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Genetic Selection of a *Plasmodium*-Refractory Strain of the Malaria Vector *Anopheles gambiae*

FRANK H. COLLINS,* RICHARD K. SAKAI, KENNETH D. VERNICK, SUSAN PASKEWITZ, DOUGLAS C. SEELEY, LOUIS H. MILLER, WILLIAM E. COLLINS, CARLOS C. CAMPBELL, ROBERT W. GWADZ

The anopheline mosquito is the target in most malaria control programs, primarily through the use of residual insecticides. A mosquito was studied that is refractory to most species of malaria through a genetically controlled mechanism. A strain of *Anopheles gambiae*, which was selected for complete refractoriness to the simian malaria parasite *Plasmodium cynomolgi*, also has varying degrees of refractoriness to most other malaria species examined, including the human parasites *P. falciparum, P. ovale*, and *P. vivax* for which this mosquito is the principal African vector. Furthermore, the refractoriness extends to other subhuman primate malarias, to rodent malaria, and to avian malaria. Refractoriness is manifested by encapsulation of the malaria ookinete after it completes its passage through the mosquito midgut, approximately 16 to 24 hours after ingestion of an infective blood meal. Fully encapsulated ookinetes show no abnormalities in parasite organelles, suggesting that refractoriness is due to an enhanced ability of the host to recognize the living parasite rather than to a passive encapsulation of a dead or dying parasite. Production of fully refractory and fully susceptible mosquito strains was achieved through a short series of selective breeding steps. This result indicates a relatively simple genetic basis for refractoriness. In addition to the value these strains may serve in general studies of insect immune mechanisms, this finding encourages consideration of genetic manipulation of natural vector populations as a malaria control strategy.

MALARIA PERSISTS TODAY AS THE most important infectious disease in sub-Saharan Africa despite repeated attempts to control the vector mosquitoes with insecticides and massive programs for the distribution of antimalarial drugs (*1*). The rapid spread of multiple drug–resistant strains of *Plasmodium falciparum* now seriously threatens the population with increased sickness and death (*2*); alternatives to drugs as the major approach to malaria control in Africa must be found. Research on the construction of malaria vaccines is under way, but concerns about the delivery of vaccine and about parasite evasion mechanisms persist. Vector control is an attractive alternative, but conventional strategies have failed in Africa and new methods must be developed. The production of genetically defined lines of vector mosquitoes refractory to the development of the parasite and thus incapable of transmitting the infection is one possible method for controlling malaria.

Genetic variation in mosquito susceptibility has been recorded, and vector strains refractory to various avian and rodent malarias have been selected and studied (*3*). We have now established a defined form of refractoriness to the human *Plasmodium* species in a major vector of malaria. Study of the susceptibility of the G3 strain of *Anopheles gambiae* (colonized in Gambia in 1975) revealed reduced susceptibility to the simian malaria *P. cynomolgi* in a portion of the mosquitoes examined. In addition to a few or no normal oocysts, the midguts of these rare mosquitoes were covered with large numbers of small melanized structures (Fig. 1). The oocyst is the stage of the parasite that begins to form about 24 hours after the mosquito ingests an infected blood meal. After fertilization in the lumen of the midgut, the newly formed ookinetes invade the midgut epithelium and develop into oocysts wherein sporozoites, the infectious stage of the malaria parasite, are formed. Encapsulation leads to death of the parasite in the mosquito and thus inability to transmit the infection.

Selection against *P. cynomolgi* resulted in the production of two mosquito lines, one that is fully refractory and one that is fully susceptible to this parasite (*4*). Ultrastructural studies of the encapsulated bodies on the midgut of the mosquitoes revealed both heme pigment granules and subcellular features typical of normal *Plasmodium* parasites. The encapsulated parasites, which are physically located between the midgut epithelial cells and the surrounding noncellular basal lamina, are enclosed by an electron-dense matrix probably composed of the protein-melanin complex typical of the immunological encapsulation reaction described in other Diptera (*5, 6*). Neither hemocytes, often involved in capsule formation, nor their fragments are evident (Fig. 1). These two mosquito lines have continued to manifest these patterns of refractoriness and susceptibility for more than 40 generations without additional selection.

The encapsulation reaction is first detected 16 hours after the refractory mosquito has ingested an infective blood meal. Electron microscopy shows that a melanin-like substance usually begins to appear on the ookinete after it has traversed the gut wall.

Assessment of the refractoriness and susceptibility of the two lines to other strains and species of *Plasmodium* indicates that the mechanism of refractoriness is a general response to most malaria parasites. Refractoriness is manifested by encapsulation. The percentage of infected mosquitoes and the number of oocysts per infected mosquito were similar in the refractory and susceptible lines (Table 1). The refractory line is almost

F. H. Collins, R. K. Sakai, K. D. Vernick, D. C. Seeley, L. H. Miller, R. W. Gwadz, Malaria Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
S. Paskewitz, Department of Entomology, University of Georgia, Athens, GA 30602.
W. E. Collins and C. C. Campbell, Malaria Branch C-22, Division of Parasitic Diseases, Centers for Disease Control, Atlanta, GA 30333.

*To whom correspondence should be addressed at Malaria Branch C-22, Division of Parasitic Diseases, Centers for Disease Control, Atlanta, GA 30333.

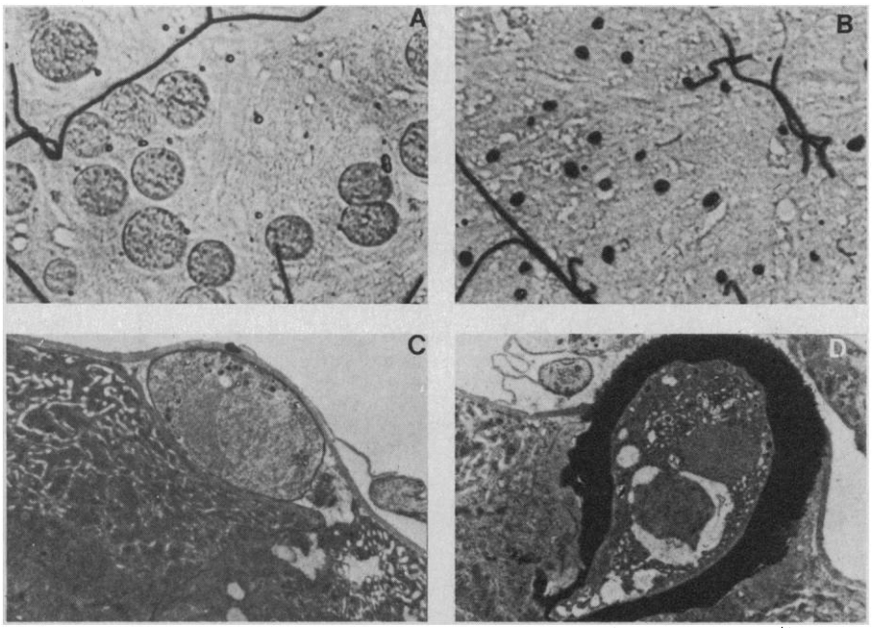

Fig. 1. (A) Normal oocysts of *Plasmodium cynomolgi* on the gut of a susceptible *Anopheles gambiae* female 6 days after the infectious blood meal. (B) Encapsulated oocysts of *P. cynomolgi* on the gut of refractory *An. gambiae* at 6 days. (C) Electron micrograph of a normal ookinete (oocyst) of *P. cynomolgi* lying beneath the basement lamina of the midgut of a susceptible *An. gambiae* 24 hours after a blood meal. (D) Encapsulated ookinete of *P. cynomolgi* beneath the basement lamina of a refractory *An. gambiae* 24 hours after a blood meal. An electron-dense melanin-like deposit surrounds the ookinete. Encapsulated ookinetes range between 5 and 10 μm in diameter.

fully refractory not only to a variety of simian *Plasmodium* species (including *P. cynomolgi, P. gonderi, P. inui*, and *P. knowlesi*) but also to rodent and avian parasites (Table 2). Although *An. gambiae* is not a natural vector of any of these parasites, the susceptible strain is fully susceptible to all of them and will support development of morphologically normal sporozoites.

When infected with human *Plasmodium* species, the refractory line exhibits a somewhat more variable response (Table 2). The line is highly or fully refractory to three isolates of *P. vivax* and one of *P. ovale*. Although the *P. vivax* isolates are all of Asian or South Pacific origin, the *P. ovale* isolate originated from an infection acquired in East Africa. Of six *P. falciparum* isolates examined, the refractory line fully encapsulates almost all ookinetes of two New World isolates from El Salvador and Brazil (SL and 7G8) and an isolate from Indochina (Indo3). It is moderately refractory to an isolate from Tanzania (TanI), but manifests only limited refractoriness to the two other African isolates examined (the Liberian clone LE5 and the physiologically and immunologically similar NF54 which originated in Holland of probable African origin). Limited data on the relative susceptibilities of the two lines to other *P. falciparum* isolates indicate that *P. falciparum* parasites from Africa are more successful in bypassing the encapsulation response than New World and Asian isolates. Sporogonic development of the quartan parasite of man, *P. malariae*, and its closely related South American relative in subhuman primates, *P. brasilianum*, is minimally inhibited in the refractory line (Table 2). The refractory *An. gambiae* are least effective in encapsulating coindigenous strains of the human malarias.

The genetic basis for refractoriness was evaluated by crossing the susceptible and refractory lines and feeding the offspring on the Ceylon strain of *P. cynomolgi*. F_1 hybrids of the refractory and susceptible lines appear phenotypically similar to susceptible individuals (Fig. 2). Although encapsulated oocysts were totally absent in the parental susceptible line, encapsulation of some parasites was present in F_1 individuals. If a single dominant gene controls susceptibility, then backcrosses of F_1 males or females to the refractory line would yield two distinct phenotypes, one corresponding to the F_1 with predominantly normal oocysts and the other to the refractory line with predominantly encapsulated ookinetes. Indeed, as shown in Fig. 2, two primary phenotypes emerge, one

Table 1. Comparative infectivity of various malarias in the susceptible and refractory lines of *Anopheles gambiae*.

Species of malaria	Strain	Source of mosquito infection	Susceptible line			Refractory line		
			Number fed	Infected (%)	Oocysts per gut [mean (range)]	Number fed	Infected (%)	Oocysts per gut [mean (range)]
P. berghei	NK	Mouse	56	91	17 (1–59)	55	80	9 (1–40)
P. gallinaceum	8A	Chicken	71	77	55 (1–330)	70	98	42 (1–112)
P. cynomolgi	Ceylon	Rhesus	137	100	251 (92–425)	116	100	131 (69–350)
	B	Rhesus	114	98	173 (11–375)	104	98	203 (15–400)
P. gonderi		Rhesus	42	98	37 (5–120)	36	100	25 (2–85)
P. inui	N34	Rhesus	93	96	60 (4–200)	76	96	44 (1–185)
P. knowlesi	H	Rhesus	96	97	50 (2–150)	107	97	42 (4–115)
P. vivax	NK	Aotus	91	58	5 (1–29)	118	48	4 (1–15)
	Chesson	Aotus	21	95	40 (8–113)	18	94	53 (2–129)
	ONG	Aotus	50	72	7 (1–21)	33	67	5 (1–20)
P. ovale	G	Human	27	96	26 (2–86)	34	94	21 (2–59)
P. falciparum	SL	Aotus	119	21	1 (1–6)	153	15	1 (1–5)
	7G8	Culture	57	28	4 (1–17)	57	37	2 (1–14)
	Indo3	Culture	47	38	3 (1–22)	59	27	2 (1–6)
	TanI	Culture	113	54	9 (1–39)	106	67	5 (1–57)
	LE5	Culture	88	17	3 (1–13)	100	20	2 (1–19)
	NF54	Culture	22	100	6 (1–18)	17	82	18 (3–49)
P. brasilianum	PI	Aotus	129	45	8 (1–94)	124	54	8 (1–60)
P. malariae	Uganda II	Aotus	121	14	2 (1–7)	126	15	126 (1–12)

Table 2. The frequency of normal and encapsulated oocysts of various malarias in the susceptible and refractory lines of *Anopheles gambiae*. Oocysts are referred to as "mixed" when both normal and encapsulated oocysts are present on the mosquito gut.

Species	Strain	Susceptible line			Refractory line		
		100% normal oocysts	Mixed oocysts	100% encapsulated oocysts	100% normal oocysts	Mixed oocysts	100% encapsulated oocysts
P. berghei	NK	100	0	0	0	2	98
P. gallinaceum	8A	100	0	0	0	0	100
P. cynomolgi	Ceylon	100	0	0	0	1	99
	B	92	8	0	0	0	100
P. gonderi		100	0	0	0	3	97
P. inui	N34	100	0	0	0	0	100
P. knowlesi	H	100	0	0	0	0	100
P. vivax	NK	100	0	0	0	7	93
	Chesson	95	5	0	0	6	94
	ONG	100	0	0	0	5	95
P. ovale	G	92	8	0	0	34	66
P. falciparum	SL	100	0	0	4	4	92
	7G8	100	0	0	5	5	90
	Indo3	100	0	0	6	6	88
	TanI	95	3	2	26	61	13
	LE5	100	0	0	85	5	10
	NF54	100	0	0	64	36	0
P. brasilianum	PI	100	0	0	75	22	3
P. malariae	Uganda II	100	0	0	95	5	0

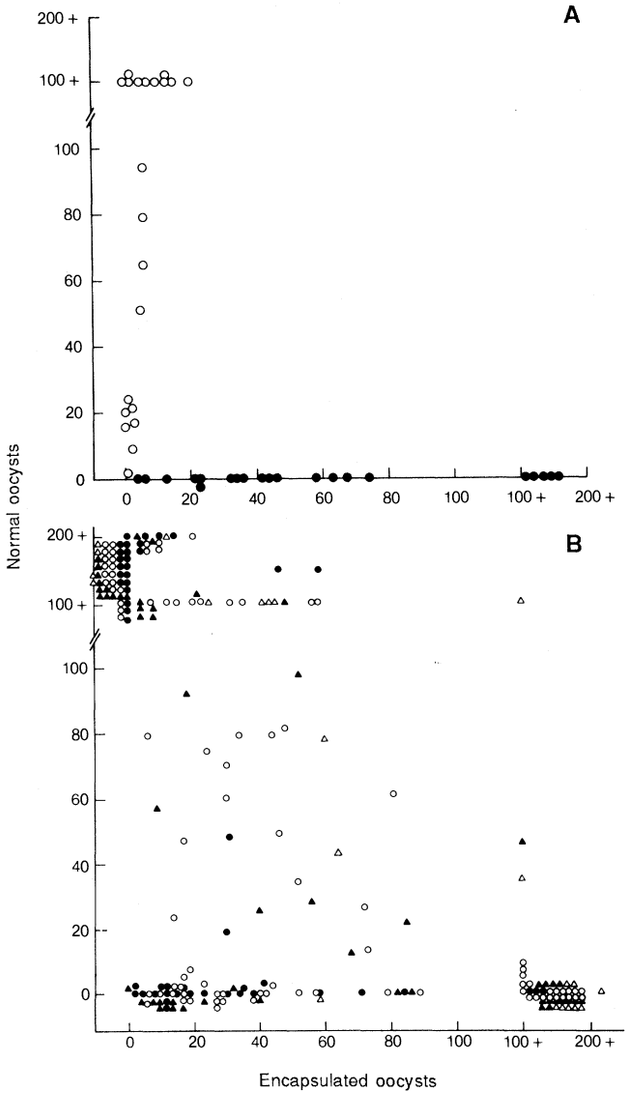

Fig. 2. (A) The number of normal oocysts plotted against the number of encapsulated oocysts in F_1 females (○) of susceptible-refractory (S/R) and refractory-susceptible (R/S) crosses, and in refractory line females (●) of *Anopheles gambiae*. (B) The number of normal oocytes plotted against the number of encapsulated oocysts in the four backcrosses of F_1 males and females to refractory line males and females. (●) SR/R; (○) RS/R; (▲) R/RS; (△) R/SR.

refractory and the other susceptible. However, a third group of intermediate individuals not clearly assignable to either group is also present. The appearance of this group prevents the clear determination of monofactorial inheritance of refractoriness; these intermediate forms could be caused by minor genes from the refractory line modifying the expression of the heterozygous genotype. A backcross of F_1 progeny to the susceptible line yielded only fully susceptible females.

Attempts to define the precise genetic basis for refractoriness have been complicated by a lack of genetic markers linked to the various characters. However, the limited number of selection steps required to establish these lines and the specific form in which refractoriness is manifested indicate a mechanism of limited physiological and genetic complexity. Evidence that the capacity to encapsulate is present in heterozygotes indicates that refractoriness is not fully recessive.

Malaria control has been primarily through methods that affect the anopheline mosquito vectors. An approach for the future may be the introduction of genes for refractoriness to malaria into vector populations.

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4. The following protocol was used in selecting the two sublines of the G3 colony of *An. gambiae* for *Plasmodium* refractoriness and susceptibility to the simian malaria parasite *P. cynomolgi*. Seventy-five G3 females infected with *P. cynomolgi* (B) were allowed to oviposit individualy and subsequently dissected to assess the proportion of parasites, in each mosquito, that were encapsulated or that developed normally. Two lines were established by pooling eggs from five mosquitoes that showed the highest level of refractoriness and six mosquitoes that showed the highest level of susceptibility. (In this and the following selection steps, there was also a selection for large numbers of parasites encapsulated in the refractory line and normal in the susceptible line.) Selection of the refractory line was achieved as follows. After a generation of amplification without selection, refractory-line females were infected with *P. cynomolgi* (Lond) and eggs from the five most refractory individuals among 50 were pooled for the next generation. This same procedure was repeated one more time with *P. cynomolgi* (Lond)–infected mosquitoes. These selection steps were followed by three generations of isofemale line selection where, for each selection event, ten isofemale lines were established, infected with *P. cynomolgi* (Lond), allowed to lay eggs, and then assessed for level of refractoriness by dissecting at least 20 females. (Isofemale lines that did not produce enough infected females were not used, and lines that showed evidence of poor fitness were not assessed for susceptibility.) The line that had the largest proportion of fully refractory females was kept for the next round of selection. By the third isofemale line–selection step, all lines were fully refractory to *P. cynomolgi* (Lond). Selection of the susceptible line was accomplished in a similar manner except that the parasite

P. cynomolgi (B) was used. One selection event involving the pooling of eggs from the five most susceptible females in a group of 50 examined was followed by four isofemale line–selection steps. Seven of the ten isofemale lines examined at the fourth isofemale line–selection step were virtually fully susceptible to *P. cynomolgi* (B). These two strains of *P. cynomolgi* were used because the G3 stock was highly refractory to the NIH strain and highly susceptible to the Lond strain of the parasite. Therefore, selection for refractoriness was felt to be more stringent if Lond was used and selection for susceptibility more stringent with *P. cynomolgi* (B). By our definition, an infected mosquito was a female with normal or encapsulated oocysts (or both) on the gut.

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Isolation and Sequence of L3T4 Complementary DNA Clones: Expression in T Cells and Brain

BÉATRICE TOURVIEILLE, SCOTT D. GORMAN, ELIZABETH H. FIELD, TIM HUNKAPILLER, JANE R. PARNES

T lymphocytes express on their surface not only a specific receptor for antigen and major histocompatibility complex proteins, but also a number of additional glycoproteins that are thought to play accessory roles in the processes of recognition and signal transduction. L3T4 is one such T-cell surface protein that is expressed on most mouse thymocytes and on mature mouse T cells that recognize class II (Ia) major histocompatibility complex proteins. Such cells are predominantly of the helper/inducer phenotype. In this study, complementary DNA clones encoding L3T4 were isolated and sequenced. The predicted protein sequence shows that L3T4 is a member of the immunoglobulin gene superfamily. It is encoded by a single gene that does not require rearrangement prior to expression. Although the protein has not previously been demonstrated on nonhematopoietic cells, two messenger RNA species specific for L3T4 are found in brain. The minor species comigrates with the L3T4 transcript in T cells, whereas the major species is 1 kilobase smaller.

MATURE MOUSE T LYMPHOCYTES can be divided into two subsets by their expression of the alternative T-cell differentiation antigens L3T4 (CD4 in humans) and Lyt-2 (CD8 in humans). The L3T4 subset consists predominantly of helper/inducer T cells and correlates best with recognition by T cells of class II (Ia) major histocompatibility complex (MHC) molecules (*1–3*). The Lyt-2 subset is made up primarily of cytotoxic and suppressor T cells and correlates best with recognition of class I (H-2K, D, or L) MHC molecules (*3, 4*). Monoclonal antibodies specific for L3T4 or Lyt-2 inhibit the functional activity (cytotoxicity, proliferation, lymphokine release) of T cells that bear these proteins (*1–6*). It has been postulated that L3T4 and Lyt-2 play a role in increasing the avidity of the interaction between T cells and antigen-presenting cells or target cells, perhaps by binding to nonpolymorphic regions of class II and class I MHC proteins, respectively (*2, 3, 6, 7*). It has alternatively been postulated that monoclonal antibodies specific for L3T4 (or CD4) inhibit function by directly transmitting a negative signal to the T cell (*8*). Although Lyt-2 and CD8 are normally known to be expressed only on particular subsets of thymocytes, T cells, and natural killer cells, CD4 has also been shown to be expressed by normal cells of the monocyte/macrophage and Langerhans lineages in both humans and rats (*9*). However, none of these proteins has been reported to be expressed on normal nonhematopoietic cells.

The genes encoding human CD8, CD4, and mouse Lyt-2 have been recently cloned, and their predicted amino acid sequences have revealed that they are evolutionarily related to immunoglobulin (Ig) variable (V) regions (*10–14*). We have now cloned the complementary DNA (cDNA) encoding mouse L3T4 and show that it too is a member of the Ig gene superfamily. We further show that the gene is expressed not only in T-lineage cells but also in brain and that the size of the mRNA in brain is different from that in T cells.

To isolate mouse L3T4 cDNA clones we screened a C57BL/Ka mouse thymocyte cDNA library with a full-length human CD4 cDNA clone (*12*) used as probe. Two mouse clones that hybridized to the human clone were isolated. The nucleotide sequence of the one (pcL3T4-C7) with the longer insert (1.3 kb) was determined (Fig. 1). Because this clone did not contain the 5′ end, it was used as a probe to isolate from the same library an additional cDNA clone (pcL3T4-14) that extended farther in the 5′ direction. The nucleotide sequence of the 5′ untranslated region, the leader, and the first ten amino acids of the mature protein were therefore determined from pcL3T4-14 (Fig. 1). The nucleotide sequence shown in Fig. 1 predicts a mature protein of 435 amino acids (predicted molecular size 48,853 daltons), with 372 amino acids external to the cell, a 25–amino acid hydrophobic transmembrane region, and a 38–amino acid highly basic cytoplasmic domain. The mature protein sequence is preceded by a 22–amino acid hydrophobic leader or signal peptide as is typically found at the NH_2-terminus of cell surface and secreted proteins. The point of cleavage of this leader was predicted by comparison with other published leader sequences.

As expected, the nucleotide sequence of the L3T4 cDNA was homologous to that of the human CD4 clone with which it was selected, and the encoded protein was also closely related (Fig. 2A). The most highly conserved region was the cytoplasmic domain (79% at the amino acid level), which may play a role in signal transduction. In contrast, the external portions of L3T4 and CD4 contained only 55% identical residues. This latter finding is similar to our previous results comparing the mouse (Lyt-2) and human (CD8) sequences of the alternative T-cell differentiation marker (*13*). The mouse L3T4 protein has four predicted *N*-linked glycosylation sites (Asn-X-Thr or Asn-X-Ser) at residues 165, 276, 301, and 370, as compared to only two in the human CD4 (*12*).

We and others observed previously that CD8 and Lyt-2 have NH_2-terminal external domains that are homologous to the Ig light chain V regions (*11, 13, 14*). A similar relation has been found for human CD4 (*12*). We therefore searched a series of data banks with the L3T4 sequence to see whether similar or additional homologies could be found. These computer comparisons indicated that L3T4 is also a member of the Ig gene superfamily. The NH_2-terminal domain of the mature protein (90 to 101 amino acids, depending on where one arbitrarily sets the border) is homologous to Ig V regions, with the greatest similarity being to light chain V regions, especially κ (up to 35%) (Fig. 2B). This domain of L3T4 has the two cysteines (residues 20 and 90) that form the characteristic disulfide loop of Ig-like homology units, as well as the structurally important tryptophan (residue 32) that is always found 12 to 15 residues downstream from the first cysteine of the disulfide

B. Tourvieille, S. D. Gorman, E. H. Field, J. R. Parnes, Department of Medicine, Division of Immunology, Stanford University Medical Center, Stanford, CA 94305.
T. Hunkapiller, Division of Biology, California Institute of Technology, Pasadena, CA 91125.