uncompensated functions of TGF-β1.

Finally, although targeted gene disruption may be a valuable tool for evaluating the roles of cytokines, oncogene products, and other proteins during embryogenesis, our data show that in some instances, a gene knockout is not equivalent to a protein knockout. The potential contribution of maternal protein to the fetal phenotype should be considered in other gene disruption experiments, particularly those targeting genes that encode secreted proteins.

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14. Mice in the newborn and neonatal periods were killed by CO_2 narcosis, and stomach contents (milk) were removed for analysis by sandwich enzyme-linked immunosorbent assay for TGF-β1 and TGF-β2 (*22*). Pooled specimens were extracted in acid-ethanol and dialyzed extensively against 4 mM HCl at 4°C, with the use of 3500 MW-cutoff Spectrapor dialysis tubing (Spectrum Medical Industries, Los Angeles, CA). Samples were clarified by centrifugation, lyophilized to dryness, and redissolved for analysis.
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Implications of *FRA16A* Structure for the Mechanism of Chromosomal Fragile Site Genesis

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Fragile sites are chemically induced nonstaining gaps in chromosomes. Different fragile sites vary in frequency in the population and in the chemistry of their induction. DNA sequences encompassing and including the rare, autosomal, folate-sensitive fragile site, *FRA16A*, were isolated by positional cloning. The molecular basis of *FRA16A* was found to be expansion of a normally polymorphic p(CCG)$_n$ repeat. This repeat was adjacent to a CpG island that was methylated in fragile site–expressing individuals. The *FRA16A* locus in individuals who do not express the fragile site is not a site of DNA methylation (imprinting), which suggests that the methylation associated with fragile sites may be a consequence and not a cause of their genesis.

Fragile sites are points on chromosomes that tend to break nonrandomly when exposed to specific chemicals or conditions of tissue culture. A major group are the rare folate-sensitive fragile sites, which segregate in families in a non-Mendelian manner (*1*). The only fragile sites that have been cloned [*FRAXA* (*2*) and *FRAXE* (*3*)] are in this group and are both on the X chromosome. One of these (*FRAXA*) is associated with the most common form of familial mental retardation, fragile X syndrome (*2*). The two X chromosome fragile sites are unstable p(CCG)$_n$ trinucleotide repeats adjacent to CpG islands that become hypermethylated when the number of copies of the repeat exceeds certain limits. None of the autosomal, folate-sensitive fragile sites have been cloned, and nothing is known of their clinical significance, perhaps because none have been reported in homozygous form. To gain further understanding of the relations between DNA sequence, position in the genome, and chemistry of cytogenetic expression of fragile sites, we have characterized the rare folate-sensitive fragile site (*FRA16A*) in band p13.11 of human chromosome 16.

A long-range restriction map in the vicinity of *FRA16A* (Fig. 1) was generated by means of anonymous DNA sequences. These sequences had been localized by physical mapping to somatic cell hybrid breakpoint intervals (*4*) and by genetic linkage analysis (*5*). Because differential methylation has been observed for the previously characterized X-linked fragile sites, DNA from normal and *FRA16A* individuals was subjected to restriction endonuclease digestion with methylation-sensitive enzymes (Not I and Bss HII).

This analysis demonstrated distinct patterns of digestion for the *FRA16A* chromosomes associated with clustered Not I and Bss HII sites, suggesting differential methylation of a CpG island associated with expression of the fragile site (*6*). Yeast artificial chromosomes (YACs) containing DNA from this region were isolated and analyzed for the presence of these clustered Not I and Bss HII sites (*7*). One of the YACs (769H1) was found to contain these rare-cutting restriction sites adjacent to a p(CCG)$_n$ hybridizing sequence. The YAC was subcloned into lambda, and clones containing the p(CCG)$_n$ repeat were isolated (Fig. 1). One of the subclones, pf16A3, was used as a probe to demonstrate instability of this sequence in pedigrees transmitting the *FRA16A* fragile site (Fig. 2).

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Southern (DNA) blot analysis of either Rsa I–digested or Not I–Pst I–digested *FRA16A* chromosomal DNAs probed with pf16A2 and pf16A3, respectively, localized the unstable *FRA16A* DNA sequence to the 650–base pair interval between the Not I and Rsa I sites (Fig. 1). This sequence was extraordinarily GC-rich and contained 20 copies of the trinucleotide repeat $p(CCG)_n$; an interrupting sequence or base substitution occurred at a minimum of six repeat intervals.

Polymerase chain reaction (PCR) analysis across the *FRA16A* $p(CCG)_n$ repeat in unrelated individuals demonstrated that the repeat was highly polymorphic (observed heterozygosity 91%), presumably because of variation in trinucleotide repeat copy number. Twenty-one different alleles were observed on normal Caucasian chromosomes with copy number ranging from 16 to 49, with a mode of 22. In two individuals, three alleles were evident, indicating some somatic instability; however, even the largest of the normal *FRA16A* $p(CCG)_n$ alleles segregated in a Mendelian manner in the normal Centre d'Etude du Polymorphisme Humain (CEPH) pedigrees.

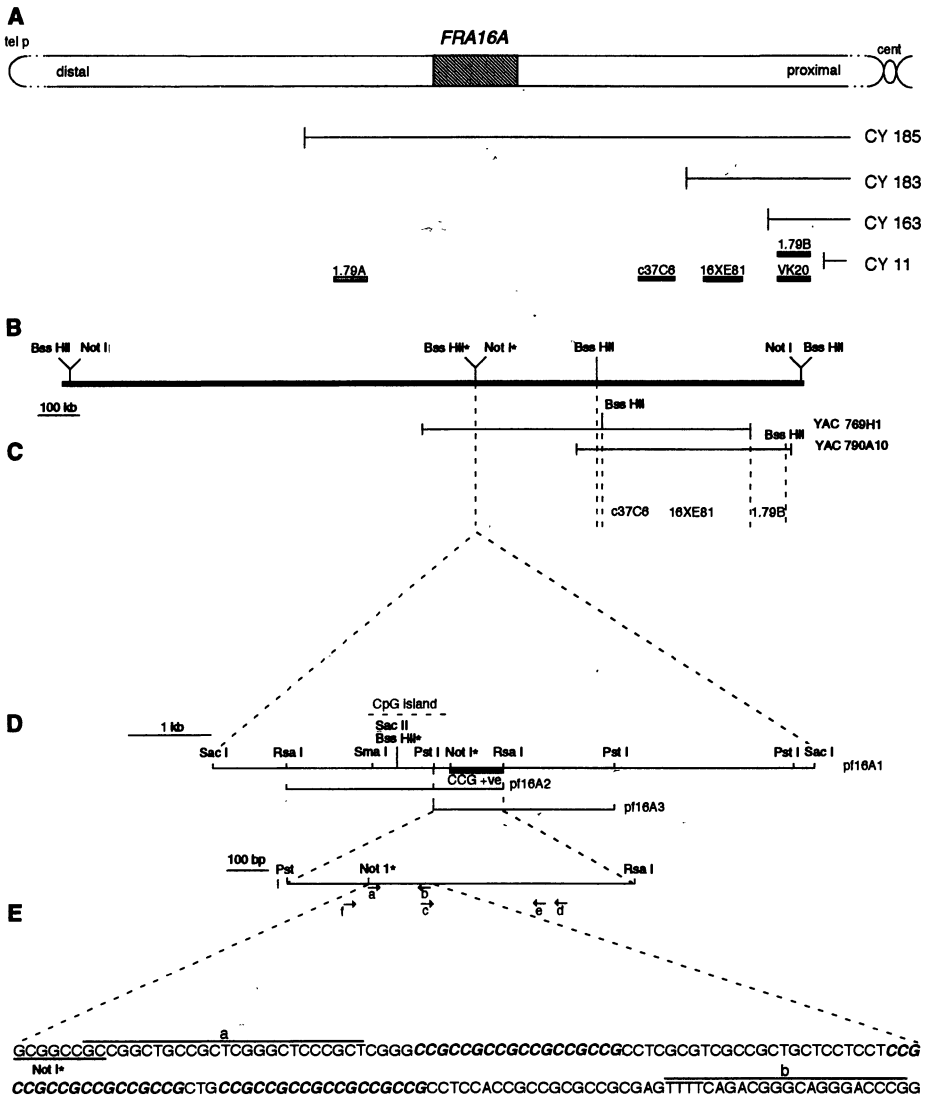

Fig. 1. Positional cloning and sequence analysis of *FRA16A*. (**A**) The extent and approximate location on the short (p) arm of human chromosome 16 of somatic cell hybrid breakpoints used to physically localize anonymous DNA probes (1.79, c37C6, 16XE81, and VK20). The probe 1.79 hybridized to sequences (designated 1.79A and 1.79B) present in two breakpoint intervals. These two sequences were also distinguished by distinct restriction fragment length polymorphisms (RFLPs) detected at each locus (*5*). (**B**) The long-range restriction map in the vicinity of *FRA16A* generated by pulse-field gel electrophoresis of Not I– and Bss HII–digested DNA from normal and *FRA16A* individuals, probed with 1.79, c37C6, 16XE81, or VK20. The Not I and Bss HII sites that exhibited differential methylation are indicated with an asterisk. (**C**) Location and restriction map of YACs 769H1 and 790A10 used to isolate *FRA16A* sequences. (**D**) Map of the *FRA16A* subclones pf16A1, pf16A2, and pf16A3 which contain the differentially methylated Not I site adjacent to a $p(CCG)_n$ repeat detected by hybridization. The location of the extensive adjacent CpG island is indicated as is the Not I–Rsa I $p(CCG)_n$ hybridizing sequence (CCG + ve) to which *FRA16A* instability localized (thickened line). (**E**) The location of PCR primers used to localize the region of *FRA16A* instability to the $p(CCG)_n$ repeat and in analysis of *FRA16A* $p(CCG)_n$ repeat copy number. Primer sequences a and b flanking the *FRA16A* $p(CCG)_n$ repeat sequence are indicated by overlines. Sequences of additional PCR primers are as follows: (c) 5′-TTTTCAGACGGGCAGGGACCCG-3′; (d) 5′-ATCCTACTCCCACTACGTCCTGAGG-3′; (e) 5′-GCCTTCCCCATCACCCTCCCCTCCA-3′; and (f) 5′-CACGCTCCGGGCCGCCGCCGCGCTC-3′.

In *FRA16A* families the PCR analysis (Fig. 3) demonstrated segregation of an apparent null allele with expression of the fragile site; we interpret this result to mean that PCR amplification was not possible (under the conditions used) across the highly expanded allele, in agreement with similar observations for *FRAXA* (*8*, *9*) and *FRAXE* (*3*). There was no evidence of a null allele in the CEPH pedigrees tested. PCR with primers e and f, which flank the a and b primers (Fig. 1E), demonstrated a similar (but higher molecular weight) pattern in CEPH pedigrees and for the apparent null allele segregating with *FRA16A* in the *FRA16A* pedigrees, indicating that the observed null allele was not due to mutation in the *FRA16A* individuals at the PCR primer annealing sites. The region of amplification associated with *FRA16A* expression was further narrowed to the polymorphic $p(CCG)_n$ repeat by single-strand conformation polymorphism (SSCP) analysis of adjacent PCR products generated with primers c and d (*10*). Finally, probes that flank the $p(CCG)_n$ repeat also flank the fragile site when used in fluorescence in situ hybridization (FISH) against *FRA16A*-expressing chromosomes (*11*).

The *FRA16A* repeat exhibits a greater degree of observable amplification, from ~3.0- to ~5.7-kb amplification in all *FRA16A*-expressing individuals, than has been observed in the *FRAXA* or *FRAXE* repeats (*2*, *3*, *8*, *9*). One *FRA16A*-expressing individual with an amplification greater than 5.7 kb appeared to have a smear of bands. The only available nonexpressing carrier of *FRA16A* had an amplification of ~0.5 kb (Fig. 2B), which is within the limit (~0.7 kb) normally associated with *FRAXA* and *FRAXE* carriers. This individual exhibited no methylation of the *FRA16A* Not I site (*6*), further supporting the correlation between methylation status and fragile site expression reported for *FRAXA* (*2*, *9*, *12*) and *FRAXE* (*3*). In general the *FRA16A* repeat appears to be more stable than its X-linked counterparts, usually being evident as a discrete single band on Southern analysis (Fig. 2). Instability was observed in the *FRA16A* pedigrees; however, this was usually more pronounced in female

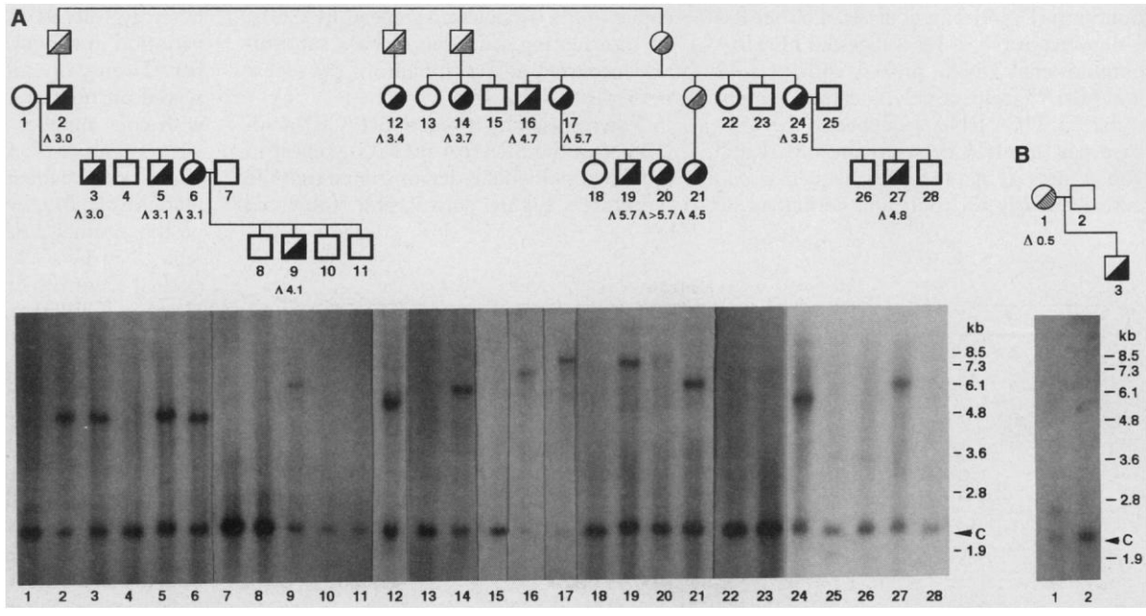

Fig. 2. Sequences adjacent to *FRA16A* detect a heritable unstable element in *FRA16A* pedigrees. (**A**) Southern blots of Pst I–digested chromosomal DNAs from members of a *FRA16A* pedigree were probed with pf16A3. Only those members shown in bold were analyzed. Members of the pedigree shown with shading are obligate carriers of unknown cytogenetic status. Crosshatching indicates *FRA16A*-expressing individuals. (**B**) Pst I digestion of chromosomal DNA from a *FRA16A* carrier (not expressing the fragile site cytogenetically) probed with pf16A3. No DNA was available from her child (now aged 7) previously shown to express *FRA16A* cytogenetically. A third unrelated family showed similar *FRA16A*-linked instability of the pf16A2 hybridizing Pst I fragment. The "C" indicates the normal allele Pst I fragment (2.2 kb). The Δ is the increase in kilobase pairs above the normal allele band length.

transmissions, as is the case for *FRAXA* and *FRAXE*. Because the *FRA16A* fragile site is not yet associated with any disorder, we have been unable to obtain various tissues of *FRA16A* individuals to assay for somatic mosaicism. The observed interruptions in the normal repeat sequence may account for the apparent relative stability of the *FRA16A* repeat, if indeed some (or all) of these interruptions are incorporated in the amplified version. The methylation status of the $p(CCG)_n$ repeat itself was examined by the use of the methylation-sensitive restriction enzyme Fnu 4HI, the recognition site (GCNGC) of which is encoded by the repeat motif (*13, 14*). *FRA16A* individuals were found to be methylated at the amplified $p(CCG)_n$ repeat and unmethylated on their normal $p(CCG)_n$ allele, whereas normal individuals were unmethylated at both alleles (Fig. 4).

Taken together these results demonstrate a remarkably similar structure for *FRA16A* to that reported for *FRAXA* and *FRAXE*—a normally polymorphic $p(CCG)_n$ repeat adjacent to a CpG island with individuals expressing the fragile site having expansion of the $p(CCG)_n$ repeat and methylation of the CpG island. They also demonstrate that fragile sites can originate at sites in the genome that are not normally associated with methylation; this suggests that the observed methylation associated with fragile sites is a consequence of the fragile site mutation, not a cause of such mutation.

Laird has hypothesized that fragile X syndrome is a result of the failure to erase the X inactivation imprint signal (*15*). The differential methylation associated with fragile X syndrome has been taken as evidence to support this hypothesis (*13, 16*) given that methylation appears to be a normal component of X inactivation. Normal chromosome 16's do not exhibit methylation at either the site of $p(CCG)_n$ amplification associated with *FRA16A* or the CpG island adjacent to it. Therefore, this locus is not a normal site of imprinting (at least by methylation status), and failure to erase methylation does not appear to be a necessary condition for fragile site genesis. The $p(CCG)_n$ repeat expansion associated with fragile sites appears to introduce a recognition site for an imprinting mechanism (de novo methylation) that is independent of normal parental imprinting (X or autosome) and results in the methylation of the $p(CCG)_n$ repeat itself and of the adjacent CpG island.

Parental bias in the mutation of unstable repeat sequences during transmission is a common property that can favor either gender (*17*). For example, individuals with the juvenile onset form of Huntington's disease inherit the disease allele from their father, whereas all individuals with the congenital form of myotonic dystrophy (DM) inherit the disease from their mother. Greater instability of the expanded *FRA16A* $p(CCG)_n$ repeat is evident in maternal transmissions than in paternal transmissions (Fig. 2A). Parental bias occurs irrespective of the autosomal location of the disease gene and in the apparent absence (demonstrated in the case of DM) (*18*) of methylation imprinting. The meth-

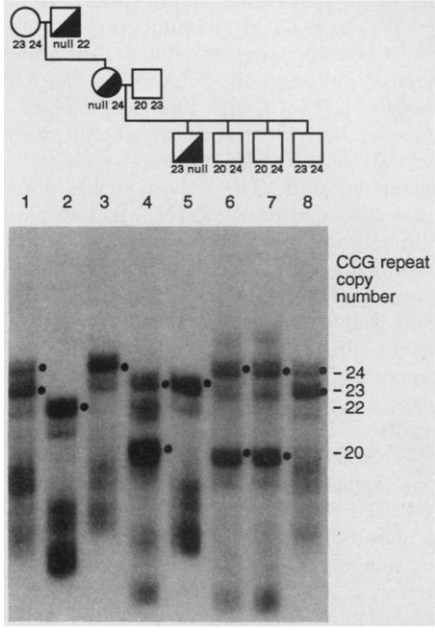

Fig. 3. Segregation of the *FRA16A* $p(CCG)_n$ PCR product as a null allele in a *FRA16A* pedigree. Lanes 1 to 8 correspond to individuals numbered 1, 2, 6, 7, 9, 8, 10, and 11 in Fig. 2A. PCR products were generated with primers a and b with the methods of Yu *et al.* (*9*). Each allele gave major and minor bands typical of simple tandem repeat PCR products. The major band scored for each allele is indicated by a dot. The variable rapidly migrating bands present in each lane are double-stranded hetero- and homoduplexes of the scored single-stranded alleles.

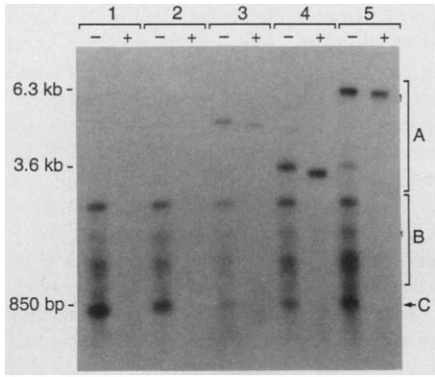

Fig. 4. Methylation status of the *FRA16A* $p(CCG)_n$ repeat assessed by Fnu 4HI digestion. Pst I–Rsa I double digests of chromosomal DNA from lymphocytes of normal (lanes 1 and 2) and *FRA16A* individuals (lanes 3 to 5) were treated with (+) or without (−) Fnu 4HI and subjected to Southern blot analysis with the 650–base pair Not I–Rsa I fragment from pf16A3. This probe detects an 850–base pair constant band (C) at the normal *FRA16A* locus, several cross-hybridizing bands located elsewhere in the genome (B), and the unstable *FRA16A* alleles in *FRA16A*-expressing individuals (A). Digestion with Fnu 4HI eliminated the normal (C) allele [and cross-hybridizing bands (B)]; however, only minor alteration is afforded to the *FRA16A* fragment (not all Fnu 4HI sites contain a methylatable CpG), indicating resistance to digestion of the majority of the sequence containing the $p(CCG)_n$ repeat because of methylation.

ylation associated with fragile sites does not appear to be a necessary condition for the mutation process, although it may be essential for the phenotypic manifestation of the disease (*19*) or the cytogenetic expression of the fragile site or both.

To explore the relation between the sequence composition of fragile sites and the chemistry of their induction, it will be of interest to determine the structure of (nonfolate-sensitive) fragile sites that are induced by a variety of chemicals and to determine whether methylation plays a role in their mutation or cytogenetic expression.

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7. YACs were isolated from the CEPH first-generation library by PCR screening for the presence of sequence-tagged site (STS) sequences from probes 1.79, VK20, and 16XE81. Total yeast DNA, including these YACs, was used as a template to generate *Alu*-PCR products for screening the Los Alamos human chromosome 16–specific cosmid library. Positive cosmids that mapped to the appropriate somatic cell hybrid breakpoint interval were subject to sequence analysis to develop further STSs. These STSs were used to screen the CEPH Mega Yac library. Positive YACs were mapped for the presence of rare-cutting restriction enzyme sites.
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10. PCR products were subject to SSCP PCR analysis according to conditions described by R. I. Richards and K. Friend [*J. Med. Genet.* **28**, 856 (1991)]. Normal Mendelian inheritance of this SSCP was demonstrated in a CEPH pedigree. Several *FRA16A* individuals were found to be heterozygous, indicating the presence of products from both chromosomes (*6*) and therefore excluding the region of instability from between the c and d PCR primers. Sequence analysis between the d primer and the Rsa I site (Fig. 1E) revealed a previously unidentified sequence deficient in repeat motifs (*6*).
11. The 2.7-kb Sac I–Not I fragment (encompassing the CpG island) from pf16A1 (Fig. 1D) was localized by FISH distal to *FRA16A* (*FRA16A* chromosomes signal scored 12 distal, 1 central, and 2 proximal). The 3.6-kb Not I–Sac I fragment [including the $p(CCG)_n$ repeat] from pf16A1 was localized by FISH proximal to *FRA16A* (*FRA16A* chromosomes signal scored 7 proximal, 1 central, and 1 distal). FISH analysis was not possible with whole YACs as probes because of the presence of duplicated sequences present on both sides of *FRA16A* (as evident by 1.79 hybridization) and also because of the presence of chromosome 16–specific repeat sequences.
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Receptor and Ligand Domains for Invasion of Erythrocytes by *Plasmodium falciparum*

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A 175-kilodalton erythrocyte binding protein, EBA-175, of the parasite *Plasmodium falciparum* mediates the invasion of erythrocytes. The erythrocyte receptor for EBA-175 is dependent on sialic acid. The domain of EBA-175 that binds erythrocytes was identified as region II with the use of truncated portions of EBA-175 expressed on COS cells. Region II, which contains a cysteine-rich motif, and native EBA-175 bind specifically to glycophorin A, but not to glycophorin B, on the erythrocyte membrane. Erythrocyte recognition of EBA-175 requires both sialic acid and the peptide backbone of glycophorin A. The identification of both the receptor and ligand domains may suggest rational designs for receptor blockade and vaccines.

The erythrocytic stage of *P. falciparum* is responsible for the death of an estimated 2 million children annually. Invasion of erythrocytes by malaria parasites requires parasite ligands and erythrocyte receptors (*1*, *2*). One ligand of *P. falciparum* is an 175-kD protein (EBA-175), the binding of which is dependent on sialic acid (*N*-acetylneuraminic acid) on the erythrocyte membrane (*3*, *4*). Evidence from a number of studies indicates that EBA-175 is a ligand for invasion (*3*–*8*), and antibodies against EBA-175 block merozoite invasion of erythrocytes in vitro (*6*). Heretofore, the binding domain on EBA-175 was unknown and its erythrocyte receptor had not been definitively identified. We report here both the identification of the domain of EBA-175 for erythrocyte binding and the erythrocyte receptor requirements for this binding.

To define the binding domain of EBA-175, we expressed different regions of EBA-175 (Camp clone) defined by homology between *P. falciparum*, *P. vivax*, and the related monkey parasite *P. knowlesi* (*8*) on the surface of COS cells as chimeric proteins using the

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