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Crystallization of Amino-terminal Domains and Domain Fragments of Variant Surface Glycoproteins from *Trypanosoma brucei brucei**

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Crystals were produced from variant surface glycoproteins (VSG) of *Trypanosoma brucei brucei* antigenic variants MITat 1.2, 1.6, and ILTat 1.22, 1.23, 1.24, 1.25, and 1.26. Purified VSGs had molecular weights from 60,000 to 68,000 on sodium dodecyl sulfate-polyacrylamide gels, whereas the crystals obtained were composed of polypeptides of approximate M_r 40,000–50,000. Amino-terminal amino acid sequences determined from the crystallized VSGs were identical to sequences obtained from the respective intact proteins, indicating that the crystals contained VSG amino-terminal fragments. Crystallization conditions and lattice dimensions of the crystals are given.

Trypanosomes are parasitic protozoa, usually transmitted to vertebrates by the bite of an infected tsetse fly, leading in man to the development of sleeping sickness and in cattle to the wasting disease Nagana. The trypanosomes are able to survive in the bloodstream of the vertebrate host through the process of antigenic variation. Each individual parasite is covered with a monolayer of about 10^7 identical glycoprotein molecules (1), visible in transmission electron microscopy as an electron-dense layer some 12–15 nM thick (2). By expressing genes encoding different glycoprotein sequences, trypanosomes modulate their antigenic profile such that under selective pressure from the immune system, antigenically different populations develop in waves (3). The glycoproteins are known as variant surface glycoproteins (VSGs).[1] VSGs are bound to the plasma membrane through a carboxyl-terminal glycophosphatidylinositol (4, 5). Rupture of the trypanosome activates a lipase (6) which removes diacylglycerol from the glycolipid (7) to release the VSG in a water soluble form which may be readily purified to homogeneity (1, 8). Sequence analyses of VSGs, or of their genes, have shown very limited amino-terminal sequence similarities. However, within the carboxyl-terminal 50 or so amino acids, sufficient homologies emerge to allow classification into two sequence classes (9, 10). VSGs are dimers in solution (11, 12), which may be cleaved by proteases to produce fragments containing an amino-terminal domain (13). For MITat 1.6, a well characterized variant, the major proteolytic product was an amino-terminal fragment carrying antigenic determinants common to the intact trypanosome (14). Here we describe the crystallization of amino-terminal fragments of VSGs of *Trypanosoma brucei brucei* produced by the action of an endogenous protease.

MATERIALS AND METHODS

Derivation of the trypanosome MITAR-1 serodeme is described in Ref. 1 and the ILTAR-1 serodeme in Ref. 15. MITat variants 1.2 and 1.6 were previously referred to as 221 and 121, respectively (1). Soluble VSGs were prepared according to Cross (1) and stored at −20 °C in lyophilized form.

The crystals were initially grown by vapor diffusion in hanging drops (16). Lyophilized proteins were rehydrated to a concentration of 40 mg/ml in 20 mM Tris, pH 7.5, containing 0.1% sodium azide. Drops containing 2 μl of protein suspension and 2 μl of well solution were equilibrated against wells containing various precipitants. Compositions of well solutions used in successful trials are shown in Table I.

Crystals of MITat 1.2 grown by the hanging drop method were difficult to harvest without cracking and showed up to 2% variability in unit cell dimensions. To overcome these problems, lyophilized protein was rehydrated to a concentration of 40 mg/ml in 200 mM Tris-HCl, pH 7.5, containing 8% polyethylene glycol 8000 and 0.1% sodium azide and equilibrated in 20-μl dialysis buttons (Cambridge Repetition Engineering, Cambridge, United Kingdom) against 10–12% polyethylene glycol 8000 in the same buffer. Within 2 weeks crystals appeared in each button, growing on the surface of the dialysis membrane. They were dislodged by delicate prodding, and the dialysis buttons were immersed in buffer used for harvesting crystals (200 mM Tris-HCl, pH 7.5, containing 20% polyethylene glycol 8000 and 0.1% sodium azide). Crystals prepared by this method were suitable for high resolution x-ray data collection.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli (17). Amino-terminal amino acid sequences of intact VSGs and protein from dissolved crystals were determined by automated gas phase Edman degradation (18, 19). In order to use crystals for sequence or SDS-PAGE analysis it was necessary to carefully wash them to remove associated protein. Each crystal was drawn into a Pasteur pipette, allowed to sink, and, when it reached the tip of the pipette, was inspected to ensure that no precipitated protein was attached. The crystal was then placed in a 20-μl dialysis button and dissolved by dialysis against H_2O containing 0.1% sodium azide.

For x-ray analyses, crystals were mounted in glass capillaries (Charles Supper) and photographed on a Supper Model 3000 preces-

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[1] The abbreviations used are: VSGs, variant surface glycoproteins; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; MITat, Molteno Institute trypanosome antigen type; ILTat, ILRAD trypanosome antigen type.

TABLE I

Crystallization conditions and crystal dimensions

All wells contained 0.1% sodium azide. PEG, polyethylene glycol; SAS, saturated ammonium sulfate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Bis/Tris, 2-bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane.

Variant	Reservoir	Morphology	Maximum crystal dimensions
			mm
MITaT 1.2	8.4% PEG 8000, 20 mM Tris, pH 7.5, 7% ethylene glycol	Bipyramidal	1.0
	20% PEG 1450, 10 mM sodium borate, pH 7.4		
	20% PEG 3350, 10 mM sodium borate, pH 7.4		
MITat 1.6	80% SAS, 50 mM Tris, pH 6.5	Bipyramidal	0.8
ILTat 1.22			
Type I	55–57% SAS, 21.5 mM Tris, pH 7.5	Bipyramidal	0.5
Type II	54% SAS, 20 mM Tris, pH 7.4	Sheets	0.3
ILTat 1.23	40% SAS, 20 mM Tris, pH 7.4	Twinned plates	0.2
ILTat 1.24			
Type I	60% SAS, 50 mM Mes, pH 6.5	Bipyramidal	0.5
Type II	60% SAS, 50 mM Mes, pH 6.5	Needles	1.0
ILTat 1.25			
Type I	42% SAS, 100 mM Bis/Tris, pH 6.5	Rhomboidal	0.1
Type II	82% SAS, 20 mM Tris, 50 mM Mes, pH 6.5	Hexagonal	0.1
Type III	82% SAS, 20 mM Tris, 50 mM Mes, pH 6.5	Rhomboidal	0.2
Type IV	82% SAS, 20 mM Tris, 50 mM Mes, pH 6.5	Needles	0.1
ILTat 1.26			
Type I	17% PEG 8000, 10 mM Tris, pH 7.5	Sheets	0.2
Type II	62% SAS, 50 mM Tris, pH 7.5	Needles	0.1

sion camera with CuKα radiation produced by an Elliot rotating anode x-ray generator operated at 40 kV and 20 mA. To determine crystal lattices, screenless precession photographs were taken at a crystal to film distance of 100 mm. Still and oscillation photographs were used to estimate crystal resolution. The 6-Å structures of ILTat 1.24 and MITat 1.2 were solved as described previously (20, 21).

RESULTS AND DISCUSSION

MITat 1.2—It was previously reported that MITat 1.2 crystals contained protein of approximately 43 kDa, whereas freshly purified MITat 1.2 migrated as 60 kDa on SDS-PAGE (21). The 43-kDa polypeptide resulted from proteolysis by an endogenous, uncharacterized protease, but a similar sized proteolytic fragment was observed after proteolysis of MITat 1.2 by trypsin (21). Amino-terminal sequence analysis established that that 43-kDa fragment had the same amino terminus as intact (60 kDa) MITat 1.2 (Table II), indicating that it was the amino-terminal "domain" as described for other VSGs by Johnson and Cross (13). Large-scale trypsin proteolysis of MITat 1.2 yielded sufficient quantities of the 43-kDa fragment for crystallization, and isomorphous crystals grew under identical conditions as observed for the fragment produced by endogenous protease. This method is currently used for production of crystallizable MITat 1.2, and solution of its x-ray structure to 3.0 Å is in progress.[2] Among the VSG variants, MITat 1.2 was easiest to crystallize and several conditions were found (Table I).

TABLE II

Relative molecular weights and amino-terminal sequences of intact and crystallized VSGs

Variant	Relative molecular weights		Amino-terminal amino acid sequence	
	Crystal	Intact	Crystal	Intact
	kDa			
MITat 1.2	41.7	52.3	AAEK	AAEKGF
MITat 1.6	51.3	64.6	TDKGAI	TDKGAI
ILTat 1.22				
Type I	51.3	63.1	*X*KNKAS	TKNKAS
Type II	30.2			
ILTat 1.23	30.0	44.7		AAGDN
ILTat 1.24	40.7	56.2	T*X*FGVKYELW	TXFGVK
ILTat 1.25				
Type I	36.3	50.1		*XX*PTTA
Type II	23.5			
Type III	23.5		AAPTTA	
ILTat 1.26	39.8	60.0	TAKAPL[a]	

[a] The ILTat 1.26 crystal sequence is for the 39.8-kDa component of a VSG preparation that crystallized. Other data were taken from washed crystals.

TABLE III

Unit cell dimensions, space groups, and resolutions of VSG crystals

The unit cell and resolution units are Ångstroms. The V_m units are cubic ångstroms/dalton. The number of monomers per crystal asymmetric unit assumed in calculating V_m is given in parentheses.

Variant	Space group	Unit cell dimensions				V_m	Resolution
MITat 1.2	$P4_12_12$	96.1	96.1	110.5		3.0 (1)	2.9
MITat 1.6	$P4_12_12$[a,b]	138	138	208		2.4 (4)	9
ILTat 1.22	$P4_12_12$[a]	83	83	121		2.0 (1)	3.5
ILTat 1.24	$P2_12_12_1$	55.1	98.9	172.9		2.9 (2)	2.7
ILTat 1.25							
Type I	C2	107	122	82	$B = 102$	3.7 (2)	2.5
Type II	$P6_3$	126	126	235		3.3 (8)	5.6
Type III	I4	176	176	87		3.6 (8)	2.8

[a] Or the enantiomorph $P4_32_12$.

[b] The assignment of the axial symmetry of MITat 1.6 is tentative because of the poor diffraction.

MITat 1.6—MITat 1.6 was the first VSG variant to be crystallized.[3] Large crystals were produced (0.8 mm, Table I), but they diffracted poorly to approximately 9-Å resolution. The protein in washed crystals migrated as 51.3 kDa on SDS-PAGE, whereas intact MITat 1.6 migrated as 64.6 kDa.

ILTat 1.22—The protein originally set up in hanging drops was 63.1 kDa on SDS-PAGE and initially grew type I crystals which contained protein migrating as 51.3 kDa on SDS-PAGE. Solutions of ILTat 1.22 kept approximately 1 year at 4 °C underwent proteolysis to produce fragments of 51.3, 30.2, 23.4, and 18.2 kDa. Bipyramids (type I) and thin sheet (type II) crystals formed in hanging drops equilibrated against 54% saturated $(NH_4)_2SO_4$ and 20 mM Tris, pH 7.5. Type II crystals were composed of the 30.2-, 23.4-, and 18.2-kDa fragments. The amino-terminal sequences of the original intact VSG and the protein obtained from type I crystals were identical (Table

[2] D. Freymann, J. Down, P. Metcalf, M. Turner, and D. C. Wiley, unpublished data.

[3] B. A. Foster, P. J. Bjorkman, M. J. Turner, and D. C. Wiley, unpublished data.

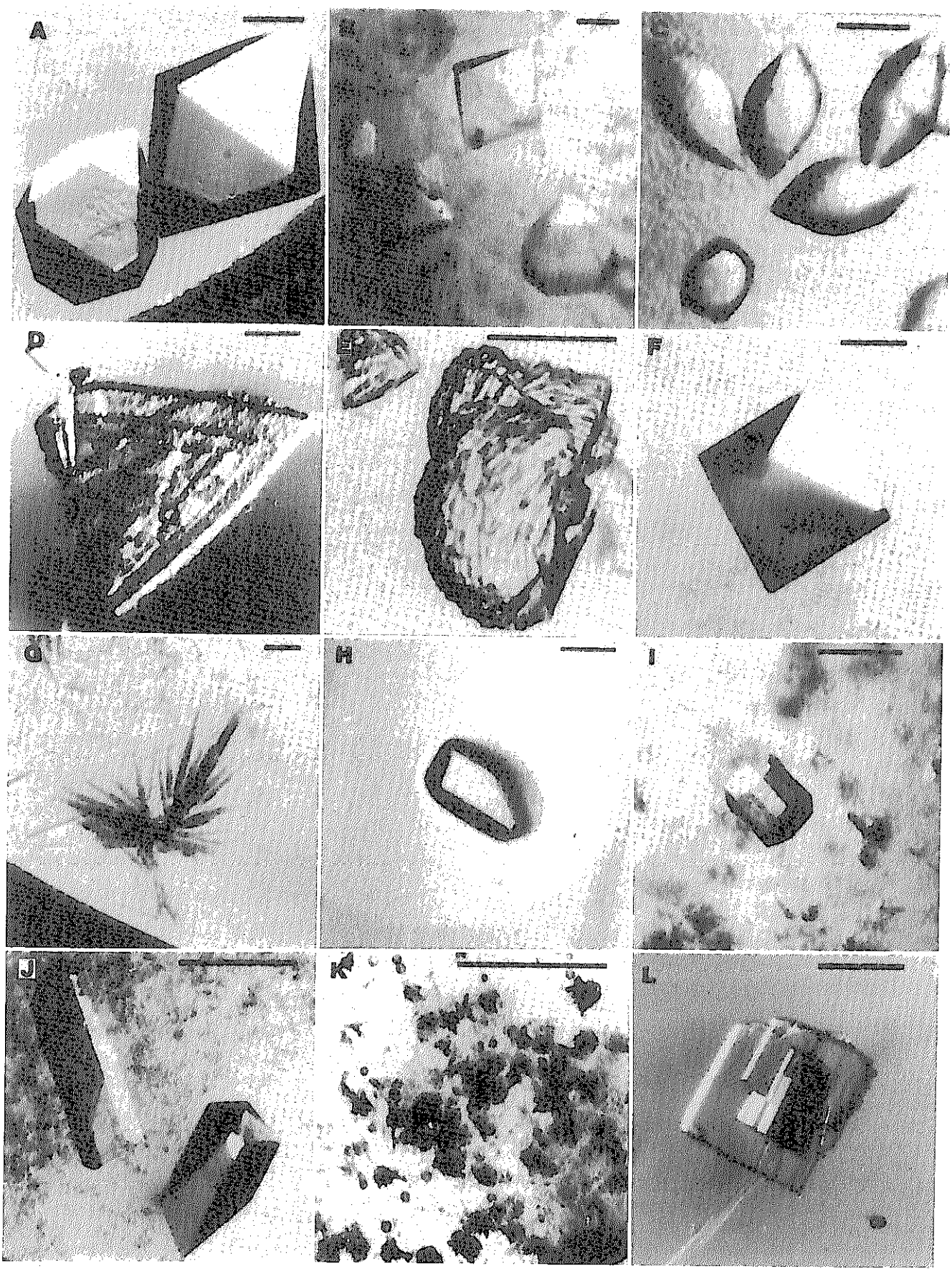

FIG. 1. **Photomicrographs of VSG crystals.** Photomicrographs were shot on a Nikon FE2 camera and Microflex PFX attached to a Nikon SMZ-2T microscope with Kodak Panatomic-X film. The *black horizontal bars* indicate 100-μm scale. *A*, MITat 1.2; *B*, MITat 1.6; *C*, ILTat 1.22, type I; *D*, ILTat 1.22, type II; *E*, ILTat 1.23; *F*, ILTat 1.24, type I; *G*, ILTat 1.24, type II; *H*, ILTat 1.25, type I; *I*, ILTat 1.25, type II; *J*, ILTat 1.25, type III; *K*, ILTat 1.25, type IV; *L*, ILTat 1.26, type I.

II). Insufficient quantities of the second crystal form were obtained to allow sequence analysis.

ILTat 1.23—Immediately after purification, ILTat 1.23 relative migrated with a molecular mass of 44.7 kDa, which was notably smaller than the 60–68 kDa observed for the other variants. The fragment produced by endogenous proteolysis was also smaller: 30 kDa compared to 40–50 kDa observed for the other VSGs. Amino-terminal sequence analysis of the initially purified ILTat 1.23 mixture was uninterpretable, a finding consistent with the presence of a "ragged" amino terminus and not seen in the other variants studied. After further purification of the intact 45-kDa ILTat 1.23 by gel permeation high performance liquid chromatography on a Waters Protein-Pak 300 column and Western transfer to polyvinylidene difluoride membranes (22), gas phase sequencing yielded the following amino-terminal sequence: Ala-Ala-Gly-Asp-Asn. Small, highly twinned crystals grew after three months at 20 °C (Fig. 1), but, to date, insufficient quantity has hindered their x-ray and sequence analyses.

ILTat 1.24—Crystals of ILTat 1.24 diffracted to 2.7 Å (Table III). The 6-Å resolution structure was reported (20), and solution of the x-ray structure to 3-Å resolution is in progress.[4] Unlike MITat 1.2, trypsin proteolysis of ILTat 1.24 did not produce fragments that crystallized like the respective amino-terminal domains produced by endogenous proteolysis. Experiments using commercially available proteases also did not produce crystallizable amino-terminal domain from intact ILTat 1.24.

ILTat 1.25—Four crystal forms of ILTat 1.25 were produced, SDS-PAGE analysis of crystal form I showed a relative molecular mass of 37 kDa, compared to 68 kDa for the intact ILTat 1.25. Despite several attempts, gas phase sequence analysis of protein from type I crystals was not interpretable. Crystal forms II, III, and IV were produced from a mixture of ILTat 1.25 that had broken down to a protein fragment of approximately 23.5 kDa after storage at 4 °C for 1 year. Gas phase Edman degradation of form II crystals yielded equal proportions of the following amino-terminal sequences: Ala-Ala-Pro-Thr-Thr-Ala-, identical to the sequence obtained from the intact ILTat 1.25 (Table II) and Gly-Glu-Asn-Thr-Glu-Ser-, corresponding to approximately amino acid residue 140 as deduced from the cloned cDNA sequence.[5] The amino-terminal domains of MITat 1.2 and ILTat 1.24 were also observed to break down to polypeptides of approximately 23 kDa as a result of endogenous protease activity, but crystallization trials on the small quantities produced to date have not produced crystals.

ILTat 1.26—Needles and sheets were produced of ILTat 1.26 under the conditions tested. The relative molecular mass of the crystallized fragment was 39.8 kDa, whereas the intact protein migrated as 60.0 kDa on SDS-PAGE (Table II).

This report describes crystallization and crystal properties of seven variant surface glycoproteins from *T. brucei brucei*. For crystals from which amino acid sequence data could be obtained, the amino-terminal sequences of the crystallized and intact species were identical. All the VSGs produced crystals containing polypeptides smaller than the original proteins and ranging in size from 37 to 50 kDa. Johnson and Cross (13) observed that similar sized fragments resulted from trypsin digestion of VSGs. They suggested that the amino and carboxyl termini of VSGs are structurally distinct, with the amino terminus being relatively resistant to trypsin and the carboxyl terminus being susceptible to proteolysis. Our results suggest that an endogenous protease in the VSG preparations liberated the amino-terminal portions of the VSGs, which subsequently crystallized. For ILTat variants 1.22 and 1.25, smaller fragments were also crystallized, and for ILTat 1.25 amino acid sequencing confirmed that the crystallized fragment contained the amino terminus. Although all the variants that were studied produced crystallizable fragments, none of those for which sequence data could be obtained contained the carboxyl terminus.

The enzyme responsible for VSG proteolysis was not identified, and its origin (*e.g.* trypanosome or rat serum) remains uncertain. Cross (8) described a protease in *T. brucei brucei* that produced a similar pattern of VSG proteolysis as observed here. However, the protease described by Cross (8) bound DE52 and was inhibited by Zn^{2+} or TLCK. In this study, the VSGs were purified through DE52, and attempts to stop protease activity with Zn^{2+} were not successful. TLCK inhibited or greatly diminished VSG proteolysis.[6] This observation and the ability of trypsin to produce crystallizable amino-terminal domain of MITat 1.2 may indicate that the endogenous protease has a substrate specificity similar to trypsin.

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REFERENCES

1. Cross, G. A. M. (1975) *Parasitology* **71,** 393–417
2. Vickerman, K. (1969) *J. Cell. Sci.* **5,** 163–193
3. Van der Pleog, L. H. T. (1987) *Cell* **51,** 159–161
4. Ferguson, M. A. J., Haldar, K., and Cross, G. A. M. (1984) *J. Biol. Chem.* **260,** 4963–4968
5. Ferguson, M. A. J., Low, M., and Cross, G. A. M. (1985) *J. Biol. Chem.* **260,** 14547–14555
6. Cardoso de Almeida, M. L., and Turner, M. J. (1983) *Nature* **302,** 349–352
7. Hereld, D., Krakow, J. L., Bangs, J. D., Hart, G. W., and Englund, P. T. (1986) *J. Biol. Chem.* **261,** 13813–13819
8. Cross, G. A. M. (1984) *J. Cell. Biochem.* **24,** 79–90
9. Holder, A. A., and Cross, G. A. M. (1981) *Mol. Biochem. Parasitol.* **2,** 135–150
10. Rice-Ficht, A. C., Chen, K. K., and Donelson, J. E. (1981) *Nature* **294,** 53–57
11. Auffret, C. A., and Turner, M. J. (1981) *Biochem. J.* **193,** 647–650
12. Strickler, J. E., and Patton, C. L. (1982) *Exp. Parasitol.* **53,** 117–131
13. Johnson, J., and Cross, G. A. M. (1979) *Biochem. J.* **178,** 689–697
14. Miller, E. N., Allen, L. M., and Turner, M. J. (1984) *Mol. Biochem. Parasitol.* **13,** 309–322
15. Miller, E. N., and Turner, M. J. (1981) *Parasitology,* **82,** 63–80
16. McPherson, A. (1985) *Methods Enzymol.* **114,** 112–125
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685
18. Hunkapiller, M. W., Lujan, E., Dreyer, J. D., and Hood, L. E. (1983) *Methods Enzymol.* **91,** 399–413
19. Hunkapiller, M. W., Lujan, E., Ostrander, F., and Hood, L. E. (1983) *Methods Enzymol.* **91,** 227–236
20. Metcalf, P., Blum, M., Freymann, D., Turner, M. J., and Wiley, D. C. (1986) *Nature* **325,** 84–86
21. Freymann, D., Metcalf, P., Turner, M. J., and Wiley, D. C. (1984) *Nature* **311,** 167–169
22. Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038

[4] M. Blum, M. Turner, and D. C. Wiley, unpublished data.

[5] M. Carrington and M. J. Turner, unpublished data.

[6] J. A. Down and A. Gurnett, unpublished data.