cell disease. Although sickle cell anemia was the first disease to be understood at the molecular level (*19*), there is still no cure or adequate treatment. If a transgenic mouse model can be developed, new drug therapies and even gene therapies could be tested. Once perfected in model systems, protocols that are safe and effective for humans could be developed.

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10. Adult animals were made anemic with phenylhydrazine (*20*) to induce reticulocytosis, anesthetized, perfused (*11*), and tissues were removed. Total RNA was prepared from frozen tissue [*Anal. Biochem.* **162**, 156 (1987)] with the following modification. The final RNA pellets were resuspended in a solution containing 1.0% SDS, proteinase K (100 mg/ml), 25 m*M* NaCl, 1.0 m*M* EDTA, and 10 m*M* tris-HCl *p*H 7.5. After digestion for 3 hours at 50°C, the samples were extracted with phenol/chloroform, chloroform, and ethanol precipitated.
11. Animals were perfused by cutting the right atrium and injecting phosphate-buffered saline into the left ventricle. The lung is not perfused in this procedure and, therefore, is contaminated with blood.
12. Blood cells were washed twice with saline and lysed in a volume of water equal to the cell pellet. One-fourth volume of carbon tetrachloride was mixed with the hemolysate, and cell membranes were extracted by brief vortexing and mirocentrifugation. The aqueous phase was removed and frozen at 20°C. Samples were subsequently thawed, diluted with an equal volume of 0.05% KCN, and separated on an agarose isoelectric focusing gel (Resolve-Hb, Isolabs Inc., Akron, Ohio) according to the manufacturer's specifications. After focusing, proteins were fixed in the gel with 10% trichloroacetic acid for 10 min. The gel was then rinsed for 1 hour with water, dried, and hemoglobin bands were visualized without staining.
13. Hemoglobin bands were cut out of the agarose IEF gel and eluted in water for 1 hour at room temperature. After dialysing against water overnight at room temperature, the samples were lyophilized and resuspended in water. Equal volumes of sample (purified hemoglobin or whole hemolysate), alkaline-urea buffer (6.0*M* urea, 15 m*M* boric acid, 0.5 m*M* EDTA, 25 m*M* tris-HCl, *p*H 8.6), and β-mercaptoethanol were mixed and an aliquot was loaded onto a cellulose acetate strip (Gelman) that had been soaked overnight in alkaline-urea buffer. The samples were then electrophoresed for 1 hour at 190 V in alkaline-urea buffer. Proteins were subsequently stained with 0.5% imido black in methanol:acetic acid (45:10). The strips were destained in methanol: acetic acid (47.5:5), dried, and photographed.
14. Although only four hemoglobin bands are observed on the IEF gel in Fig. 2B, nine hemoglobins representing all possible combinations of mouse and human α- and β-globin polypeptides probably exist inside the cell. During electrophoresis oxy-hemoglobin tetramers ($\alpha_2\beta_2$) dissociate into dimers ($\alpha_1\beta_1$) that are separated by charge differences. Therefore, hemoglobin tetramers composed of dimers of unlike charge are not detected (*21*).
15. Mouse, human, and hybrid hemoglobins synthesized by transgenic mice were separated by preparative IEF on 4.0% acrylamide gels containing 2.0% Pharmalyte *p*H 5 to 8. Each of the four bands was sliced from the gel, homogenized, and the hemoglobin was eluted in 0.1*M* potassium phosphate buffer. The isolated fractions were concentrated with Amicon filters (YM 10).
16. Hemoglobins were maintained in the carbon monoxide (CO) form during separation and concentration procedures to avoid auto-oxidation. Prior to functional studies the hemoglobins were converted to the oxy-state by photolysis and vacuum removal of CO. The oxygen equilibrium curve of each hemoglobin fraction was determined using a Hemox Analyzer (TCS, Southhampton, PA) in 0.1*M* potassium phosphate buffer, *p*H 7.0 at 20°C (*22*). All samples were analyzed four times and the curves were drawn in continuous mode. The maximum error of measurement of the P_{50} values is ±1 mmHg [*Crit. Care Med.* 7, 391 (1979)].
17. OEC of whole blood and unfractionated hemolysates from transgenic mice were also determined and compared to mouse and human controls. The curve for whole blood of 5393 transgenic progeny is virtually identical to the mouse control, while the curve for an unfractionated transgenic hemolysate is shifted to the left of the mouse hemolysate control. The left shift of the transgenic hemolysate OEC can be attributed to the presence of high-affinity hybrid and human hemoglobin species. The similarity of the whole blood OEC for transgenic and control mice may be due to adaptive responses, such as an increase in allosteric effectors of oxygen affinity, in the transgenic mice.
18. Quantitative solution hybridizations of blood RNA from the seven transgenic lines indicate that mouse α- and β-globin mRNA levels (picograms of total RNA per microgram) are not decreased in mice expressing high levels of human α- and β-globin mRNA.
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23. We thank N. Martin, J. Askins, and M. Avarbock for excellent technical assistance, J. Prchal for providing human reticulocyte RNA, K. Hall for instructions on electrophoresis of hemoglobins on denaturing cellulose acetate strips, and J. Engler for synthesizing the human α-, human β-, mouse α-, and mouse β-globin oligonucleotides. Supported in part by grants HL-35559, HD-09172, HL-38632, and HD-23657 from the National Institutes of Health and predoctoral training grant T32 CA-09467 from NIH (to T.R.).

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Circumsporozoite Protein Heterogeneity in the Human Malaria Parasite *Plasmodium vivax*

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Phenotypic heterogeneity in the repetitive portion of a human malaria circumsporozoite (CS) protein, a major target of candidate vaccines, has been found. Over 14% of clinical cases of uncomplicated *Plasmodium vivax* malaria at two sites in western Thailand produced sporozoites immunologically distinct from previously characterized examples of the species. Monoclonal antibodies to the CS protein of other *P. vivax* isolates and to other species of human and simian malarias did not bind to these nonreactive sporozoites, nor did antibodies from monkeys immunized with a candidate vaccine made from the repeat portion of a New World CS protein. The section of the CS protein gene between the conserved regions I and II of a nonreactive isolate contained a nonapeptide repeat, Ala-Asn-Gly-Ala-Gly-Asn-Gln-Pro-Gly, identical at only three amino acid positions with published nonapeptide sequences. This heterogeneity implies that a *P. vivax* vaccine based on the CS protein repeat of one isolate will not be universally protective.

MALARIA, A DISEASE CAUSED BY A mosquito-borne protozoan parasite of red blood cells, is so widespread and causes disability so severe that many strategies to control it have been devised. Recently much effort has been focused on the construction of vaccines designed to elicit a host immune response to sporozoites, the parasite stage injected into humans by mosquitoes. The predominant surface, or circumsporozoite (CS), proteins of sporozoites are characterized by tandemly repeated peptide units that occupy about one-third of each molecule and are immunogenic (*1–3*). In *Plasmodium vivax*, one of four *Plasmodium* species naturally infecting humans, the unit has been found to be a nonapeptide repeated about 20 times (*2*, *3*).

For a vaccine based on the repeat region to be feasible, the repeats must, at a minimum, be substantially conserved among isolates. Heterogeneity has been found in the repeat units of two monkey malarias, *P. cynomolgi* (*4*) and *P. knowlesi* (*5*), which are related to *P. vivax* (*6*), but no significant CS protein heterogeneity has been thought to occur in *P. vivax* (*7*). We report here that over 14% of the microscopically determined *P. vivax* cases in a western province of Thailand produced sporozoites that were not recognized by antibodies either to *P. vivax* sporozoites from different geographic areas or to a recombinant CS protein being tested as a potential vaccine (*8*). The reason for this nonreactivity is a different repeating nonapeptide.

Sporozoites were produced by feeding laboratory-reared *Anopheles dirus* mosquitoes on men (*9*) who visited either of two malaria clinics in Kanchanaburi Province and were found, on examination of a thick blood film, to have an uncomplicated *P. vivax* infection that included gametocytes, the stage infective to mosquitoes. Sporozoites were harvested from salivary glands of infected mosquitoes 14 days after feeding. Enzyme-linked immunosorbent assays (ELISAs) were used to screen for *P. vivax* and *P. falciparum* CS proteins (*10*); cohorts positive for *P. falciparum* indicated a subpatent mixed infection and were discarded.

Between November 1987 and November 1988, sporozoites were tested from mosquitoes fed on 162 men infected with an intraerythrocyte parasite morphologically identified as *P. vivax* (*11*). Twenty-three cases (14.2%) produced sporozoites that did not react in the *P. vivax* ELISA (*10*). Both the nonreactive and reactive cases occured predominately at the beginning of the annual rainy season, April to June, and appear from clinical histories to have been contracted at loci from eastern Burma to south-central Thailand. Our dependence on walk-in patients limited both the geographic area sampled and our knowledge of the clinical course of the infections.

An assortment of monoclonal antibodies (MAbs) developed to *P. vivax* sporozoites tested positive for reactive and negative for nonreactive sporozoites by indirect fluorescent antibody tests (IFATs) (*12*). Sera from squirrel monkeys (*Saimiri sciureus boliviensis*) immunized with a recombinant *P. vivax* candidate vaccine consisting of the CS-protein repeat (*8*) also did not bind to the nonreactive sporozoites in IFATs. These

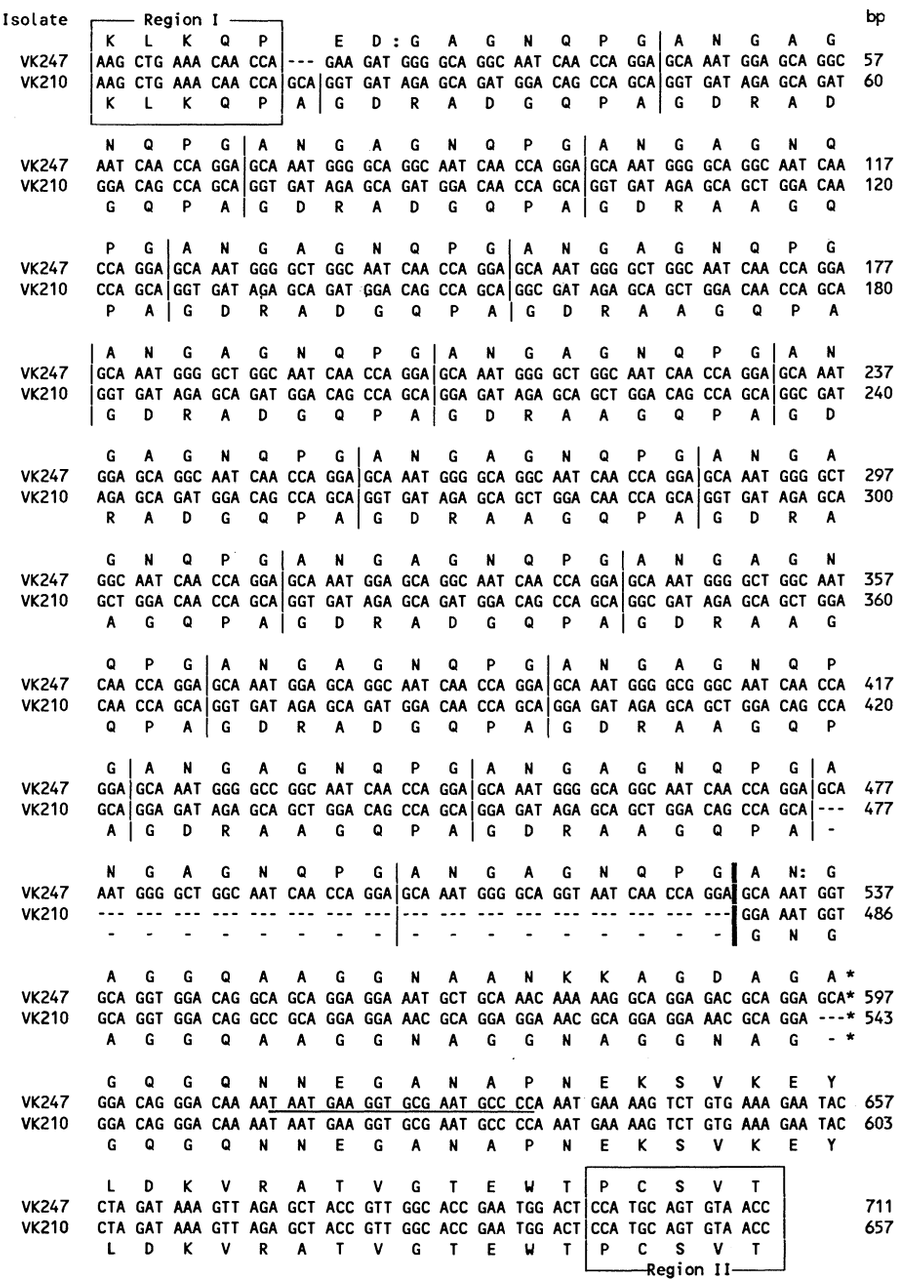

Fig. 1. The sequences of the genes encoding the circumsporozoite proteins of *P. vivax* isolates VK247 and VK210 bounded by region I and region II. Oligonucleotide primers homologous to regions I and II were used in a PCR reaction (*18*) to synthesize this segment of the genes from total genomic DNA. The sequences are aligned to best fit complete nonapeptide tandem repeat units at the nucleic acid level; each repeat is bordered by (|). There are 18 complete and one partial repeat of ANGAGNQPG in the VK247 sequence. However, 19 complete repeats can be seen, bounded by (:), starting the repeat unit at the first G downstream of region I and ending two AA after the terminal repeat. There are 17 repeating GDRA (A/D)GQPA peptide units in the VK210 isolate. The 3′ post-repeat variable region is between (▮) and (*); the 3′ post-repeat conserved region starts at (*) and continues to region II. The region I and II sequences shown here are assumed to be correct for these isolates because the primers used in the PCR reactions were of adequate homology to hybridize and prime the extension with the polymerase. The underlined sequence in the VK247 isolate, base pairs 613 to 632, indicates the compliment of the sequencing primer Pv3 (*18*). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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results implied that the CS protein repeat region of the nonreactive sporozoites differed significantly from those already sequenced (*2, 3*).

It was possible, despite microscopic evidence, that the nonreactive sporozoites represented malaria species other than *P. vivax*. *Plasmodium malariae* and *P. ovale* are human parasites sometimes encountered in Thailand, and *P. knowlesi* and *P. cynomolgi* are Asian monkey malarias transmissible to man (*13*). All nonreactive sporozoites tested were, however, negative in IFATs with MAbs for *P. falciparum*, *P. malariae*, *P. ovale*, *P. knowlesi*, *P. cynomolgi*, and other sporozoites (*12*). Blood-stage parasites from patients who infected mosquitoes with reactive (VK244) (*14*) and nonreactive (VK247) sporozoites were tested with ribosomal DNA probes specific for *P. vivax*, *P. falciparum*, *P. malariae*, and *P. ovale* (*15*); both blood samples were positive for *P. vivax* probes only.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting (*16*) were performed on reactive (VK311) and nonreactive (VK247) sporozoites. Both MAbs NSV3 and WR1, which recognize different epitopes on the CS protein (*12*), bound to a triplet of proteins (38, 40, and 46 kD) on blots from the reactive sporozoites. No binding was detected in this molecular size range with either NSV3 or WR 1 on blots from the nonreactive sporozoites (*17*).

The composition of the CS protein of one reactive (VK210) and one nonreactive (VK247) case were deduced by cloning and sequencing the CS gene portion flanked by regions I and II with the polymerase chain reaction (PCR) method (*18*). The deduced amino acid (AA) sequence of the reactive clone contained the same nonapeptide repeat, GDRA(D/A)GQPA, published for other *P. vivax* isolates (*2, 3*) (Fig. 1). The repeat unit of VK247 was also a nonapeptide, ANGAGNQPG, but differed from the reactive repeat at six of nine AA positions. Nucleotide variations in VK247 sometimes occurred at codon positions 3, 4, and 5, but all changes were synonymous. The respective nonamer units were tandemly repeated 19 times in VK247 and 17 times in VK210 (Fig. 1). The DNA from a second isolate of nonreactive sporozoites, VK293, was sequenced and found to be identical to VK247. The repeat units of VK247 and VK293 could not have resulted from simple frame shifts, deletions, or gene conversions during meiotic recombination of GDRA(D/A)GQPA and must have evolved separately.

Immediately following the 3′ terminal repeat units of isolates VK247 and VK210 are nearly identical repeat-related nonamers (ANGAGGQAA and GNGAGGQAA, respectively) that also appear as repeat terminals in the North Korean [(NK) the only other Old World *P. vivax* sequenced (*3*)] and Belem isolates; these are similar to the terminal nonamer of Sal-1 (Fig. 2) (*2, 3*). In all five *P. vivax* isolates there are short variable sequences between these terminal or post-repeat nonamers and the beginning of the 33 AA constant region that precedes region II. The 15 AA post-repeat variable region of VK247 and the 13 AA's of VK210 have more homology with portions of the 21 AA variable region of NK than with each other or with the American isolates (Fig. 2). This post-repeat variation may result from meiotic crossovers that occurred during the brief diploid phase that malaria undergoes during each mosquito passage.

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                  18        19
VK247    ANGAGNQPG|ANGAGNQPG‖ANGAGGQAA GGNAANKKA--------GDAGA [GQGQ...]

            :  ::     :  :    :::::::: :::::::::        : ::   ::::
                  18        19        20
  NK     GDRAAGQPA|GDRAAGQAA|GNGAGGQAA‖GGNAANKKAEDAGGNAGGNAG- [GQGQ...]

            :  ::     :  ::   :::::::: :::               : ::   ::::
                  16        17
VK210    GDRAAGQPA|GDRAAGQPA‖GNGAGGQAA GGN--------AGGNAGGNAG- [GQGQ...]

            :  ::     :  ::     : ::::                   : ::   ::::
                  18        19        20
Sal-1    GDRAAGQPA|GDRAAGQPA|GDSAAGQAA‖----------------GGNAG- [GQGQ...]

            :  ::     :  :    ::::::::                   : ::   ::::
                  18        19        20
Belem    GDRAAGQPA|GDRAAGQAA|GNGAGGQAA‖----------------GGNAG- [GQGQ...]
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Fig. 2. A comparison of AA homologies for terminal nonapeptide repeat units and post-repeat variable regions of VK247, NK, VK210, Belem, and Sal-1 *P. vivax* CS proteins (*2, 3*). The end of a repeat unit is represented by (|) and the end of the terminal repeat unit by (‖); the numbers indicate the number of the unit repeat. The post-repeat variable sequences have been shifted to positions of maximum homology with VK247, represented by (:). The post-repeat constant regions are identical in all five CS proteins and are not shown beyond GQGQ.

Further evidence that VK247 is not a *P. cynomolgi* or *P. knowlesi* isolate comes from the 33 AA post-repeat conserved region that starts with GQGQ and continues to region II. This sequence is 100% conserved in all five *P. vivax* isolates, even at the nucleotide level, but has less than 80% AA homology with *P. cynomolgi* and *P. knowlesi* (*3–5*).

The phylogenetic correspondence within *P. vivax* as well as between *P. vivax* and the Asian monkey malarias is complex, and the status of CS protein polymorphism in Thailand will require careful study of parasite morphology, distribution, clinical course, and genome. The dangers of representing large geographical areas with single isolates are obvious (*19*), but often ignored.

There are two practical implications of CS repeat heterogeneity. The widespread and growing use of the ELISA and other immunological methods (*10*) for incriminating vectors should first be conducted with concomitant mosquito dissections to establish the correlation between the two procedures. Second, as we have shown, monkey antibody to a candidate *P. vivax* CS repeat vaccine (*8*) did not bind to nonreactive sporozoites, and it is unlikely that antibodies to another similar vaccine (*8, 20*) will be any more efficacious. Heterogeneity in the *P. vivax* CS protein repeat implies that a *vivax* vaccine based on this repeat will not be universally protective.

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9. Procedures were approved by the human use committees of the Ministry of Public Health, Thailand, and the U.S. Army Medical Research and Development Command. Men ≥20 years old signed a consent form describing the experiment and its possible risks. A history was taken from each volunteer, blood was drawn, and up to 200 mosquitoes were fed for 10 min on his forearms. Treatment for *P. vivax* was 0.5 g of chloroquine base for 3 days and 15 mg of primaquine for 14 days.
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11. Blood-stage species identification was first made by a Thai Malaria Division microscopist, then confirmed by an Armed Forces Research Institute of Medical Sciences microscopist. Ten coded slides, five each from blood-producing reactor and nonreactor sporozoites, were examined by malariologists

at the Malaria Branch, Centers for Disease Control, Atlanta, GA. All were identified as *P. vivax*; blood-stage parasites producing reactor and nonreactor sporozoites could not be distinguished morphologically.
12. The IFATs (*10*) were done with nonreactive sporozoites and anti–*P. vivax* MAbs: Navy Medical Research Institute (NMRI) NSV3 and NSV4; New York University (NYU) 2F2 (*1*, *7*); Centers for Disease Control (CDC) CDC1; and Walter Reed Army Institute of Research WR1, 3, 7, 9, 21, and 28. These MAbs recognize at least three CS protein epitopes (R. A. Wirtz *et al.*, unpublished data). MAbs to other sporozoite species were also used: *P. malariae* 109–179.4 and *P. ovale* 110–54.3 from CDC; *P. cynomolgi* Ceylon Cy6A4.E11, NIH-13E.11C9, Gombak GB1G11.G1, and *P. knowlesi* 2G3 from NYU; and *P. berghei* (3.28, 3.116, 3.135, 3.213), *P. yoelli*, and *P. inui* from NMRI. Not all MAbs were tested against nonreactive sporozoites from the same patient mosquito feed. Some MAbs were tested in pools. MAbs were protein A purified (*10*) and tested at 2.5 or 5 μg/ml and were IFAT positive with the homologous sporozoite.
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15. Species-specific regions of the small subunit rRNA were detected as described by A. P. Waters and T. F. McCutchan, *Lancet*, in press. The oligonucleotides have been described (*15*) with the exception of the diagnostic for *P. vivax*, which is designated 165R and has the sequence: 5′-AAATCA(A/T)CC(G/A)(G/A)ATTCAGTCCCACGT-3′. The probe used to diagnose *vivax* would distinguish between *P. cynomolgi* and *P. vivax*.
16. PAGE and Western blotting were performed as described (*7*) with the following modifications. The nitrocellulose paper was treated with 0.3% Tween 20, and strips were incubated overnight with the specific antibody diluted in 10 m*M* tris, *p*H 8, containing 0.05% Tween 20, they were washed, and then incubated with alkaline phosphatase–conjugated antibody to mouse antigen (Promega). The paper was washed and developed with nitro blue tetrazolium (Promega).
17. R. A. Wirtz *et al.*, unpublished data.
18. Oligonucleotide primers PvRI (5′-GTCGGAATT CAATAAGCTGAAACAACCA-3′ and PvRII (5′-CAGCGGATCCACAGGTTACACTGGTGG-3′) corresponding to regions I and II, respectively, of the published *P. vivax* sequence (*2*, *3*) were synthesized. In order to facilitate unidirectional subcloning, primers were designed to contain either an Eco RI (PvRI) or Bam HI (PvRII) site starting five bases proximal to their 5′ ends. DNA was phenol-chloroform extracted from 2.6×10^5 sporozoites, in the presence of 20 μg of carrier yeast transfer RNA, and amplified by PCR 5 min at 94°C, then amplified through 25 cycles [incubated (1.5 min at 94°C, 2 min at 45°C, and 4 min at 72°C), followed by 5 min at 72°C] (GeneAmp Kit, Perkin-Elmer). DNA was precipitated, resuspended, and digested with Eco RI and Bam HI. Amplified DNA was agarose-purified by electrophoresis, excised (GeneClean, Bio 101), and ligated into Bluescript plasmid SK^+. A single insert-containing clone was selected and sequenced (Sequenase, U.S. Biochemical). Sequencing primers were M13 and T3, which flanked the insert site in the SK^+ vector and an oligomer, Pv3 (5′-GGGGCATTCGCACCTTCATTA-3′), the reverse complement of bases 613 to 632 in the conserved post-repeat region of isolate VK247. To check for sequencing errors, PCR was repeated on VK247 genomic DNA. The sequences were identical. Sequences have been deposited in the GenBank database (IntelliGenetics) (accession numbers M25759 and M25760 for VK210 and VK247, respectively).
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Fitness Differences Among Remnant Populations of the Endangered Sonoran Topminnow

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Four correlates of fitness were measured in three stocks of the endangered Sonoran topminnow, *Poeciliopsis occidentalis*, from Arizona. Survival, growth, early fecundity, and developmental stability were greatest in laboratory-reared fish from the most heterozygous natural population studied. Conversely, all four traits were poorest in fish from a population with no electrophoretically detectable genetic variation. These results emphasize the need for genetic as well as demographic information for the development of comprehensive species recovery programs.

A SURVEY OF GENETIC DIVERSITY IN the endangered Sonoran topminnow, *Poeciliopsis occidentalis occidentalis* (Atheriniformes: Poeciliidae), revealed that remnant populations in Arizona contain low levels of genetic variation (*1*). A hatchery stock derived from one of these populations, Monkey Spring, was being used in a species recovery effort involving reintroductions within the Gila River drainage; however, these topminnows were not the best choice

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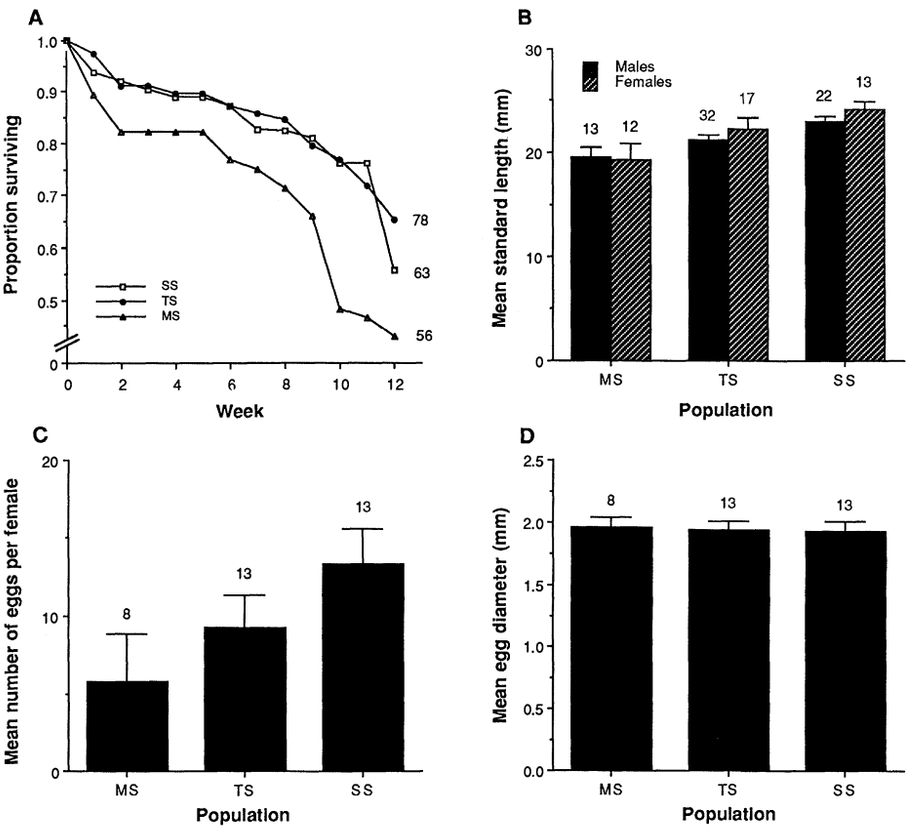

Fig. 1. Fitness differences among Sonoran topminnow populations. (**A**) Survival to 12 weeks. (**B**) Growth (mean standard length). (**C**) Fecundity at 12 weeks of age (least-squares means adjusted for standard length). (**D**) Mean egg diameter. Error bars represent 95% confidence intervals of the means. Sample sizes are shown.