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20. By means of an air-pressure delivery system, 1% CTB dissolved in 0.9% saline (~1 nl) was injected stereotaxically through a glass micropipette into the TMN region of chloral hydrate–anesthetized rats (350 mg/kg). Coordinates from the Paxinos rat atlas were: AP, −4.0 mm (from bregma); DV, −9.0 mm (from cortical surface); and RL, +1.5 mm (from midline).
21. After processing preoptic tissue for FOS immunoreactivity to identify activated cells (Co^{2+}-enhanced DAB; black nuclei), and posterior hypothalamic tissue for adenosine deaminase immunoreactivity (1:50,000) to delineate the tuberomammillary nucleus for injection site localization (Co^{2+}-enhanced DAB; black cell bodies) [E. Senba *et al., J. Neurosci.* **5**, 3393 (1985)], sections were incubated for 18 hours at room temperature in goat anti-CTB primary antibody (List Biol. Labs, 1:100,000), followed by a 2-hour incubation in biotinylated donkey anti-goat secondary antibody (Jackson, 1:1000). An ABC kit (Vector Labs, 1:500) was used to visualize retrograde CTB labeling and CTB injection sites (DAB alone; brown cytoplasmic granules).
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28. We thank Q.-H. Ha and M. Magner for technical assistance, R. Burstein, J. Elmquist, T. Scammel, M. Greenberg, and J. A. Hobson for helpful discussions, and R. Kellems for ADA antiserum. Supported by USPHS grants NS22835, MH10709, and NS30140 and by a Grant-in-aid from the American Heart Association (94013110) and the Department of Veterans Affairs Medical Research Service.

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Plasmodium Hemozoin Formation Mediated by Histidine-Rich Proteins

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The digestive vacuole of *Plasmodium falciparum* is the site of hemoglobin degradation, heme polymerization into crystalline hemozoin, and antimalarial drug accumulation. Antibodies identified histidine-rich protein II (HRP II) in purified digestive vacuoles. Recombinant or native HRP II promoted the formation of hemozoin, and chloroquine inhibited the reaction. The related HRP III also polymerized heme, and an additional HRP was identified in vacuoles. It is proposed that after secretion by the parasite into the host erythrocyte cytosol, HRPs are brought into the acidic digestive vacuole along with hemoglobin. After hemoglobin proteolysis, HRPs bind the liberated heme and mediate hemozoin formation.

Plasmodium falciparum, the etiologic agent of severe malaria, ingests more than 75% of its host cell hemoglobin in a short period during the trophozoite stage of its intraerythrocytic cycle (*1*). Proteolysis occurs inside an acidified digestive vacuole where aspartic and cysteine proteases degrade the hemoglobin, releasing the toxic heme moiety (*2*). Lacking heme oxygenase, plasmodia detoxify heme by polymerization into an insoluble crystalline substance called hemozoin (*3*), in which the iron of one heme is coordinated to the propionate carboxylate group of the next heme (*4*). Hemozoin can be made in vitro by adding whole trophozoite lysate to acidified heme at pH 5 to 6 (*5*). The observed properties of this reaction suggested the existence of an enzymatic activity, but the identification and purification of the proposed enzyme (named heme polymerase) have remained elusive. Hemozoin appears to be structurally identical to β-hematin (*4*), which can form spontaneously under nonphysiological conditions without the addition of protein (*6*). Purified hemozoin devoid of proteins or β-hematin can seed the polymerization reaction under more physiological conditions (*7*); this finding raised the possibility that hemozoin formation may be a nonenzymatic process, but it shed no light on the mechanism of initiation of heme polymerization before the first crystals exist. Chloroquine, a mainstay of antimalarial therapy, inhibits hemozoin formation during chemical synthesis (*6*), hemozoin-initiated reaction (*7*), or trophozoite extract–mediated production (*5*). In vivo, the consequences of free heme accumulation during chloroquine treatment are membrane lysis (*8*) and inactivation of the hemoglobin-degrading proteases (*9*). This appears to be the mechanism of action of 4-aminoquinoline antimalarials (*5*, *10*).

When a ring-stage complementary DNA (cDNA) *P. falciparum* library was screened with rabbit antisera to purified digestive vacuoles or to hemozoin (*11*), 67 of 104 clones that were isolated encoded the sequence for HRP II. A specific monoclonal antibody (mAb) identified HRP II in purified digestive vacuoles by both protein immunoblot and immunofluorescence (Fig. 1, A and B). In contrast, neither preimmune sera nor antibody to knob-associated HRP I recognized the vacuoles. In whole intraerythrocytic trophozoites, the previously reported pattern of intense HRP II staining of the erythrocyte cytosol and paucity of staining in the trophozoite (*12*) was confirmed. However, staining could also be seen internal to the parasite, over the digestive vacuole (Fig. 1C). Thus, a substantial portion of the parasite's HRP II is internalized into the digestive vacuole. This new route contrasts with HRP II's previously established path of secretion into the bloodstream, which forms the basis of the recent ParaSight test that detects HRP II in lysed finger-prick samples (*13*).

HRP II contains 51 repeats of the tripeptide His-His-Ala (*14*); together, histidine and alanine make up 76% of the mature protein. Recent work with human histidine-rich glycoprotein (HRG) demonstrated the existence of a heme-binding site in the histidine-rich domain (*15*). Its heme-binding motif, Gly-His-His-Pro-His-Gly (*16*), has similarities to the repetitive HRP II sequence Ala-His-His-Ala-His-His-Ala-Ala-Asp (*14*). We hypothesized that HRP II could bind heme in the digestive vacuole and might play a role in hemozoin formation. To examine this hypothesis, we ligated a cDNA clone of HRP II in-frame into a pET 15b vector for *Escherichia coli* expression (*17*). The recombinant protein was collected by nickel chelation chromatography. Similarly, native HRP II was purified from *Plasmodium* culture supernates (*18*). Each molecule of HRP II bound an estimated 17 molecules of heme in 100 mM sodium acetate at pH 4.8 (Fig. 2A). In an in vitro

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heme polymerization assay, purified recombinant and native HRP II promoted synthesis of hemozoin, whereas other proteins such as bovine serum albumin (BSA) and lysozyme, or amino acid polymers such as polylysine and polyasparagine, did not (Fig. 2B). Human HRG also did not polymerize heme. Polyhistidine trapped heme as a red pellet and did not yield spectra consistent with hemozoin. Chloroquine inhibited the HRP II–mediated synthesis of hemozoin, consistent with the proposed action of this drug on heme polymerization (*5*, *8*, *10*). Product formation with HRP II increased with time, protein concentration, and initial heme concentration, reaching 20 times the base-line amount (Fig. 2C). The reaction rate was optimal around pH 4.0 and slowed near pH 6.0, close to the pK_a (where K_a is the acid constant) of the histidine imidazole group. HRP II could be inactivated by boiling, with a half-time ($t_{1/2}$) of ~25 min. The choice of buffer did not influence the reaction, but increasing the ionic strength accelerated the production of hemozoin. Fourier transform infrared spectroscopy (FTIR) of the HRP II reaction product (Fig. 3) showed characteristic absorption bands at 1660 and 1210 cm^{-1}, which are present in hemozoin or β-hematin but are absent in free heme (*4*).

HRP III is a homologous protein with 27 dihistidine repeats, mostly His-His-Ala but some His-His-Asp (*14*). Its hydrophobic leader peptide is 90% identical to that of HRP II. Wild isolates of *P. falciparum* that have been studied do not lack HRP II. Laboratory clones that have a subtelomeric deletion of HRP II retain HRP III (*19*, *20*). Native and recombinant forms of HRP III were also capable of binding and polymerizing heme in a chloroquine-inhibitable fashion (Fig. 2D). A single clone, 3B-D5, which was generated in a genetic cross, lacks both HRP II and III (*20*) but still makes hemozoin. This strain, like its parents, has a 10-kD protein in vacuole preparations, which reacted with HRP II mAb 2G12 in a protein immunoblot analysis (Fig. 4A). A candidate for this protein is HRP IV, an 84–amino acid molecule with a predicted molecular mass of 10.6 kD that contains 31% histidines (*21*). HRP IV is present in clone 3B-D5 and its parents, and it is expressed in intraerythrocytic stages (Fig. 4B). *Plasmodium falciparum* thus has redundant HRPs, at least two of which can mediate hemozoin formation. Redundancy is the rule rather than the exception for important *Plasmodium* functions that have been studied. Multiple proteins perform the same role in hemoglobin degradation (*2*), erythrocyte and hepatocyte invasion (*22*), and cytoadherence (*23*, *24*). The selective pressure for this phenomenon in vivo is unknown.

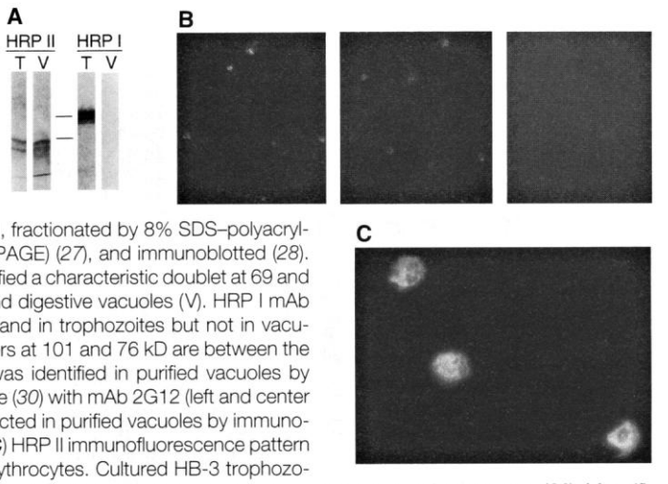

Fig. 1. HRP II is found in purified digestive vacuoles. (**A**) Protein immunoblot analysis. Purified digestive vacuoles (*26*) and mature trophozoites from *P. falciparum* clone HB-3 were counted by hemocytometer, and 10^6 of each were lysed, denatured, fractionated by 8% SDS–polyacrylamide gel electrophoresis (PAGE) (*27*), and immunoblotted (*28*). HRP II mAb 2G12 (*29*) identified a characteristic doublet at 69 and 72 kD in trophozoites (T) and digestive vacuoles (V). HRP I mAb 89 distinguished a 92-kD band in trophozoites but not in vacuoles. Molecular mass markers at 101 and 76 kD are between the pairs of lanes. (**B**) HRP II was identified in purified vacuoles by indirect immunofluorescence (*30*) with mAb 2G12 (left and center panels). HRP I was not detected in purified vacuoles by immunofluorescence (right panel). (**C**) HRP II immunofluorescence pattern in *P. falciparum*–infected erythrocytes. Cultured HB-3 trophozoites were fixed, incubated with mAb 2G12, and processed for indirect immunofluorescence (*30*). Magnifications in (B) and (C), ×600.

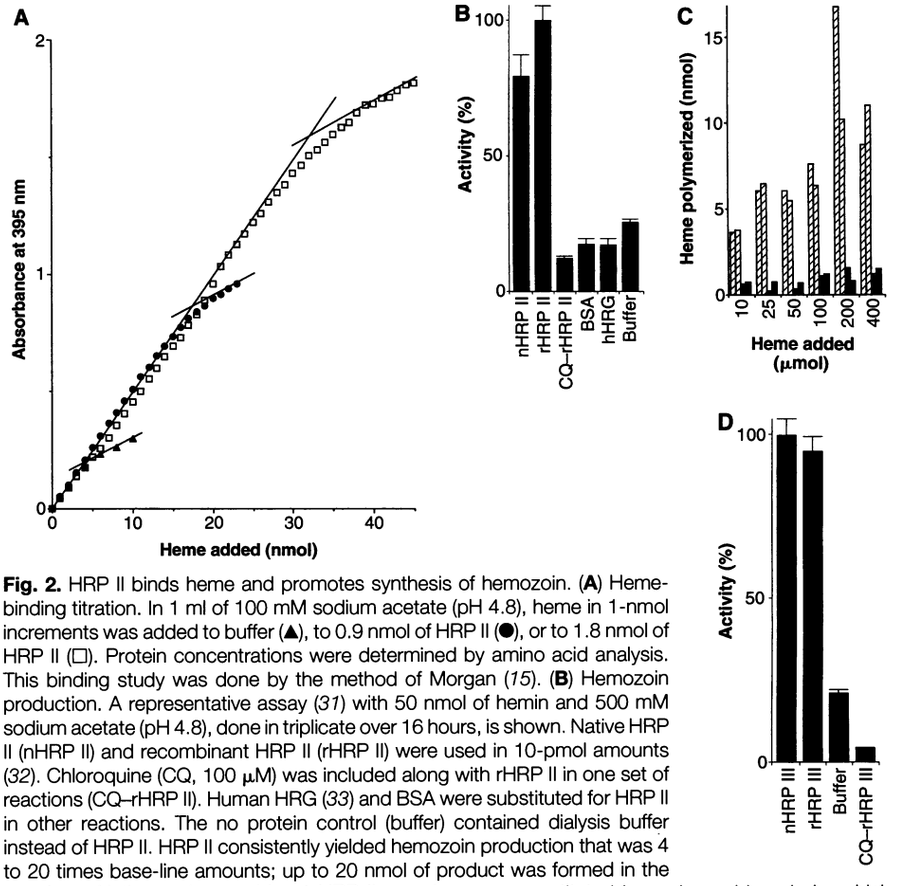

Fig. 2. HRP II binds heme and promotes synthesis of hemozoin. (**A**) Heme-binding titration. In 1 ml of 100 mM sodium acetate (pH 4.8), heme in 1-nmol increments was added to buffer (▲), to 0.9 nmol of HRP II (●), or to 1.8 nmol of HRP II (□). Protein concentrations were determined by amino acid analysis. This binding study was done by the method of Morgan (*15*). (**B**) Hemozoin production. A representative assay (*31*) with 50 nmol of hemin and 500 mM sodium acetate (pH 4.8), done in triplicate over 16 hours, is shown. Native HRP II (nHRP II) and recombinant HRP II (rHRP II) were used in 10-pmol amounts (*32*). Chloroquine (CQ, 100 μM) was included along with rHRP II in one set of reactions (CQ–rHRP II). Human HRG (*33*) and BSA were substituted for HRP II in other reactions. The no protein control (buffer) contained dialysis buffer instead of HRP II. HRP II consistently yielded hemozoin production that was 4 to 20 times base-line amounts; up to 20 nmol of product was formed in the reactions. Native and recombinant HRP II samples were quantitated by amino acid analysis, which indicated >90% purity. (**C**) Increase in product with increasing substrate. Increasing micromolar concentrations of heme were added to the reaction. Polymerized heme was quantitated as in (B). Striped bars represent two independent reactions with heme plus HRP II (450 pmol). Stippled bars represent two independent controls lacking HRP II. (**D**) Production of hemozoin with HRP III. Conditions and abbreviations are as in (B).

Hemozoin has long fascinated malariologists. It is present in crystals within the parasite digestive vacuole, where it appears to serve as a detoxified storage form for the heme released during hemoglobin catabolism (*3*). Because the hemozoin is scavenged by reticuloendothelial macrophages and appears to impair functions such as

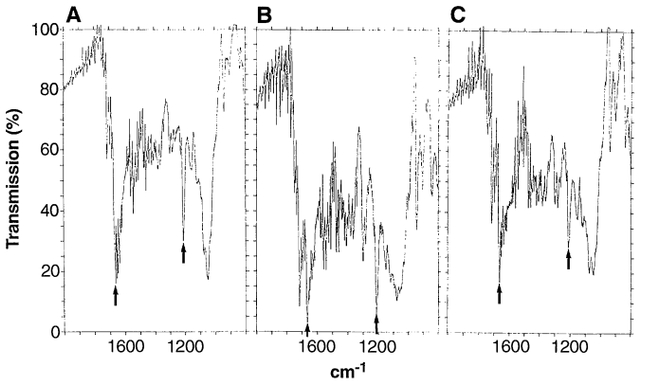

Fig. 3. FTIR spectra. (**A**) HRP II incubation product (*34*); (**B**) authentic parasite hemozoin; (**C**) synthetic β-hematin. The unique peaks at 1210 and 1660 cm^{-1} (*4*) are indicated by arrows.

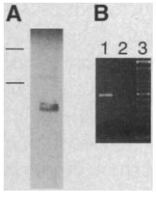

Fig. 4. (**A**) An antibody to HRP II (mAb 2G12) reacts with a 10-kD protein, found in the digestive vacuoles of clone 3B-D5, that lacks HRP II and HRP III. Protein immunoblot analysis was done as in Fig. 1, except that an 18% SDS-PAGE gel was used. Markers at 36 and 19 kD are indicated. The band visualized is the same as the fast-migrating band in Fig. 1A. (**B**) Polymerase chain reaction of reverse-transcribed mRNA from clone 3B-D5 with the use of oligonucleotide primers to HRP IV (*35*). Lane 1, reverse transcriptase added; lane 2, control (no reverse transcriptase); lane 3, 100-bp markers. A band at the predicted size of 680 bp is seen.

phagocytosis and oxidative burst (*25*), it may play a more active role after the parasite has burst out of its host erythrocyte. Hemozoin formation appears to be the target of numerous antimalarials, as first proposed by Fitch and co-workers (*10*) and later demonstrated by Slater and Cerami (*5*). Heme polymerization can be achieved in vitro under extreme conditions, such as incubation in 4.5 M acetate or heating at 70°C (*6*). In 0.5 M acetate at 37°C, the reaction was 50% complete after 7 days, which suggests that a mechanism is required to facilitate the in vivo reaction. A heme polymerization activity was detected in crude trophozoite extracts (*5*) but was not identified. Addition of preformed hemozoin can nucleate the production of further hemozoin (*7*), although this observation again indicated that a protein was needed to initiate the hemozoin formation.

Our findings that HRP II (and possibly HRP III and IV) exist in the digestive vacuole, and that HRP II and III are capable of mediating hemozoin formation, tie together the previous data in a physiological context. HRP II is secreted by the parasite into the erythrocyte cytosol (*12*); this would appear to be an unusual route for vacuolar targeting, as it would enable continuous delivery alongside its source of substrate, hemoglobin. We do not know whether HRPs function as enzymes by binding substrate and releasing product, or whether they play a nucleation role. Detailed kinetic analysis will be required to distinguish these possibilities. The reaction can polymerize at least 2000 hemes per molecule of HRP II; however, the fact that preformed hemozoin can promote further hemozoin synthesis (*7*) suggests that HRPs may be more important in the initiation of hemozoin polymers than in their extension. Whichever mechanism applies, we have identified a critical target for the development of new antimalarial agents.

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31. A 10 mM fresh stock solution of hemin (Sigma) in 0.1 N NaOH was prepared. Protein samples were assayed for hemozoin formation by incubation for 12 to 16 hours in a final volume of 1 ml with 500 mM sodium acetate (pH 4.8) and 50 nmol of hemin. After incubation, 10 μl of 10% SDS was added and the tubes were vortexed. After centrifugation at 15,000*g* for 1 hour, the pellets were resuspended in 1.5 ml of 2.5% SDS and 0.1 M sodium bicarbonate (pH 9.1). The SDS and sodium bicarbonate solubilized heme, whereas hemozoin remained insoluble. The pellet was sonicated on the lowest setting for 10 s, with care taken to sonicate the sides of the tube, where hemozoin is adherent. After 5 min, the suspension was vortexed and then centrifuged for 1 hour. The pellet was suspended in 1.5 ml of 2.5% SDS and sonicated as above. After 5 min, the tubes were vortexed and centrifuged at 15,000*g* for 1 hour. The remaining pellet was suspended in 1 ml of 2.5% SDS for qualitative spectral analysis. To quantitate, we added 20 mM NaOH and measured absorbance at 400 nm (*5*) on a Beckmann DU-64 spectrophotometer. A range of concentrations from 10 to 1000 pmol of alternative proteins [BSA and lysozyme, Boehringer-Mannheim; polyasparagine, polylysine, and polyhistidine, Sigma; human HRG, see (*33*)] was also assessed for hemozoin production.
32. A cDNA clone encoding HRP II was digested with Rsa I and Bam HI; the pET 15b vector (Novagen) was cut with Xho I [J. Sambrook, E. F. Frisch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. A blunt end ligation was performed after filling in bases with Klenow polymerase. The recombinant clone in pET 15b begins with amino acid 20 of HRP II to bypass the hydrophobic leader peptide. The plasmid was electroporated into *E. coli* expression host BI 21/DE3 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (*17*) for 6 hours. The pellet from 2 liters of induced culture of recombinant HRP II was sonicated in 20 mM tris (pH 8.0), 500 mM NaCl tris-saline (TS), and 50 mM imidazole. The soluble supernatant was filtered and then passed over 4 ml of an activated nickel column (Novagen) at 10 ml/hour. The column was washed with 10 bed volumes of 50 mM imidazole in TS followed by 8 bed volumes of 200 mM imidazole in TS. Recombinant HRP II was eluted with 1 M imidazole and dialyzed for 48 hours in sodium acetate (pH 4.8) with three to five changes of dialysate. Concentrated HRP II was insoluble in water or in 20 mM tris (pH 7). Recombinant HRP III was prepared in a similar fashion. To obtain native HRP II, we grew *P. falciparum* clone HB-3 in O^+ human erythrocytes at 2% hemocrit, using RPMI 1640 medium with 10% human serum [W. Trager and J. B. Jensen, *Science* **193**, 673 (1976)]. Extracellular medium was collected after 12 hours of incubation. Imidazole was added to a concentration of 50 mM, and the culture medium was processed over a 2-ml nickel column, as with recombinant HRP II. Native HRP III was obtained from infected red blood

cell lysates of clone Dd2 and processed as above, except that the nickel column was washed with 100 mM imidazole before elution.

33. Human HRG was extracted from serum by passage over a nickel column, as described (*18*). It was eluted with 200 mM imidazole and was dialyzed against water for 48 hours with three changes.
34. Recombinant HRP II (100 pmol) was incubated for 14 hours with 600 nmol of hemin in 500 mM sodium acetate (final volume, 12 ml). Insoluble material was collected by centrifugation as above, and was processed by washing in 2.5% SDS and 0.1 M sodium bicarbonate, then in 2.5% SDS, and finally three times in water. Isolation of parasite hemozoin and production of β-hematin were as described (*4*). After lyophilization, each pellet was placed on a sodium chloride disk, and data were acquired for five cycles on a FTIR spectrometer (Perkin-Elmer 1710).
35. RNA was isolated from *P. falciparum* clone 3B-D5 trophozoites [K. A. Creedon, P. K. Rathod, T. E. Wellems, *J. Biol. Chem.* **269**, 16364 (1994)] and was reverse-transcribed after extensive deoxyribonuclease digestion (*24*). The HRP IV–specific sequence GTAATAGTCCAAAAAGATATTG was used as a primer. This oligonucleotide and one corresponding to the downstream sequence CATCAAATTCTTCTAAGCC were used for polymerase chain reaction amplification; products were analyzed on a 1.3% agarose gel. A control incubation, taken through the same steps but omitting reverse transcriptase, was performed; a band of the predicted size was found from the reverse transcriptase incubation only. Similar reactions with nested primers also gave bands of the predicted size.
36. We thank D. Taylor for mAbs, T. Wellems for *Plasmodium* clones 3B-D5 and Dd2, D. Covey for help with FTIR analysis, T. Steinberg for fluorescence microscope use, K. Luker for advice on amino acid analysis, A. Oksman for parasite culture, and S. Francis and D. Minning for critical reading of the manuscript. Supported by NIH grant AI-31615. D.E.G. is a Charles E. Culpeper Medical Scholar.

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TECHNICAL COMMENTS

Polypropylene Tube Surfaces May Induce Denaturation and Multimerization of DNA

Claire Gaillard and François Strauss (*1*) found that DNA fragments containing the sequence $(CA)_{30}$ spontaneously associated in a series of products of reduced mobility, and that these products bond to the nuclear nonhistone high mobility group (HMG) proteins 1 and 2. We have obtained evidence strongly suggesting that this association is mediated by partial denaturation of the DNA resulting from interaction with certain polypropylene tube surfaces. This phenomenon has implications for the possible role of surfaces (including cellular surfaces) in affecting the conformation of DNA and should be taken into consideration by investigators conducting experiments that are sensitive to low levels of denaturation.

We incubated a ^{32}P-labeled 124-bp fragment of DNA containing the sequence $(CA)_{30}{\cdot}(GT)_{30}$(fCA30) (*2*) in ordinary polypropylene (pp) microfuge tubes (*3*) and produced a ladder of apparently multimeric complexes as observed by Gaillard and Strauss; however, no ladder was seen when the incubation was carried out in borosilicate glass tubes (*3*) or in pp tubes that have been commercially coated to have low-binding properties ("lube" tubes; Marsh Biomedical Products, Rochester, New York). Moreover, the association was inhibited when an excess amount of a single-stranded oligonucleotide (lacking any significant homology to the DNA fragment) was present in the solution before adding the fragment.

To determine whether the association actually required the presence of a pp surface, as opposed to its being a solution phenomenon that was simply inhibited by contaminants released from the glass and lube tube surfaces, we preincubated fCA30 in a glass tube for several hours, then transferred the DNA to a pp tube and continued the incubation for another several hours. If the glass emitted some diffusible inhibitor of the reaction, there should have been no association after transferring to the pp tube. However, association did in fact occur after transfer, indicating that the pp surface was required. Because single-stranded DNA binds more tightly to many surfaces than does double-stranded DNA, it is likely that the inhibitory effect of the oligonucleotide results from its binding to putative sites on the pp surface that mediate the association. Similar experiments involving transfer from lube tubes into pp tubes did not result in association, indicating that a diffusible inhibitor *was* present in the lube tubes. It is likely that, on transferring the solution, this inhibitor also prevents association by binding to active sites on the pp surface. Thus, it is important to avoid the use of lube tubes for manipulating or storing DNA in order to observe this phenomenon. To minimize background levels of denaturation and complexation while using pp tubes, it is best to store DNA samples in the frozen state and avoid small volumes that increase the ratio of pp surface to sample volume.

Parallel experiments were performed using a 62-bp control fragment (fC) lacking any long repeats and another, approximately 120-bp fragment (fGA37) (*4*) containing an internal $(GA)_{37}$ repeat instead of a CA repeat. Multimerization was observed with fGA37, but not with fC.

When various surfaces and inhibitors were used, the same conditions that led to multimerization of fCA30 and fGA37 also led to the appearance, in the case of fC and fGA37, of a faster migrating fraction that comigrated with the denatured fragment. (In the case of fCA30, the denatured fragment was poorly resolved from the duplex under our conditions.) When fCA30 was deliberately subjected to alkaline denaturation followed by renaturation (performed in lube tubes, or in the presence of an excess of oligonucleotide to disallow the pp-dependent association, or in both) the resulting pattern of products was similar to that seen for spontaneous association in pp tubes.

The simplest explanation of these results is that the formation of slower migrating products is mediated by partial or complete denaturation followed by misaligned annealing at the repetitive sequence. Further intermolecular pairing of single-stranded ends could lead to multimeric branched structures, which would be expected to bind HMG1 and HMG2, as observed by Gaillard and Strauss, because those proteins are known to bind to branched or distorted DNAs (*5*).

Because we observed little or no denaturation or multimerization using tubes from one supplier (*3*), it may be that minor constituents of the plastic that vary with manufacturing procedures contribute to the surface activity. A fuller account and discussion of these results will be presented elsewhere (*6*).

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REFERENCES AND NOTES

1. C. Gaillard and F. Strauss, *Science* **264**, 433 (1994).
2. Fragment fCA30 was cut from a plasmid kindly provided by F. Strauss.