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DIAGNOSIS OF DRUG RESISTANT *PLASMODIUM FALCIPARUM* BY DNA PROBES

Principal Investigator: Yaakov Pollack, Ph.D.
Grantee Institution: Ben-Gurion University
Collaborator: Michael Alpers, Ph.D.
Institution: Papua New Guinea Institute of Medical Research

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EXECUTIVE SUMMARY

The purpose of this project was develop DNA probes for analyzing drug resistant malaria infections. Within this framework, it was also intended to acquaint the staff of the Papua New Guinea Institute of Medical Research with the DNA technologies involved in diagnosing resistant and nonresistant malaria cases, as well as the application of these techniques on Papua New-Guinea (PNG) field isolates.

The work was initiated with two defined probes which were used to assess the relationship between two biological phenomena and drug resistance. This approach yielded some novel interesting results concerning the biology of the parasite (see appendix 2) but was found unsuitable for the purpose of drug resistance diagnosis. This and new findings concerning drug resistance, especially that of chloroquine, initiated the second stage of the project which was aimed at studying the involvement of a new protein molecule (metallothionein) in drug resistance. The rational of this approach lay in findings originating from other biological systems which indicated that this molecule may be involved in some forms of drug resistance. This approach yielded interesting novel findings including the proof that *Plasmodium falciparum* contains gene(s) coding for this protein. The most exciting finding, however, is the fact that we have proved that this molecule is involved in protecting the parasite from the damage caused by oxidative stress, which is the basis of the therapeutic effects of some antimalarials (primaquine, artemisinine and in part of that of chloroquine). These findings open new avenues in understanding the mode of action of some antimalarials, drug resistance, and designing new natimalarials. This study is still continuing and we hope that in the near future we will be able to use the gene coding for this protein as a probe for malaria and drug resistance diagnoses.

Parallel to this study the technology of diagnosing malaria and malaria drug resistance was introduced into the Papua New Guinea Institute of Medical Research. This was achieved by reciprocal visits of both collaborating groups in Papua New Guinea and

Israel. The basic techniques studied and applied are isolation of DNA, restriction enzyme analysis, Southern blotting, DNA hybridization, labeling of probes, and PCR (polymerase chain reaction).

During the period of the project, samples of infected blood were collected from different areas in PNG (see appendix 1). These samples were tested for the species of the parasite as well as its sensitivity to chloroquine and, in a smaller number of cases, to pyrimethemaine. DNA was prepared from the samples according to the procedure practiced in the first stage of the project. These samples are now ready for diagnosis, using hybridization and/or PCR. This will be done as soon as the appropriate probe will be found. As mentioned earlier, the gene coding for metallothionein may turn out to be a good candidate for that purpose.

In conclusion, while the study of some issues raised in this project continues, the major objectives put forward were achieved. Thus, members of the Papua New Guinea Institute of Medicine are fully acquainted with the techniques needed for diagnosis of malaria and malaria drug resistance. The development of appropriate probe(s) for that purpose, although not yet finalized, is at a very advanced stage.

RESEARCH OBJECTIVES

Malaria is diagnosed today in the same manner in which the causing agent of the disease—*Plasmodium falciparum*—was discovered over one hundred years ago, namely, by examination of blood smears under the light microscope. This is a very simple and easy-to-operate assay which can be operated relatively easily under field conditions. However, in spite of this, it turns out that in endemic areas where large numbers of samples have to be processed, this assay turned out to be much less accurate than expected, as reflected by the high percentage of false positive results. This initiated the search for a more reliable diagnostic assay. The need for a different diagnostic assay was augmented by the rapid spread of drug resistant parasites which necessitated, in addition to the identification of the parasite itself, also assessment of its sensitivity to antimalarial drugs. This, obviously, could not be achieved by light microscopy. We, as well as others (1-7), had shown in the mid-1980s that DNA probes can be used for diagnosing malaria. The objective of this

project was to develop DNA probes for diagnosing drug resistance. The project was planned as a collaborative effort in which the Israeli group would concentrate on the development of the probe, whereas the Papua New Guinea side would be involved in collecting *P. falciparum* isolates, assessment of drug sensitivities of parasites, preparation of DNA from selected isolates, and analysis of these DNA samples, using DNA probes by techniques introduced to PNG by the Israeli group.

The part of the project which dealt with the development of DNA probes was supported by both the Israel Academy of Sciences and Humanities and the Israeli Ministry of Health.

METHODS AND RESULTS

Development of probes for drug resistance

Two markers were initially studied as candidates for diagnosing drug resistance. These were, a) Methylation pattern, and b) RFLP sites. These markers were studied using two probes: BS11, a repetitive sequence (8) and pI IBE6.1, a clone containing 5' upstream sequences and part of the DI IFR gene. The results of this approach were summarized in a paper (see appendix 2). Briefly, in this approach we were able to discover that the genome of *Plasmodium falciparum* contains a vertebrate methylation pattern, namely, only cytosine residues were found to be methylated and only those preceding guanine residues.

This approach failed however to show a correlation between the two markers and drug resistance. Thus another approach was undertaken. The rapid development in the field of chloroquine resistance had influenced the nature of this approach. Foote et al. (9) and Wilson et al. (10) reported the amplification of a plasmodial mdr gene (pfmdr) in some resistant isolates, implicating this gene in chloroquine resistance. However, Wellems et al. (11) could not find a linkage between this gene and the resistant phenotype in genetic crosses. To add to the complexity, Foote et al. (12) were able to identify correctly 34 out of 36 drug resistant isolates using point mutations in the pfmdr as markers, thus indicating that this gene is involved somehow in drug resistance. It was accepted then (as it still is today) that an additional gene is involved in this resistance. The gene we thought might be involved is the one coding for metallothionein. The rationale for that relies on the one hand on some properties of metallothionein and on the other hand on some chemical properties of several antimalarial drugs. Metallothioneins

are low molecular weight, cystein rich (33 mole %) proteins found abundantly in lower and higher eukaryotes (for a review see 13). One of the cellular functions attributed to metallothionein is protecting the cell from the damage caused by reactive oxygen species (ROS) and especially from hydroxyl free radicals (14). It is this property which interested us regarding drug resistance, as it is suggested that some antimalarial drugs may confer their therapeutic effect by enhancing ROS production, e.g. artemisinin (15,16), primaquine (17,18), quinine and chloroquine (Profs. M. Chevion and J. Golenser, Hebrew University, personal communication). The ability of the metallothionein gene to be induced or amplified under various stress conditions is well documented (13) and in several cases it was shown to be the reason for the resistance against several drugs. We therefore decided to verify whether *Plasmodium falciparum* contains genes coding for metallothionein, and if found, the way they are involved in the action/resistance of antimalarial drugs. Presented below are the results obtain from this approach.

Studies at the DNA/RNA levels

To verify whether the plasmodial genome contains MT gene(s), PCR was applied. Using two oligomers corresponding to conserved regions of the vertebrate MT genes (amino acids 1–7 and 43–53), two sequences were found to be amplified (Fig. 1, lane 1). No amplification was observed in the absence of DNA (lane 3) or in the presence of human DNA (lane 2), which is the most likely candidate to contaminate *P. falciparum* DNA preparations.

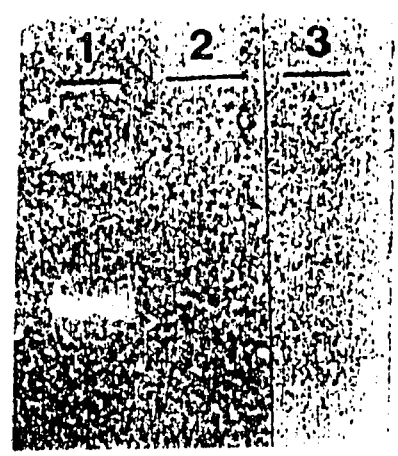

Fig. 1. PCR analysis of *P. falciparum* DNA. Two oligomers corresponding to amino acids 1-7 and 43-53 of MT which are conserved through evolution were used. 1–*P. falciparum* DNA; 2–human DNA; 3–No DNA.

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Fig. 2. Mucleotide sequence of PCR fragments obtained by metallothioneon primers.

No 121

```
          10        20        30        40        50        60        70
5' ATGGATCCTAACTCCTCATGCAGTGTGAAACAACATCAAAATCTATTTAATCAAAATATAAAGTATAAAAATTTT
3' TACCTAGGATTGACGAGTACGTCACACTTTCTTGTACTTTTACATAAATTAGTTTTATATTTCATATTTTTAAAA
     80        90       100       110       120       130       140       150
TTAAATAATAAATATAATACATTTGATAATAAGGAAGTACCTATAAATGAATATCGTTTCTTGGAGAGTCAACGA
AATTTATTATTTATATTATGTAAACTATTATTCCTTCATCCATATTTACTTATAGCAAAGAACCTCTCAGTTGCT
        160       170       180       190       200       210       220
ACAAACGAAGATAACCAAATATATAAAAATAGTGATAAGCTTATCTATGAAAATGTTTATGATGAGAGTTTTATA
TGTTTGCTTCTATTGCTTTATATATTTTTATCACTATTCCAATAGATACTTTTACAAATACTACTCTCAAAATAT
    230       240       250       260       270       280       290       300
TTTGATCGTAAAGTTTGCACTAATAATACCTGCGTGTGTATTATTAATCAGAGTAGTAATAGTGATAGTAGGAAA
AAACTAGCATTTCAAACGTCATTATTATGCACGCACACATAATAATTAGTCTCATCATTATCACTATCATCCTTT
        310       320       330       340       350       360       370
TTAAATTGTTTTATAAAAAATGAAATATATGATTTGGGATGTAACCAAAGGAGAGAAGCAAATCGGAATAGTGAA
AATTTAACAAAATATTTTTTACTTTATATACTAAACCCTACATTGGTTTCCTCTCTTCGTTTAGCCTTATCACTT
    380       390       400       410       420       430       440       450
AGATCATATGGTATAATGTATAATGAAAATGATATGAAAATTAATTTGGGGATGTTATTAAGAAATGATATATCA
TCTAGTATACCATATTACATATTACTTTTACTATACTTTTAATTAAACCCCTACAATAATTCTTTACTATATAGT
        460       470       480       490       500
CTATTTAATAGGAGTAGAAATGTGCACAAGGATGTGTATGTAAAGGAGCA 3'
GATAAATTATCCTCATCTTTACACGTGTTCCTACACATACATTTCCTCGT 5'
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No 89, No 117, No 136, No 162

```
          10        20        30        40        50        60        70
5' ATGGATCCTAATTGCTCATGTTGGTCGGTGTACTGAAGGTTTTGTCGCTCAAAAGGACACTACAATTGATAAATT
3' TACCTAGGATTAACGAGTACAACCAGCCACATGACTTCCAAAACAGCCACTTTTCCTGTGATGTTAACTATTTAA
     80        90       100       110       120       130       140       150
TCTCGAAAAGGAATTACAGGAAGCCGAAAAATGCCTACAAACCCACAAAGAAAAATGCGAAGAACCACCAAAACC
AGAGCTTTTCCTTAATGTCCTTCGGCTTTTTACGGATCTTTGGGTGTTTCTTTTTACGCTTCTTGGTGGTTTTGG
        160       170       180       190       200       210       220
AAAACCCGCCGATAAAGGTGCCGCTGGAGAAAGTCGTGGTCGTTCGGACATCGGCTCACCCGACATCATAATCCC
TTTTGGGCGGCTATTTCCACGGCGACCTCTTTCAGCACCAGCAAGCCTGTAGCCGAGTGGGCTGTAGTATTAGGG
    230       240       250       260       270       280       290       300
ACGTGCTGATGACAACGATAACGGCTCAGGTAGCGATTCCGAAGGCTCAGAAGACGATGACGAAGAAGAAGATGA
TGCACGACTACTGTTGCTATTGGCGAGTCCATCGCTAAGGCTTCCGAGTCTTCTGCTACTGCTTCTTCTTCTACT
        310       320       330       340       350       360       370
CGACGAAGACGGTGAAGGCGACCACCAACAACAGCAGGAAGAGGAGGAAGAGGAAAGGGCAAAGGAGACCACACA
GCTGCTTCTGCCACTTCCGCTGGTGGTTGTTGTCGTCCTTCTCCTCCTTCTCCTTTCCCGTTTCCTCTGGTGTGT
    380       390       400       410       420       430       440       450
GATAACAAAAGAAGACGGGGTGAAGGCATGTGACATAGTGGAAACTATTTAAAGATGGCAGTAGTGTCCAAGA
CTATTGTTTTCTTCTGCCCCACTTCCGTACACTGTATCACCTTTGTGATAAATTTCTACCGTCATCAGAGGTTCT
        460       470       480       490       500       510       520
TGCGTGTCGCCAAAAATATGGCGGAAACAACTCACGTTTAGGTTGGAAATGTGCACAAGGATGTGTATGTAAAGG
ACGCACACGGGTTTTTATACCGCCTTTGTTGAGTGCAAATCCAACCTTTACACGTGTTCCTACACATACATTTCC
    530
AGCAC 3'
TCGTG 5'
```

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Cloning of the smaller (~500 bp) amplified fragment in pUC118 yielded after DNA sequencing, two different clones (500 bp and 530 bp), showing high homology at certain regions and a lower homology at other regions (Fig. 2). Using these two clones as probes in a restriction enzyme - Southern blot analysis of *P. falciparum* DNA yielded positive results (Fig. 3), confirming that these sequences reside in the plasmodial genome.

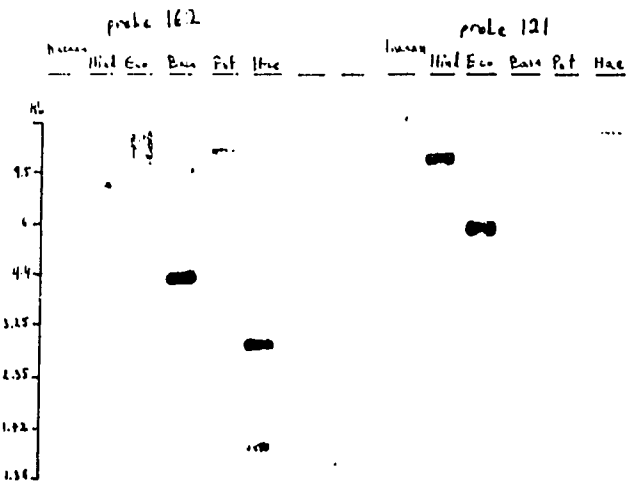

Fig. 3. Restriction analysis of *P. falciparum* DNA using cloned MT fragments.

1µg parasite DNA was digested with restriction enzyme Hind III (Hind); EcoRI (Eco); BamHI (Bam); Pst I (Pst) and HaeIII (Hae). Human DNA served as a control (Human). Two clones (162, 121) used as probes, originated from the 500 bp amplified PCR fragment (see Fig. 1), λ-size marker.

Both sequences were also expressed at the RNA level.

Fig. 4 shows the results obtained in a Northern blot analysis using the 530 pb clone as a probe. A signal is observed in lane 1 which contained plasmodial RNA, but no signal was observed with human RNA (lane 2).

1 2

Fig. 4. Northern blot analysis.

RNA was prepared from unsynchronized cultures.

1-Parasite RNA; 2-human RNA.

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Another approach for verifying the existence of MT sequences in the plasmodial genome was to use the reverse transcriptase-PCR (RT-PCR) system. The results of such an approach are shown in Fig. 5. Using either oligo $dT_{(15)}$ or a 43bp oligomer (corresponding to amino acids 43 to 53 of the protein and identical to the one used in the PCR in Fig. 1) as the first primer for RT, resulted in the amplification of a sequence (lanes 1 and 2, respectively), identical in size to the one obtained when either DNA from a λ gt10 genomic library of *P. falciparum* (lane 4) or *P. falciparum* genomic DNA (lane 5) were used. No amplification was observed in the absence of RT (lane 3).

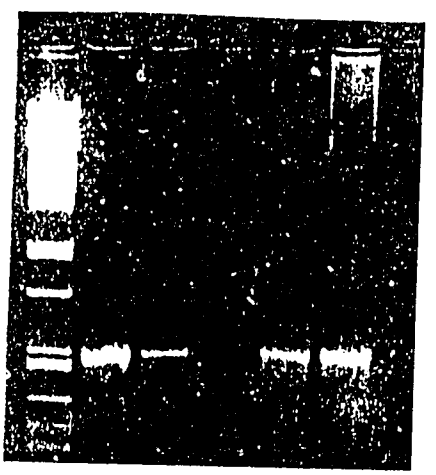

Fig. 5. RT-PCR of *P. falciparum* RNA.
P. falciparium RNA was reverse transcribed with oligod (T)15 (lane 1), MT corresponding oligomer (lane 2) followed by PCR with the appropriate MT oligomers. Lanes 3-5 are PCR controls of *P. falciparum* RNA in the absence of RT, λgt10 genomic *P. falciparum* DNA and *P. falciparum* genomic DNA, respectively.

Studies at the protein level

The ability of MT to bind cadmium (Cd) serves as a simple assay for determining its presence in biological samples. Table 1 summarizes an experiment aimed to verify whether or not *P. falciparum* extracts contain Cd binding proteins. As can be seen, the *P. falciparum* extract exhibits a 4 to 5 fold increase in Cd binding capacity compared to background levels (= no extract or extract prepared from non infected RBC). Moreover, if the extract was prepared from parasites cultivated for 24 hrs in the presence of 1μM Cd, there was a 40% increase in this capacity (even without taking into consideration the fact that only one third of the parasites survived under these conditions). This result is in accordance with the known inductive effect Cd has on MT expression.

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Extract	%Survival	Cadmium bound (p moles)
-	-	6.00
non-infected RBC	-	5.63
free parasites	100	25.70
free parasites grown in 1μM cadmium	30	36.50

Table. 1: Cadmium binding capacity of *P. falciparum* extract. Extracts were prepared from uninfected RBC and free parasites grown in the absence and 1 μM cadmium . MT was determined by a modified Cd - heme radioassay.

A Western blot analysis of *P. falciparum* extracts, also confirmed the previous finding (Fig. 6). Thus, *P. falciparum* extracts (lanes 1-5) contain Cd binding proteins migrating similarily to those of rabbit MT (MT). Only a very low level of Cd binding was observed in the region where haemoglobin, a probable contaminant of *P. falciparum* extracts, migrates (Hb).

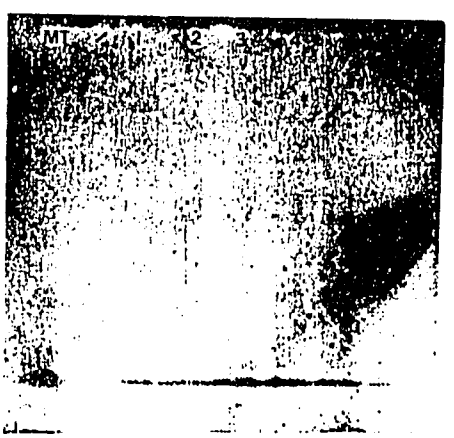

Fig. 6. Western blot analysis of *P. falciparum* extracts for the presence of MT.

Lanes 1-5: increasing quantities of *P. falciparum* extracts. MT = rabbit metallothionein; Hb = human Haemoglobin.

Physiological experiments

As mentioned previously, cells which over produce MT exhibit Cd resistance and in several cases, also show an increase in resistance to oxidative stress. To study whether such phenomena also occur in the parasite, we selected for a Cd resistant *P. falciparum* strain by culturing the parasites in the presence of increasing concentrations of Cd.

Starting at a concentration of 5nM, we now have a strain which grows successfully at 500 nM. Fig. 7 shows the growth curves (as judged by H^3 hypoxanthine incorporation) of both resistant and sensitive lines in the presence of 500nM Cd. As can be seen, the growth of the sensitive line is strongly inhibited, while the growth of the resistant line was not affected.

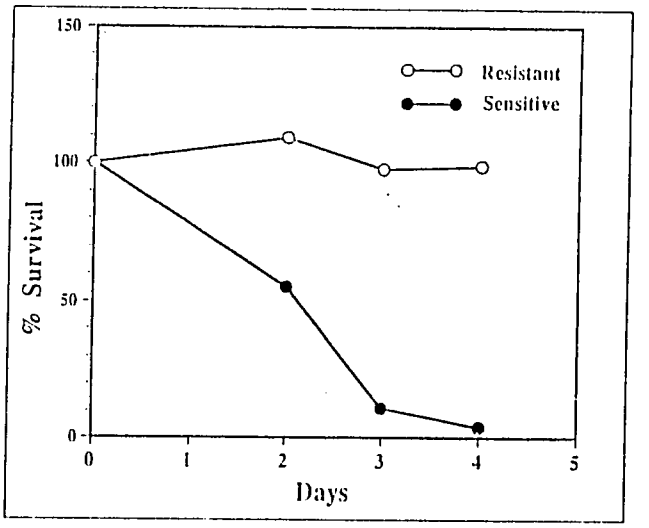

Fig. 7. Effect of Cd on the growth of Cd sensitive and Cd resistance *P. falciparum*.
The Cd resistant line originates from the sensitive line which was cultured in the presence of increasing concentrations of Cd. 500 nM Cd was used in this experiment. Results are expressed as % of control in the absence of Cd.

When the capacity of the resistant line to cope with oxidative stress was measured by culturing in the presence of primaquine, it was found (Fig. 8) that the growth of the resistant line was not affected at 10^{-5} and 10^{-6}M drug concentration, whereas the growth of the sensitive line was dramatically inhibited. Thus, Cd resistance also confers oxidative stress resistance in *P. falciparum*.

In conclusion, the results presented above strongly support the notion that MT exists in *P. falciparum* and participates in its protection from oxidative stress.

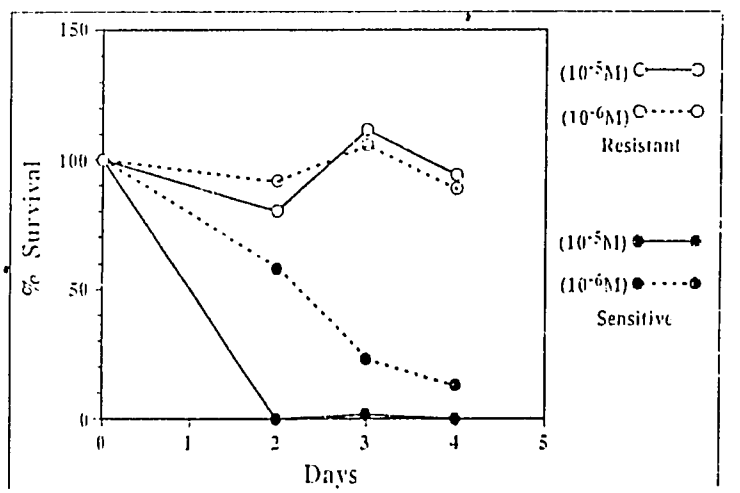

Fig. 8. Effect of primaquine on the growth of Cd sensitive and Cd resistant *P. falciparum*.
The same two lines described in Fig. 7 were used in this experiment. 10^{-5}M and 10^{-6}M primaquine were used. No cadmium was added. Results are expressed as % of control in the absence of primaquine.

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Analysis of PNG isolates

The introduction of the techniques required for this objective was achieved by mutual visits in PNG and Israel of members of both collaborating groups. Detailed protocols of all the techniques needed for this objective were written and practiced. These include DNA isolation, Southern blot analysis, DNA hybridization, dot blot hybridization, labeling of probes, and PCR analysis.

In parallel, infected blood samples from different regions of PNG (see appendix 1 for a map) were collected and assessed for chloroquine and pyrimethamine sensitivity. DNA was prepared from selected samples. These DNA samples await the final diagnosis with the appropriate probe, which we believe will be the gene coding for metallothionein.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

Although malaria and drug resistant malaria are still diagnosed worldwide by light microscopy and by *in vitro* culturing, respectively, we have no doubt that in the near future they will be replaced, in endemic areas, by DNA-based techniques. The reason for this is that these techniques are faster and more accurate, can handle larger numbers of samples, and more than one parameter can be analyzed in a single assay (19,20). It is therefore of great importance that the health authorities and institutions in malaria-endemic areas, who are responsible for malaria health and control programs, will acquire the knowhow of these technologies. This was one of the objectives of this project, and it was fulfilled. The PNG Institute of Medical Research now has the capability of conducting malaria diagnosis using DNA based techniques.

Our approach yielded interesting results also regarding the development of suitable probe(s). However, these are still to be finalized, but it seems to us that an additional, new route in the understanding and diagnosing of drug resistance has been opened.

PROJECT ACTIVITIES/OUTPUTS

Publications

1. Pollack, Y., Kogan, N., Golenser, J. (1991) Expt. Parasitol. 72:334.

Special Seminars

1. Molecular and Cellular Parasitology Research Group, Dept. of Biology, Imperial College, U.K., Sept. 1992.

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2. Division of Biological Science, Institute of Cell, Animal and Population Biology, Univ. of Edinburgh, Sept. 1992.

Congresses and Meetings

1. Israel Society of Parasitology & Protozoology Annual Meeting, May, 1994.
2. Sixth G.I.F. Meeting on Host-Pathogen Interaction—A multisystem approach. May 29–June 2, 1994. Greifswald, Republic of Germany.
3. VIII International Congress of Parasitology, October 10–14, 1994, Izmir, Turkey.
4. Israel Society of Microbiology, Annual Meeting, Feb. 6–7, 1995.

PROJECT PRODUCTIVITY

While some of the objectives are still not finalized, we believe that the basic goals were achieved. Thus, transfer of technology regarding DNA based diagnostic techniques was made. Collection of infected blood sample from different regions of PNG and assessment of drug sensitivity was achieved as well. The development of a suitable probe for diagnosing drug resistance is in an advanced stage. It is worthwhile noting that as of now the exact nature of chloroquine resistance is still not clear, in spite of the great efforts made in many laboratories around the world.

FUTURE WORK

The characterization of the plasmodial metallothionein gene(s) continues and includes physical characterization of the gene, its protein product and its physiological role. The data obtained will be used to construct a probe which will be used for diagnosing the PNG isolates.

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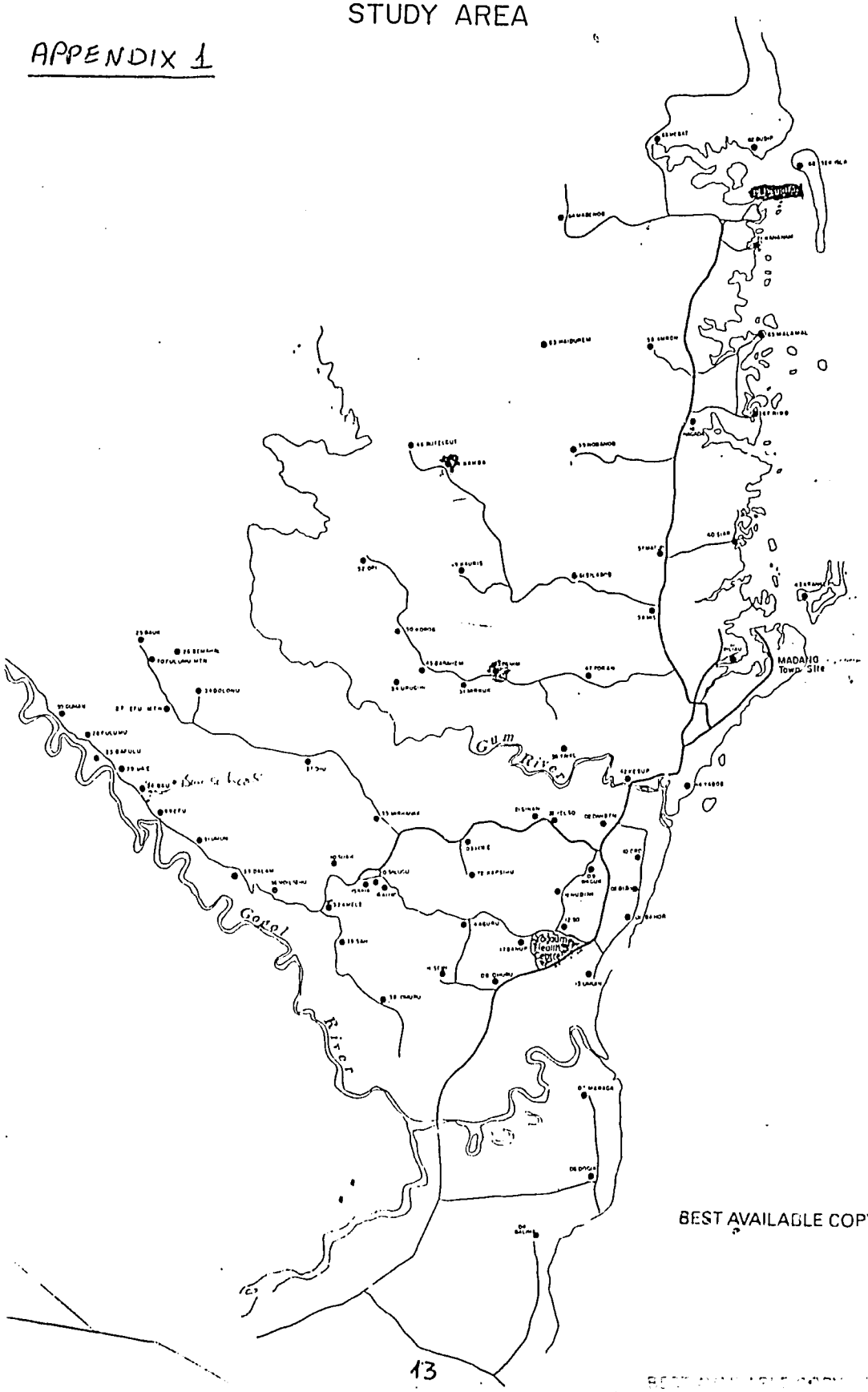
MALARIA RESEARCH PROGRAM
STUDY AREA

APPENDIX 1



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APPENDIX 2

EXPERIMENTAL PARASITOLOGY 72, 339–344 (1991)

Plasmodium falciparum: Evidence for a DNA Methylation Pattern

YAAKOV POLLACK,* NINA KOGAN,* AND JACOB GOLENSER†

*Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel; and †Department of Parasitology, The S.F. Kuvin Center for the Study of Infectious and Tropical Diseases, The Hebrew University Hadassah Medical School, Jerusalem, Israel

POLLACK, Y., KOGAN, N., AND GOLENSER, J. 1991. *Plasmodium falciparum:* Evidence for a DNA methylation pattern. *Experimental Parasitology* 72, 339–344. © 1991 Academic Press, Inc.

The methylation status of the adenine and cytosine residues in the genome of *Plasmodium falciparum* was studied using restriction enzymes exhibiting differential activity dependent on the methylation state of these residues in their recognition site. The gene coding for the enzyme dihydrofolate reductase–thymidylate synthase was studied for that purpose. No methylated adenine residues were observed in this gene in four strains tested. However, partial methylation of cytosine residues was observed in all strains. This methylation occurred at a specific site of the gene and was of the eukaryotic type, namely at a CpG sequence. © 1991 Academic Press, Inc.

INDEX DESCRIPTIONS AND ABBREVIATIONS: *Plasmodium falciparum;* DNA methylation; 6-Methyladenine; 5-Methylcytosine; DHFR-TS gene; Dihydrofolate reductase–thymidylate synthase (DHFR-TS).

INTRODUCTION

Modified bases occur, with very few exceptions, in the DNA of all prokaryotes and eukaryotes (Razin and Friedman 1981). In *Escherichia coli* DNA, both 6-methyladenine and 5-methylcytosine are found (Doskocil and Sormova 1965) in GATC and CCA/TGG sequences, respectively (Razin *et al.* 1980). Methylated adenine residues were also observed in several lower eukaryotic protozoans (Ray and Steele 1978). In higher eukaryotes, 5-methylcytosine is the only methylated base (Wyatt 1951) and is present only in the dinucleotide CpG (Sinshimer 1955). There is ample evidence suggesting that methylation plays a significant role in gene expression (Razin *et al.* 1984; Ceder 1988). Very little is known about methylation of the plasmodial genome. Previously, we reported the lack of both 6-methyladenine and 5-methylcytosine (Pollack *et al.* 1982). This conclusion was based on HPLC (high performance liquid chromatography) analysis, as at that time, specific DNA probes were not available. Taking into consideration the extremely low level of the G + C content of the parasite's DNA (18%), (Pollack *et al.* 1982), and the fact that there is a 50% underrepresentation of the dinucleotide CpG in the genome (Hyde and Sims 1987; Weber 1987), HPLC may not be sensitive enough to detect low levels of methylation. Here we describe the results of a restriction enzyme analysis of the methylation status of cytosine and adenine in the DHFR-TS gene of *P. falciparum*.

MATERIALS AND METHODS

P. falciparum cultures. P. falciparum strains FCR-3, HG-2G1, and D6 were cultured according to Trager and Jensen (1976) in tissue culture flasks in RPMI 1640 medium (GIBCO), supplemented with 10% human serum (blood group AB), 25 m*M* Hepes buffer, pH 7.3, 0.2% $NaHCO_3$, and 50 μg/ml gentamycin. Normal blood group 0 human erythrocytes were added to a final concentration of 3%. Growth was initiated by inoculation of parasitized erythrocytes to a final 1% parasitaemia. The medium was changed daily for 4–5 days, at which time parasites were harvested.

DNA preparation. DNA was prepared from free parasites as described by us previously (Pollack *et al.* 1982). DNA from the 3A-B4 strain was kindly provided by Dr. T. E. Wellems.

Probes. pHBE 6.1, kindly provided by Dr. T. E.

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Wellems, contains the first 1421 nucleotides of the plasmodial DHFR-TS gene and an approximately 2.3-kbp fragment upstream to the 5' end of the gene (see Fig. 1).

Hybridization. Restriction conditions, Southern blotting, P^{32}-labeling of probes, and hybridization conditions were as described previously (Pollack *et al.* 1984).

RESULTS

The most commonly used restriction enzymes for the detection of methylated cytosines in eukaryotic DNA are *Msp*I and *Hpa*II. Both recognize the sequence CCGG; however, while *Msp*I is indifferent to the state of methylation of the internal cytosine residue, *Hpa*II will not cleave a methylated site. Thus, a comparative analysis of DNA digested with these enzymes can reveal the existance of methylated cytosine residues within the region of DNA tested. In the case of *P. falciparum*, this approach may be less informative due to its very low (18%) G + C content (Pollack *et al.* 1982) and to the underrepresentation of the dinucleotide CpG (which is part of the recognition sequence of these enzymes) in its genome (Hyde and Sims 1987; Weber 1987). This assumption was confirmed when identical restriction patterns were obtained in a *Msp*I/*Hpa*II analysis of the DHFR-TS gene and its neighboring regions (data not shown). It was also evident that these enzymes hardly cut the DNA, yielding high molecular weight fragments which were poorly separated on the gel, thus preventing us from reaching a definite conclusion. To overcome this problem, other restriction enzymes exhibiting differential sensitivity to cytosine methylation, were used. *Mbo*I, *Sau*3AI and *Dpn*I all recognize the sequence GATC. Their activity is, however, affected differently by the state of methylation of the cytosine and the adenine residues in their recognition site (Roberts 1988). The presence of a methylated cytosine in the GATC sequence results in the inability of *Sau*3AI to cleave this site, while not affecting the activity of *Mbo*I and *Dpn*I. The state of methylation of the adenine residue in the GATC sequence affects the activity of these enzymes in a different manner. While *Mbo*I does not cleave this sequence when the adenine residue is methylated, *Dpn*I cleaves it only if it is methylated; whereas the activity of *Sau*3AI is unaffected by the state of methylation of the adenine. A comparative DNA restriction analysis using *Sau*3AI, *Mbo*I, and *Dpn*I can therefore be informative regarding the methylation status of both cytosine and adenine residues in the GATC sequence. Furthermore, if the sequence of the DNA under study is known, the position of the methylated bases can also be determined.

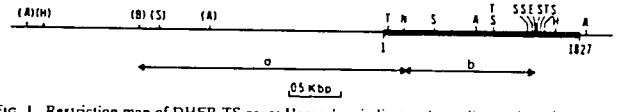

FIG. 1. Restriction map of DHFR-TS gene: Heavy line indicates the coding region of the gene and the numbers below denote the first (1) and last (1827) nucleotides of the gene. Restriction sites in this region were deduced from the known sequence of the gene (Bzik *et al.* 1987; Cowman *et al.* 1988). Tentative localization of restriction sites in the region upstream to the 5' end of the gene were determined in this study and are indicated in parentheses. The pHBE 6.1 probe used in this study is presented below the restriction map. Indicated are two fragments of the probe produced by *Nco*I cleavage producing a 2.5-kbp *Bcl*I–*Nco*I fragment (a) and a 1279-bp *Nco*I–*Eco*RI fragment (b). Symbols of restriction enzymes (site of cleavage): A, *Acc*I (−3450, −1700, 811, 1905); B, *Bcl*I (−2300); E, *Eco*RI (1418); H, *Hha*I (−3300, 1621); N, *Nco*I (139); S, *Sau*3AI (−2150, 442, 1030, 1360, 1396, 1441, 1529); T, *Taq*I (26, 1032, 1496). An additional *Sau*3AI site not shown in the figure is localized very close to the *Bcl*I site, probably on its 5' side.

As the number of fragments, as well as their sizes, obtained upon complete digestion of this DNA is known, any deviations from the expected restriction pattern may be a result of methylation. By determining the size of the unexpected fragments, the position of the methylated sequence can be established.

Figure 2 describes a *Sau*3AI, *Mbo*I, and *Dpn*I restriction analysis of the DHFR-TS gene using two DNA fragments corresponding to this region as probes. Upon complete digestion of this gene, whose sequence is known (Bzik *et al.* 1987; Cowman *et al.* 1988), by either one of the enzymes, three fragments (330-bp, 588-bp, and 3-kbp in size) should be observed when using probe b, and two fragments (190-bp and 3-kbp in size) should be observed when probe a is used (see Fig. 1 for restriction map and probes). It can been seen in Figs. 2A and 2B that *Mbo*I digestion of DNA of the four strains tested (lane 2, a,b,c, and d) yielded only the expected fragments, indicating the absence of 6-methyladenine. This co sion was further supported by the lac any detectable digestion of the gen *Dpn*I (lane 3, a,b,c, and d). On the o hand, the *Sau*3AI digest of DNA fro strains tested was found to contain a bp-long fragment (Fig. 2A, lane 1, a,b,c d) which could not be accounted for the known sequence of the gene. To out the possibility that this fragmen sulted from a lower affinity of the en toward a certain GATC site, the cou its appearance was studied in the pres of excess enzyme concentration. As c seen in Fig. 3, the intensity of the 91 fragment, as well as that of all the expected fragments (330-bp, 588-bp, a kbp), is already maximal after 1 hr of d tion and is not changed upon further tion (lane 1 vs lanes 2, 3, and 4, re tively). This result argues strongly ag the above mentioned possibility as, if was the case, the intensity of the 91 fragment should have diminished in t

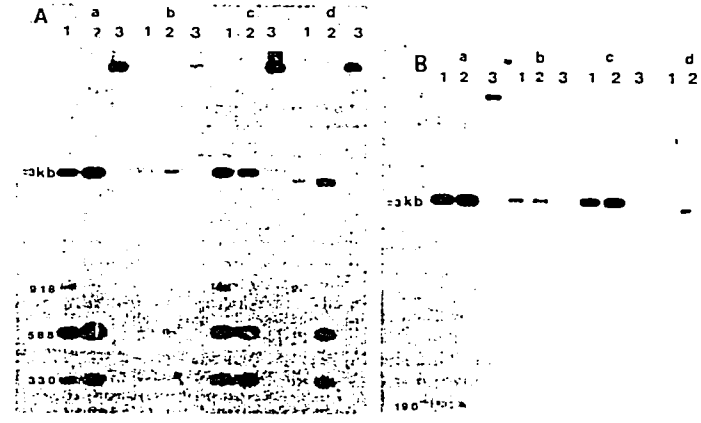

FIG. 2. *Sau*3AI/*Mbo*I/*Dpn*I analysis of DHFR-TS gene: DNA (1.2 μg) from strains (a) FCR-3, (b) IIG-2G1, (c) D6, and (d) 3A-B4 was cleaved with *Sau*3AI (lane 1), *Mbo*I (lane 2) and *Dpn*I (lane 3) an analyzed by Southern blotting using (A) probe b, and (B) probe a.

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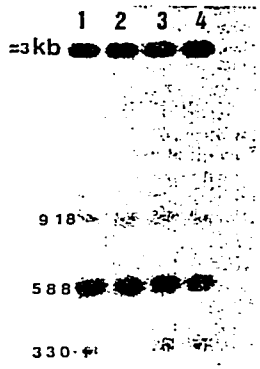

FIG. 3. Kinetics of appearance of the 918-bp fragment: DNA (4.8 μg) from FCR-3 was cleaved by excess concentration of *Sau*3AI. Aliquots containing 1.2 μg DNA were withdrawn after 1, 2, 3, and 4 hr of incubation (lanes 1–4, respectively) and analyzed by Southern blotting using fragment b of the pHBE 6.1 probe (Fig. 1).

This fragment could therefore be a result of the existance of a methylated cytosine in a GATC site. The most probable GATC site is that at position 1030, since methylation at this site would result, in a *Sau*3A digestion, in the appearance of a 918-bp fragment (nucleotides 442 to 1360), which is similar in size to the one observed in the experiment. In addition, from all of the GATC sites present in the coding region of the gene, this site is the only one to be succeeded by a G residue, thus producing the CpG sequence which is the only sequence in eukaryotes where methylated cytosine is found. To prove it, advantage was taken of the fact that the GATC site at position 1030 comprises part of an adjacent *Taq*I site (-GATCGA-), which is insensitive to cytosine methylation. If the 918-bp is the result of methylation at this position, it should be cleaved upon further digestion with *Taq*I yielding the 588- and 330-bp fragments. In Fig. 4, it can be seen that this was the case. The 918-bp fragment (lane 1) was further cleaved by *Taq*I (lane 4), yielding the expected fragments (the 416-bp fragment in lane 4 resulted from the cleavage of the 3-kbp fragment by *Taq*I at position 26). It should be noted, however, that this site is only partially methylated as the expected fragments produced by *Sau*3AI cleavage of this site do also appear (330 and 588-bp, lane 1, Figs. 2A and 4).

Two additional CpG-containing sites (*Hha*I) were found to be unmethylated (data not shown).

DISCUSSION

Phylogenically, *P. falciparum* is classified as a lower eukaryote. However, comparison of the amino acid sequence of several plasmodial proteins with that of their functionally equivalent proteins from other

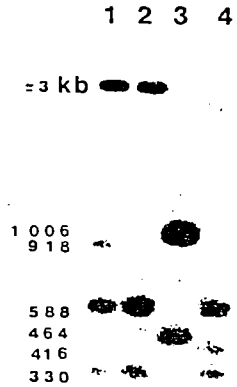

FIG. 4. Localization of methylated GATC site in DHFR-TS gene: DNA (1.2 μg) from FCR-3 strain was cleaved by *Sau*3AI (lane 1), *Mbo*I (lane 2), *Taq*I (lane 3) and *Sau*3AI + *Taq*I (lane 4) and analyzed as described in the legend to Fig. 3, using probe b.

organisms revealed a higher degree of homology with the vertebrate sequences rather than with those from lower eukaryotes (Hyde and Sims 1987; Bzik *et al.* 1987; Bianco *et al.* 1986; Yang *et al.* 1987; Ardeshir *et al.* 1987). Another property of the plasmodial genome, which is typical to vertebrate genomes, is the underrepresentation of the dinucleotide CpG (Hyde and Sims 1987; Weber 1987). This fact may be indicative of the presence of 5-methyl cytosine in the plasmodial genome as it was shown that in eukaryotes, there is a direct correlation between the degree of CpG underrepresentation and the extent of cytosine methylation of this sequences: the higher the degree of underrepresentation, the higher the level of methylation (Bird 1980). Previous attempts to detect 5-methyl cytosine using either a chemical procedure (Pollack *et al.* 1982) or a comparative *Msp*I/*Hpa*II restriction analysis of four plasmodial genes (Weber 1987) failed to show it. Although these procedures are reliable, they cannot be conclusive when applied to genomes extremely low in their G + C content and having very low CpG-containing restriction sites for enzymes which are used for that purpose. For example, the circumsporozoite gene, whose methylation status was studied using *Msp*I/*Hpa*II analysis (Weber 1987) does not contain any such site in its coding region (Dame *et al.* 1984). Neither are there any *Msp*I/*Hpa*II restriction sites in the DHFR-TS gene (Bzik *et al.* 1987; Cowman *et al.* 1988). It is therefore obvious that *Msp*I/*Hpa*II analysis cannot be used to exclude definitively the existance of methylated cytosine residues in the genome of *P. falciparum* and that other enzymes whose activity is sensitive to methylation should be used. In this study, in addition to *Msp*I and *Hpa*II, we used several such enzymes. Using the enzymes *Sau*3AI and *Mbo*I, we were able to detect in all the isolates tested, a CpG site in the DHFR-TS gene which was partially methylated.

Restriction enzymes are the most commonly used tool for the detection of methylated bases. Some caution should be taken however when interpreting the results, as it has been shown, in a small number of cases, that a restriction enzyme may exhibit a lower affinity than normal toward a certain recognition site in the DNA molecule (Brown and Smith 1977; Forsblum and Philipson 1976; Gingeras and Brooks 1983; Thomas and David 1975). This may lead to a slower rate of cleavage of this site, or, in extreme cases, to the lack of its cleavage. However neither one is the case in this study as (a) the 918-bp fragment behave kinetically in a manner similar to that of the other fragments; (b) the GATC site, whose methylation results in the appearance of this fragment, is cleaved by *Sau*3AI.

In conclusion, evidence is presented for the existance of 5-methylcytosine in *P. falciparum* DNA. The methylation is of the eukaryotic type by virtue of its appearance in a CpG site and by the fact that it was partial.

ACKNOWLEDGMENT

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