, lane 2). The DNA sequence of the 2.3-kb fragment has been determined (*8*). The 2.3-kb fragment from the 35 percent formamide reaction has a site about 80 bp 5′ to the start of the gene and a site approximately 1000 bp 3′ to the gene.

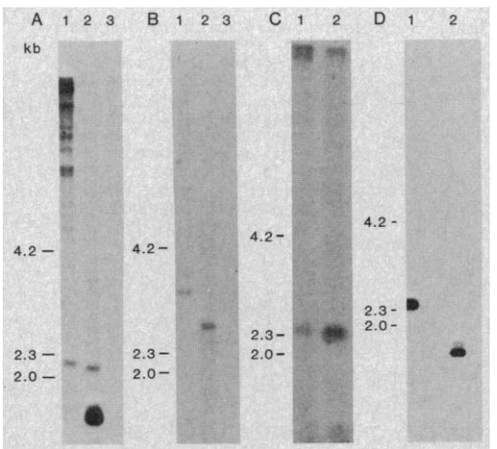

Fig. 1. Analysis of DNA fragments released from genomic DNA by mung bean nuclease digestion. *Plasmodium* or rhesus monkey DNA (1 μg) was digested with mung bean nuclease in various concentrations of formamide. *Plasmodium falciparum* and *P. lophurae* DNA digestions were best in 30 to 40 percent formamide while *P. knowlesi* DNA digestions were best in 40 to 45 percent formamide. The DNA was then subjected to electrophoresis through 0.8 percent agarose for 22 hours at 2 V/cm. DNA was transferred to nitrocellulose and hybridized with a radioactively labeled probe. (A) Hybridization to a labeled fragment of a plasmid, pA1, containing a chicken β-actin gene (*1*). (Lane 1) *Plasmodium knowlesi* DNA in 40 percent formamide; (lane 2) *P. knowlesi* DNA in 45 percent formamide; (lane 3) rhesus monkey DNA in 45 percent formamide. (B) Hybridization to a labeled DNA plasmid probe, β37, containing the β-tubulin gene from *Chlamydomonas* (*2*). (Lane 1) *Plasmodium knowlesi* DNA in 40 percent formamide; (lane 2) *P. knowlesi* DNA in 45 percent formamide; (lane 3) rhesus monkey DNA in 45 percent formamide. (C) Hybridization to a labeled oligonucleotide with sequence similarity to the histidine-rich protein of *P. lophurae* (*6*). (Lane 1) *Plasmodium lophurae* DNA in 30 percent formamide; (lane 2) *P. lophurae* DNA in 35 percent formamide. (D) Hybridization to a plasmid, pmPf5 (*8*), containing the complete coding region of the CS gene of *P. falciparum*. (Lane 1) *Plasmodium falciparum* DNA in 35 percent formamide; (lane 2) *P. falciparum* DNA in 40 percent formamide.

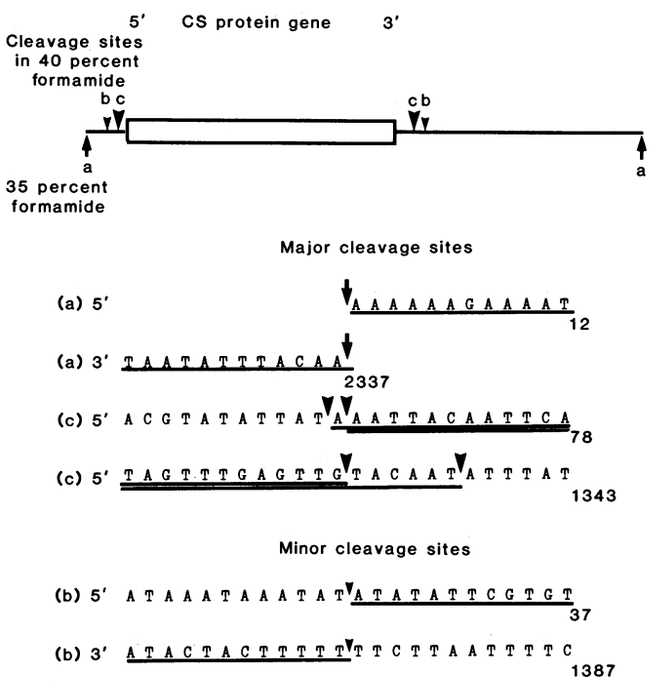

Fig. 2. (Top) A map of mung bean cleavage sites in the *P. falciparum* circumsporozoite protein (CSP) gene (above). The coding region is boxed; noncoding regions are represented by a line. Arrows show cleavage sites in 35 percent formamide (a). Large arrowheads show the predominant cleavage sites in 40 percent formamide (c). Small arrowheads show minor cleavage sites in 40 percent formamide (b). A bacteriophage λ library containing mung bean nuclease–treated *P. falciparum* DNA inserts was constructed as described (*9*) in the vector λgt11 (*18*). Clones containing the CSP gene were selected by screening with a monoclonal antibody (*8*). (Bottom) Primary sequences of DNA on both sides of the termini of several clones. The number after each line of sequence indicates the position of this nucleotide in the total sequence of the CSP gene (*8*). The underlined part of the sequence was derived by sequence analysis of the termini of clones. (a) pmPf1; (b) pmPf15; (c) pmPf5, pmPf8, and pmPf13.

Both the 5′ and 3′ termini of other clones were sequenced and compared to the sequence of the 2.3-kb fragment (Fig. 2). The three 1.3-kb fragments have sites either 10 or 11 bp from the start of the gene and sites either 27 or 35 bp from the 3′ end of the gene. One clone has been sequenced which contains a minor fragment 1.35 kb from the 40 percent formamide reaction. This fragment is cut 52 bp in front of the gene and 60 bp after it. Sequences at the termini of these clones represent sequences spared by the nuclease (Fig. 2). We do not know the extent to which the DNA is cleaved beyond these fragments. There is no apparent sequence identity either 5′ or 3′ to the cuts. Although the cut sites are rich in dA·dT they have no more dA·dT than surrounding areas both inside and outside the gene that are not cut. Further, the sequence 5′ to a given site is no more dA·dT rich than the sequence 3′ to a site. Sequences of independent clones of the predominant 1.3-kb fragment in the 40 percent reaction are interesting because they show a slight variability in the point of cleavage that was not apparent by Southern blot analysis.

Our data indicate that sites of cleavage depend on the structure of naked DNA. A cloned DNA sequence synthesized in and isolated from *Escherichia coli* yields the same cleavage products as genomic DNA from *Plasmodium*. Mung bean nuclease cleavage of cloned *Plasmodium* ribosomal genes in formamide yields fragments of defined size that correspond to the coding areas for the small ribosomal RNA (rRNA), the 5.8*S* RNA, and the large rRNA (*10*). Figure 3 shows a map of a cloned plasmid, pPbSL7.8, which contains an Eco RI DNA fragment from *P. berghei* with the coding region for the entire small rRNA, the 5.8*S* RNA, and 2.2-kb of the large ribosomal gene which is interrupted by an Eco RI cleavage site (*11, 12*). Figure 3A shows the products of cleavage of the cloned restriction fragment with either a restriction nuclease or mung bean nuclease. In Fig. 3B, mung bean nuclease cleavage products of cloned ribosomal genes are directly compared with those from total genomic DNA. The major *Plasmodium* derived fragments are all the same size. This suggests that mung bean nuclease cuts these cloned *Plasmodium* DNA's

nearly quantitatively and yields the same products as genomic DNA. The fact that the cloned and genomic DNA's react identically indicates that the DNA has not been cut previously by *Plasmodium* nucleases. Therefore, cleavage depends on the structure of the DNA clone.

We are interested in understanding what type of DNA sequence constitutes a mung bean nuclease cleavage site in genomic DNA. Since the enzyme cleaves single-stranded DNA, cleavage might be directly related to denaturation of local regions of DNA and cutting of single-stranded areas. Thus, since the ease of denaturation of DNA by heat or chemicals is thought to be directly related to the concentration of dA·dT base pairs in its sequence, cleavage might also be related to dA·dT richness. The finding that the sequence of 1000 bp to the 3′ end of the CS gene is, over all, more than 80 percent rich in dA·dT and yet is not cleaved in 35 percent formamide reactions argues against this. Further, S_1 nuclease, another single-strand–specific nuclease, cleaves single-stranded DNA under these conditions but does not substitute for mung bean nuclease in the cleavage of *Plasmodium* DNA. We also tested linear and nicked plasmids of known sequences under the conditions described for *Plasmodium* DNA. One plasmid, pkH47 (BRL) containing 100 consecutive deoxyadenosines at the Pst I site of pBr322, was cleaved in the run of deoxyadenosines in 45 percent formamide, but not in 40 percent formamide (data not shown). Some sites in *P. lophurae* and *P. falciparum* DNA are sensitive to cleavage in formamide that is 10 to 15 percent less concentrated than that needed for cleavage of pkH47. This suggests that most cleavage is not directly related to denaturation of regions of DNA on the basis of dA·dT richness.

We also analyzed DNA from other organisms. Cleavage that yielded fragments of defined size was not uncommon even in DNA from higher eukaryotic organisms (Fig. 1A, lane 3). Since introns may or may not be cleaved by mung bean nuclease, analysis of these data may be complicated by their presence. Some DNA's, like that of the parasitic trematode *Schistosoma mansoni*, yielded single, small fragments that hybridized either to actin or tubulin probes (data not shown). This indicates that *Plasmodium* DNA is not unique in its susceptibility to this type of cleavage. Cleavage of linear or nicked bacterial plasmids or bacteriophage λ did not occur under these conditions, although under different conditions there is evidence that mung bean nuclease recognizes a structural uniqueness of the lactose operator in bacteriophage λ (*13*).

The potential of localized regions of DNA to assume different conformations may be a factor in regulating certain protein-DNA interactions such as chromatin packing and transcription. It is reasonable to expect certain nucleases to recognize these structures. For example, micrococcal nuclease appears to prefer sites outside rather than inside genes in the initial stages of a DNA cleavage reaction. A large number of roughly equivalent sites spaced about 200 nucleotides apart outside genes are statistically among the first sites cleaved (*14, 15*). S_1 nuclease is even more specific in that it recognizes and cleaves sites 5′ to many eukaryotic genes when the genes are cloned as part of supercoiled plasmids, but these sites are not cleaved when the plasmids are linear (*16*).

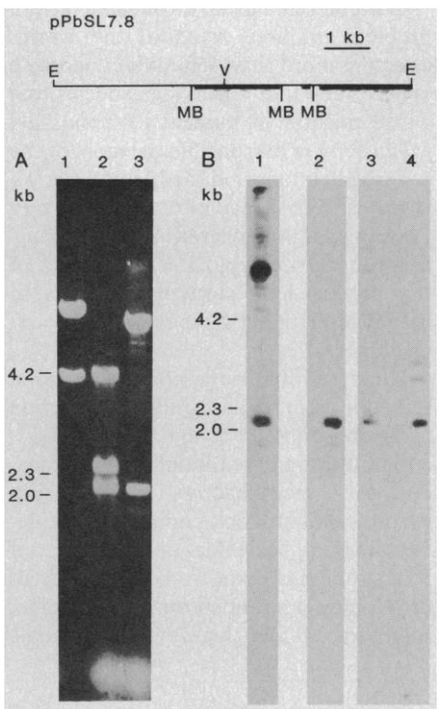

Fig. 3. (Top) A map of the plasmid pPbSL7.8 insert shows restriction sites as well as three mung bean cleavage sites that occur in reactions containing 45 percent formamide. The thick lines represent, from left to right, the coding area for the small rRNA, the 5.8*S* RNA, and 2.2 kb of the large rRNA of *P. berghei*. (A) Mung bean nuclease sites in a cloned fragment of the ribosomal gene of *P. berghei*. Portions (0.5 μg) of pPbSL7.8 DNA (*11*) were treated with Eco RI (lane 1), mung bean nuclease and Eco RI sequentially (lane 2), or mung bean nuclease alone (lane 3). Products from reactions were then subjected to electrophoresis through 1.2 percent agarose at 2 V/cm for 18 hours, and the DNA was visualized by staining with ethidium bromide. (B) Comparison of digestion products resulting from mung bean nuclease cleavage of cloned and genomic DNA. DNA from pPbSL7.8 (lane 1) and λPb27 (lane 2) (*11*) were digested with mung bean nuclease as described above. Genomic DNA (5 μg) was digested as above with mung bean nuclease in 40 percent (lane 3) or 45 percent (lane 4) formamide. The products were compared by Southern blot analysis with the use of a radioactively labeled plasmid (pPbSL5.6) probe (*11*) that contains only the gene for the small rRNA subunit. The band at 6.7 kb in lane 1 results from hybridization of the probe to a pBr322-derived sequence on the blot.

Our sequence analysis of clones of the CS gene of *P. falciparum* places a cleavage site 80 bp before the coding region and a site 1000 bp 3′ to the gene in 35 percent formamide reactions. In 40 percent formamide the cleavage sites are nearly as precise but closer to the gene. Southern blot analysis of four other genes suggests that they are cleaved in a similar telescoping pattern with increasing formamide concentration. Sequence analysis of several different cleavage sites gives no indication that the nuclease recognizes a particular primary sequence. The fact that a point of cleavage that is totally spared in one concentration of formamide is completely cleaved in another also indicates that conformational transitions are induced by formamide and that the cleavage is not the result of primary sequence recognition. The data may, however, reflect the potential of DNA for conformational flexibility. Other DNA structural variations have been described but little is known of their significance or how the structures relate to DNA-protein interactions. The ease with which the putative conformational signposts described here are detected in genomic DNA makes it likely that their biological significance will be discovered.

It should now be possible to produce recombinant libraries that contain mostly complete gene fragments. In such libraries the representation of any gene will relate directly to its copy number in the genome. This is of importance with regard to parasites that produce, at different stages of their life cycle, certain proteins that are not available in quantity. In this article we have described an application of this technique, and in the accompanying research article (*8*) we describe the gene for the CS protein which is produced by *P. falciparum* when it is in its insect host but which was cloned from DNA obtained from blood stage parasites. The intact gene, which occurs as a single copy in *P. knowlesi* (*17*), was found by immunological screening with monoclonal antibodies in about one in ten to one in 20,000 insert-containing phage λ. This approach

should allow cloning of genes that are difficult to isolate in *Plasmodium* and other organisms.

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4. Mung bean nuclease (P-L Biochemicals) reactions were done in a volume of 100 μl with 1 unit of enzyme per microgram of DNA. Reactions were incubated at 50°C for 30 minutes in various concentrations of formamide containing 0.2*M* NaCl, 1 m*M* $ZnSO_4$, and 30 m*M* sodium acetate, *p*H 4.6. The solution was then diluted fourfold with 0.01*M* EDTA, extracted with phenol, and precipitated with ethanol before subsequent treatment.
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9. The λgt11 library was constructed as follows. *Plasmodium* DNA (5 to 10 μg) was digested with mung bean nuclease as described (*5*) in 30, 35, 40, and 45 percent formamide. The reaction product from each of the four reactions was diluted fourfold with 0.01*M* EDTA, treated with phenol, and precipitated in 2.5 volumes of ethanol. A portion of each DNA was analyzed by the Southern blot technique with a DNA probe homologous to either actin or tubulin. The DNA from the reactions containing "gene sized" fragments homologous to the probe were combined and used as a source of fragments to ligate into λgt11. We combined the 35 and 40 percent reaction products for *P. falciparum* libraries. (In this case the DNA was treated with Klenow fragment, but in the production of other libraries we have eliminated this step.) Eco RI linkers [Bethesda Research Laboratories (BRL)] were blunt-end–ligated to the treated fragments. After digestion with Eco RI, free linkers were separated from larger fragments by using a 1.5 by 20 cm Sepharose 4B column. λgt11 (*18*) was self-ligated and digested with Eco RI. The *P. falciparum* fragments were ligated to the prepared λgt11 DNA overnight at 12°C with T4 DNA ligase (BRL) under the conditions recommended by the supplier. The ligation reaction products were packaged into infectious phage in vitro (Promega Biotec). From an initial 3 μg of starting genomic DNA, 4×10^5 packaging events were scored by detectable interruption of the β-galactosidase gene of λgt11 on RY1090 growing on LB agar supplemented with Xgal and isopropylthiogalactoside (IPTG). A *P. knowlesi* DNA library was prepared in the same way except that we used the mung bean nuclease cleavage products obtained in 40 and 45 percent formamide.
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19. We thank R. Howard for providing *P. knowlesi* and *P. falciparum* and C. Rodi for help with the synthesis of the oligonucleotide used to detect the histidine-rich protein of *P. lophurae*. We thank F. C. Eden, L. H. Miller, E. P. Reddy, M. Singer, and A. Rabson for critical review of the manuscript.

5 June 1984; accepted 29 June 1984

DNA Cloning of *Plasmodium falciparum* Circumsporozoite Gene: Amino Acid Sequence of Repetitive Epitope

Abstract. *A clone of complementary DNA encoding the circumsporozoite (CS) protein of the human malaria parasite* Plasmodium falciparum *has been isolated by screening an* Escherichia coli *complementary DNA library with a monoclonal antibody to the CS protein. The DNA sequence of the complementary DNA insert encodes a four–amino acid sequence: proline-asparagine-alanine-asparagine, tandemly repeated 23 times. The CS* β*-lactamase fusion protein specifically binds monoclonal antibodies to the CS protein and inhibits the binding of these antibodies to native* Plasmodium falciparum *CS protein. These findings provide a basis for the development of a vaccine against* Plasmodium falciparum *malaria.*

Malaria remains a formidable health problem in large areas of the world, affecting more than 150 million people a year. Of the four plasmodial species that cause malaria in humans, *Plasmodium falciparum* is responsible for most of the severe infections and the highest mortality rates. The spread of drug-resistant *P. falciparum* in many areas and the occurrence of severe epidemic outbreaks of this disease lend particular urgency to recent attempts at developing a malaria vaccine (*1*).

Under normal conditions, a malarial infection is initiated by the introduction of sporozoites into the host's bloodstream through the bite of infected mosquitoes. Hence, inactivation of the sporozoites would block completely the development of this infection. A number of recent findings point to the feasibility of developing an antisporozoite vaccine. Sporozoites are highly immunogenic and, more important, capable of eliciting a protective immune response in several host species, including humans [reviewed in (*2*)]. The immunogenicity of sporozoites resides largely, if not exclusively, in a single antigen, the circumsporozoite (CS) protein (*3*), which covers the entire parasite surface (*4*). In turn, the immunogenicity of the CS protein is restricted almost entirely to a single epitope (*3*) that is identically or quasi-identically repeated several times in tandem (*5, 6*).

Monoclonal antibodies against the CS protein (molecular weight 58,000) of *P. falciparum* sporozoites abolish the infectivity of these parasites (*7*). These monoclonal antibodies bind to a repeated epitope, which is common to different isolates of the parasite obtained from 11 areas in South East Asia, Central and South America, and West and East Africa (*8*). A prerequisite for the develop-

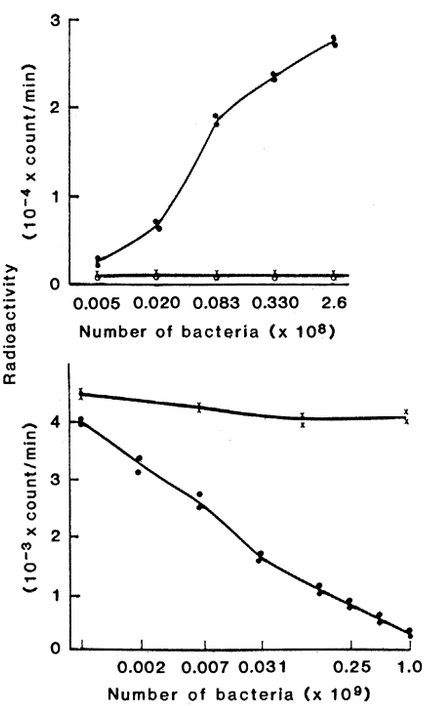

Fig. 1. (A). Two-site immunoradiometric assay. This assay was performed as described elsewhere (*17*). Briefly, wells of flexible microtiter plates (Dynatech) were coated with *P. falciparum* monoclonal antibody 2A10 to *P. falciparum*. After repeated washes with phosphate-buffered saline containing 1 percent bovine serum albumin, the wells were incubated with twofold serial dilutions of lysates of *E. coli* LE392 containing either ● p277-19 or (○) pBR322. After a 2-hour incubation at room temperature, the wells were washed and 30 μl of ^{125}I-labeled monoclonal antibody 2A10 (1×10^5 count/min) were added. After a 1-hour incubation at room temperature, the wells were washed, dried, and counted in a gamma counter. Lysates of *E. coli* LE392 containing plasmid p277-19 were also tested by using plates coated with either monoclonal antibody 2A10 or an unrelated ^{125}I-labeled monoclonal antibody (x). (B). Inhibition assay. Ten microliters (5×10^4 count/min) of ^{125}I-labeled monoclonal antibody 2A10 were incubated with 30 μl of twofold serial dilutions of lysates of *E. coli* LE392 containing either (●) p277-19 or (x) the pBR322 vector. After a 30-minute incubation at room temperature, 30 μl of these mixtures were transferred into microtiter plates that had been coated with an extract of *P. falciparum* sporozoites (*3*). After 1 hour of incubation, the wells were washed, dried, and counted in a gamma counter.