# *Trypanosoma congolense*: Structure and Molecular Organization of the Surface Glycoproteins of Two Early Bloodstream Variants†

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ABSTRACT: The complete primary structures of two variant specific glycoproteins (VSGs) of the nannomonad *Trypanosoma* (*N.*) *congolense* are presented. These coat proteins subserve the function of antigenic variation. The secondary structure potentials of both VSGs have been calculated. The amino acid sequences and secondary structure potentials of these VSGs have been compared with the primary structures and secondary structure potentials of several *Trypanosoma brucei* complex VSGs. In homologous regions, the *T. brucei* complex VSGs show a pattern of sharply contrasting secondary structure potentials. It has been suggested previously that this pattern gives rise to different folding structures in different members of this polygene protein family. Thus, different short regions of the polypeptide sequence are exposed as antigenic "caps" on the solvent-exposed surface of intact trypanosomes. A sharply contrasting secondary structure potential pattern is also found in regions of the two *T. congolense* VSGs. However, there is little homology of primary structure between each of the two *T. congolense* VSGs and any member of the *T. brucei* complex VSG polygene family whose primary structure has been determined.

The variant specific glycoproteins (VSGs)[1] of the African trypanosomes comprise a highly antigenic surface coat covering the parasitic organism. Expression of alternative VSGs, having different primary structures, results in the phenomenon of antigenic variation. In relapsing waves of parasitemia, the majority of parasites in each wave is covered by a different, antigenically non-cross-reactive VSG. It is believed that antigenic variation, i.e., the ability to produce new populations of parasites not recognized by antibodies to previous trypanosome populations, is the key mechanism which permits trypanosomes to establish themselves in their mammalian host. In addition to primary structure alterations, there is preliminary evidence that antigenic variation is also accomplished by restricting the region of the protein which may be exposed to the solvent-accessible exterior of the organism and that the exposed region of the primary structure may differ in different serotypes (Lalor et al., 1984; Cohen et al., 1984).

It is known that the strategy of antigenic variation is not confined to trypanosomes and that prokaryotes such as spirochetes and salmonella are capable of changing their surface coats (Stoenner et al., 1982; Silverman et al., 1979). We here present data on the amino acid sequences and secondary structure potentials of two *Trypanosoma congolense* variant specific glycoproteins. We discuss the structural basis of antigenic variation in light of these data.

## Materials and Methods

*Trypanosomes and VSG Isolation.* The Lister 1/148 stock of *T. congolense* and details of the cell cloning and fly passages have been previously described (Rosen et al., 1981). The *T. congolense* variants used have been given the acronym Yale (Nannomonas) antigen types 1.1 and 1.3 (YNat 1.1 and 1.3). An earlier set of papers (Rosen et al., 1981; Onodera et al., 1981; Bogucki et al., 1982) referred to YNat 1.1 and 1.2. The YNat 1.1 variant is the identical variant described in the cited papers. However, the YNat 1.2 variant was lost. The variant YNat 1.3 described in this paper was isolated independently from another BALB/c mouse which had been infected with YNat 1.1 and whose infection was allowed to relapse once only. Before the current study was begun, YNat 1.1 and YNat 1.3 were recloned by the triple-cloning procedure (Rosen et al., 1981). Following two expansions in irradiated rats, working stabilates of the clone were maintained in the vapor phase of a liquid nitrogen freezer. Thus, populations used for protein isolation were all derived from the third passage of the parasite

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[1] Abbreviations: VSG, variant specific glycoprotein; YNat, Yale Nannomonas antigen type; AnTat, Antwerp Trypanozoon antigen type; ILTat, International Laboratory Trypanozoon antigen type; MITat, Molteno Institute Trypanozoon antigen type; IoTat, Iowa Trypanozoon antigen type; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; CNBr, cyanogen bromide; Tris-HCl, 2-amino-2-(hydroxymethyl)aminomethane hydrochloride; ATP, adenosine 5′-triphosphate; dNTP, deoxyadenosine, deoxyguanosine, thymidine, or deoxycytidine 5′-triphosphate; SDS, sodium dodecyl sulfate; APS, aminopolystyrene; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; UTR, untranslated region; ds, double stranded; IEF, isoelectric focusing; bp, base pair(s).

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grown in lethally X-irradiated outbred, male retired breeder Sprague-Dawley rats. Infected blood was obtained by either cardiac puncture or aortic cannulation and perfusion (Rosen et al., 1978) during late log phase of the parasite's growth. Trypanosomes were purified by glycerol lysis of rat red blood cells and DEAE-cellulose chromatography, as described previously (Rosen et al., 1978; Lanham & Godfrey, 1970), or by the Percoll method (Bwaye & Hirumi, 1980). The procedure was modified in that the DEAE-cellulose column was run in Bicine–saline–glucose buffer (50 mM Bicine, 7 mM glucose, 5 mM KCl, 4 mM $MgCl_2$, 50 mM NaCl, 55 mM sucrose, 20 mM $CaCl_2$, and 35 mM NaOH, pH 8.0) (Lanham & Godfrey, 1970). The purified trypanosomes were suspended at $5 \times 10^9$ cells/mL in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM tosyllysine chloromethyl ketone, and 0.8 mM phenylmethanesulfonyl fluoride and snap frozen in dry ice–ethanol. The frozen cells were stored at −70 °C. Lysis occurred on rapidly thawing the cell suspension at 37 °C. The lysate was further disrupted in a Dounce homogenizer. The VSG was isolated by lectin affinity chromatography and DEAE-cellulose chromatography as previously described (Bogucki et al., 1982; Strickler et al., 1978). Rabbit antisera raised against purified YNat 1.3 glycoprotein agglutinate living *T. congolense* organisms of the VSG 1.3 serotype and do not agglutinate living YNat 1.1 organisms. The agglutination observed is characteristic of VSG-mediated agglutination (data not shown).

*Amino Acid Sequence Procedures.* The general strategy of protein sequence analysis outlined previously (L'Italien & Strickler, 1982) was followed. Briefly, the reduced carboxamidomethylated protein was digested with CNBr, trypsin, *Staphylococcus aureus* protease, or chymotrypsin. Peptides were purified by reverse-phase HPLC on a Waters μBondapak C-18 column. The tryptic, *Staph. aureus* protease, and CNBr peptide maps for YNat 1.1 have been published as well as preliminary sequence data on some of the peptides (L'Italien & Strickler, 1982). Amino acid compositions were determined on a Beckman 121M amino acid analyzer. Proteins and peptides were immobilized on solid supports and subjected to automatic Edman degradation on a Sequemat Mini-15 solid-phase sequencer equipped with a P-6 autoconverter and a 65 min/cycle program (Laursen, 1971). PTH–amino acids were analyzed by HPLC on an Altex Ultrasphere ODS reverse-phase column (L'Italien & Strickler, 1982).

*Construction of cDNA Libraries.* The methods for preparation of RNA, poly(A) RNA, and plasmid cDNA libraries have been described in detail (Binder, 1984) and are outlined here. Total RNA was isolated by the urea–LiCl method of Auiffray and Rougeon (1980), with minor modifications. Poly(A) RNA was prepared by three cycles of enrichment on oligo(dT)–cellulose. The preparation of double-stranded copy DNA followed the methods outlined in Maniatis et al. (1982).

Fifty nanograms of purified dC-tailed ds cDNA was annealed with *PstI*-cleaved, dG-tailed plasmid pUC 8 (Messing & Viera, 1982) and was used to transform *Escherichia coli* RR1 cells to ampicillin resistance. Separate plasmid libraries for YNat 1.1 and YNat 1.3 were constructed. Relative to transformation with undigested pUC8 plasmid, the efficiency of transformation of RR1 cells by the annealed cDNA–plasmid constructs was approximately 0.44% for both variants. White recombinant plasmid-containing colonies were distinguished from blue, intact vector-transformed colonies by use of the isopropyl β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside indicator system (Messing & Viera, 1982).

*Recovery of VSG cDNA Clones.* White colonies were picked and used to prepare master plates for each library. Duplicate masterplates were blotted with nitrocellulose filters (Grunstein & Hogness, 1975); blotted filters were prepared for hybridization analysis and were prehybridized and incubated with cDNA probe in 50% recrystallized formamide, 5× Denhardt's solution, 5× SSPE, and 0.1% SDS. Filters from each YNat variant library were screened with probes prepared from mRNA of both YNat variants. Probe consisted of single-stranded cDNA synthesized from oligo(dT)-primed, poly(A) RNA from the respective variant, $\alpha$-$^{32}$P-labeled dATP and dNTPs, and avian myeloblastosus virus reverse transcriptase (Rowecamp & Firtel, 1980). Putative VSG-encoding recombinant plasmids were selected by the differential hybridization method (Hoeijmakers et al., 1980). A total of 4000 colonies were screened for each library; 150 differentially reactive colonies were identified from the YNat 1.1 library, and 80 such colonies were identified in the YNat 1.3 library. After a second screening, 50 differentially reactive colonies from each library probe were selected for further analysis. Plasmid DNA from each colony was digested with *PstI* and fractionated on 1% agarose gels. Recombinant plasmids with relatively long inserts were selected. Southern blotting analysis confirmed the differential reactivity with the two probes described above.

For the YNat 1.1 library (A series), plasmids A.08, A.09, and A.51 were mapped and subcloned into M13 mp8, mp9, mp18, or mp19 for DNA sequencing (Messing, 1983). Positive identification of VSG cDNA clones was confirmed by comparison of open reading frames inferred from DNA sequencing with known protein sequence obtained from analysis of VSG-derived peptides. The plasmid A.09 includes the entire coding region of YNat 1.1 VSG mRNA. For the YNat 1.3 library (B series), plasmids B.16, B.17, B.20, and B.23 were used for subcloning and sequence analysis. The B.17 plasmid includes the entire coding region of YNat 1.3 VSG mRNA.

None of these plasmids extended to the 5′ end of the respective mRNA, as inferred from analysis of the corresponding mRNAs (data not shown).

*DNA Sequence Analysis.* The DNA sequencing methods of Sanger et al. (1977), Biggin et al. (1983), and Maxam and Gilbert (1980) were employed. For Maxam and Gilbert sequencing of inserts in M13 vectors, M13 universal primers (New England Biolabs) and synthetic primers (New England Biolabs) and synthetic primers were labeled at the 5′ end with $T_4$ polynucleotide kinase and [$\gamma$-$^{32}$P]ATP, annealed with M13 single-stranded phage templates, and extended with the Klenow fragment of *E. coli* DNA polymerase I and dNTPs (Hong, 1981; Tschudi et al., 1985). After cleavage with appropriate endonucleases, extension products were run on an 8% polyacrylamide–7 M urea gel. Bands containing extension products were cut out and extracted with 0.3 M ammonium acetate. The labeled fragments were ethanol precipitated with carrier tRNA prior to DNA sequencing (Tschudi et al., 1985).

*Calculation of Secondary Structure Potential.* The analysis of secondary structure potential was by the methods of Chou and Fasman (1974a,b, 1978) and Garnier et al. (1978). In addition, the CHOU program of Novotny and Auffray (1984) was used for the analysis of secondary structure potential, hydrophobicity, and charged residue distribution.

*Homology Searches.* Homologies between protein sequences were compared with the MATRIX program of Novotny (1982). This dot matrix analysis compares two sequences, *m* and *n* residues in length, in the form of a matrix composed of *m* rows and *n* columns. Each sequence element, e.g., amino

acid residue or base, of one sequence is compared to every element of the second sequence. The matrix element $m$ with coordinates $i$ and $j$, i.e., $m(i,j)$, is a dot if the corresponding $i$th and $j$th elements are identical. All computer-assisted computations were performed on a VAX 11/750 computer.

*Circular dichroism measurements* were performed on a Cary 16 spectrophotometer. A first approximation of the average value of $\alpha$-helix content in VSG YNat 1.1 was calculated from the following relationship: $\%\ \alpha$ helix = $(\theta_{208nm} - 4000)/(33000 - 4000)$ where $\theta$ = mean residue ellipticity (Greenfield & Fasman, 1969).

## Results

The complete amino acid and DNA base sequences of the coding regions of YNat 1.1 and 1.3 are shown in Figure 1. The results are compiled from direct amino acid sequencing and DNA base sequence analysis of cloned cDNA fragments of YNat messengers. A partial restriction map indicating fragments and direction of DNA sequencing as well as the protein fragments used to assemble the data is shown in Figure 2.

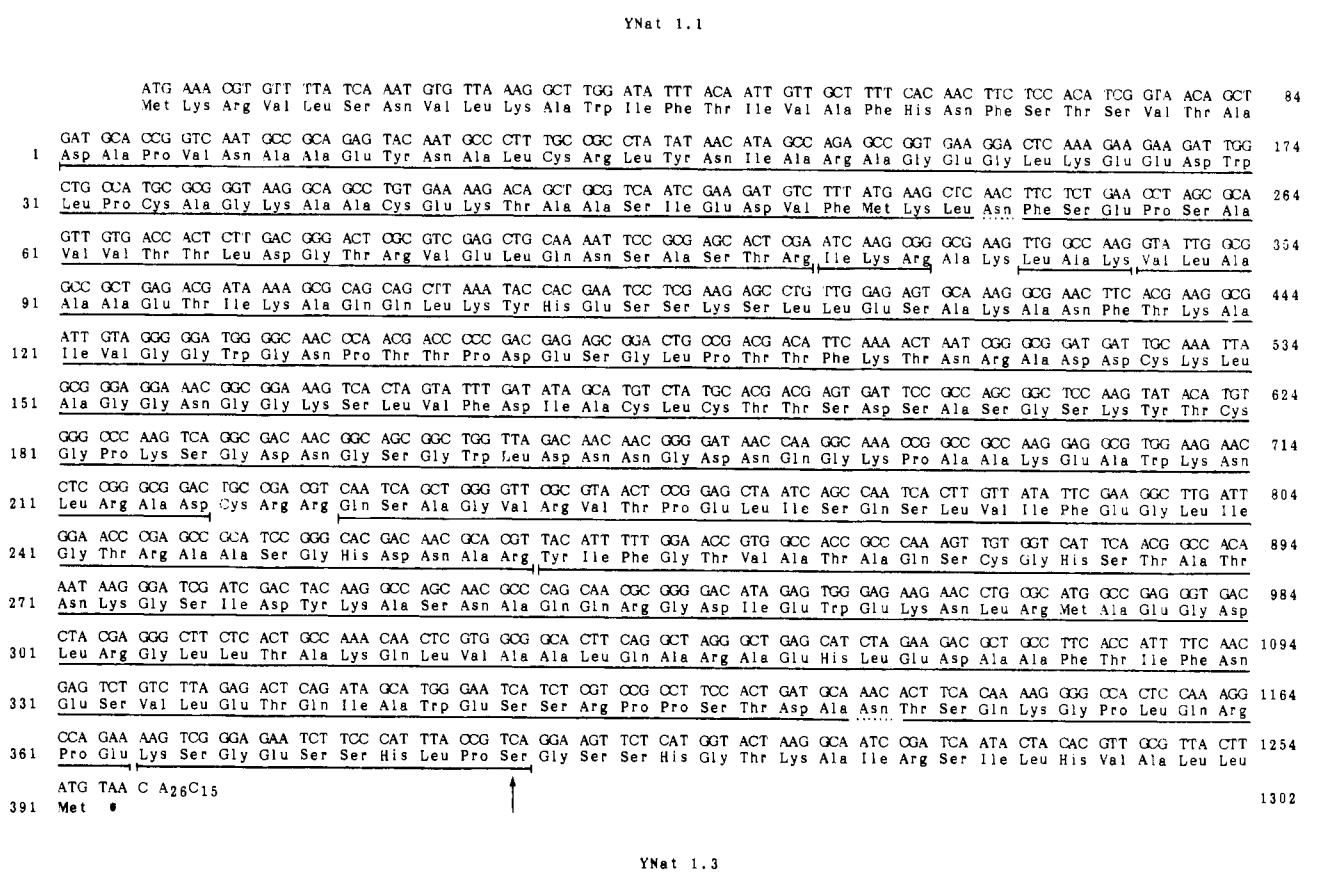
Both YNat VSG messengers encode a putative N-terminal leader sequence. For the YNat 1.1 DNA sequence, the ATG codon for the initiator methionine of the leader sequence is 28 codons upstream of the GAT triplet that encodes the known N-terminal aspartic acid residue of the mature VSG. Similarly, the YNat 1.3 messenger encodes a putative 22-codon leader sequence upstream from the ACT codon for the N-terminal threonine residue of the mature YNat 1.3 VSG. The sizes of these putative leader sequences are within the range observed for the *T. brucei* complex VSG leader sequences (Donelson & Rice-Ficht, 1985). In YNat 1.1 cDNA clone A.09, there is an open reading frame starting at the initiator methionine of the leader, extending 419 codons and followed by a TAA stop codon. This reading frame is confirmed in 87% of the codons. The YNat 1.3 cDNA clone B.17 contains an open reading frame starting with the N-terminal leader, extending 413 codons, and followed by a TAA stop codon. The reading frame is confirmed for 24% of the residues by amino acid sequence analysis of peptides derived from N-terminal, middle, and C-terminal regions of the protein. Digestion of the respective VSGs with carboxypeptidase Y provided no evidence of a free carboxyl terminus for either VSG. For the YNat 1.1 variant, protein sequence studies confirm the reading frame to serine-372 (Figure 1), thus leaving 19 codons unconfirmed before the TAA stop. Ser-372 is tentatively assigned as the C-terminus of the mature protein since (i) by composition the tryptic peptide beginning with Ser-364 contained neither Lys nor Arg and (ii) every amino acid calculated from the composition is accounted for by the peptide Ser-364 to Ser-372. In three separate attempts, this tryptic peptide coupled in exceptionally low yield to APS (<10%), which would be consistent with a C-terminally blocked or partially blocked peptide. The YNat 1.3 reading frame is last confirmed at Arg-315 (Figure 1), thus leaving the last 75 codons unconfirmed. Multiple DNA sequencing methods have been applied to these regions. Also, in the case of YNat 1.3, DNA sequence analysis of three independent cDNA clones confirms the sequence in the C-terminal region.

The calculated molecular weight of the unglycosylated pre-protein, including the leader sequence, for YNat 1.1 is 44825; the corresponding value for YNat 1.3 is 44255. These values are somewhat higher than the experimental molecular weights recently reported for in vitro translated *T. congolense* VSG variants AmNat 1.1 and AmNat 3.1 of 39000 and 43000, respectively (Cook et al., 1985). SDS–PAGE analysis of YNat 1.1 and 1.3 VSGs purified to homogeneity from freeze–thaw lysates, in the presence of protease inhibitors, gave apparent molecular weights of 53000 and 58000, respectively, for the mature, glycosylated forms (data not shown); a similar SDS–PAGE analysis of the mature AmNat 1.1 and AmNat 3.1 variants revealed apparent molecular weights of 51700 and 49900, respectively (Cook et al., 1985). Therefore, with respect to estimated polypeptide chain length and apparent molecular weight on SDS–PAGE, *T. congolense* VSGs as a group appear to be smaller glycoproteins than the VSGs of the *T. brucei* complex, which are composed of approximately 500 amino acids and have molecular weights of 55000–65000 in the mature forms (Rice-Ficht et al., 1981; Donelson & Rice-Ficht, 1985).

When the protein sequences of the YNat variants are aligned with respect to the known N-termini of the mature VSGs, and the distributions of selected residues are mapped (Figure 3), some possible amino acid residue and sequence conservation is suggested. Seven out of nine cysteines appear in similar positions in each variant. The cysteines at positions 13 and 14 in YNat 1.1 and YNat 1.3, respectively, are similar to the conserved cysteine which is seen near the N-terminus of *T. brucei* VSGs (Donelson & Rice-Ficht, 1985). There is a shared pattern of five cysteines in the middle of the sequences (YNat 1.1, positions 148, 165, 167, 180, and 215; YNat 1.3, positions 151, 173, 175, 187, and 217). This suggests that the cysteines may stabilize a domain structure. At least three of the six tryptophan residues in YNat 1.3 are in similar locations in both proteins. Each of the three tyrosine residues in the YNat 1.3 appears to have a counterpart in the YNat 1.1 sequence. The significance of conserved aromatic residues is unclear, although they may also be involved in stabilizing the protein structure.

Potential carbohydrate attachment sites of the (N-X-S/T) type are located in C- and N-terminal regions in both proteins, and in the middle region of the YNat 1.1 molecules (Figure 3). Amino acid sequence analysis revealed that three of the potential sites in YNat 1.1 are not utilized. Asn residues were positively identified at positions 116, 127, and 187, in yields comparable to those of the residues immediately preceding and following the respective Asn residues. No residue was identifiable at position 54, 330, or 351, consistent with glycosylation at these positions. Some of the attached carbohydrates may be on the surface of the molecule, since some *T. congolense* variants can be agglutimated with concanavalin A and other lectins (Jackson et al., 1978; Rautenberg et al., 1980). The carbohydrate side chain at Asn-351 of YNat 1.1 is sensitive to endoglucosaminidase H (L'Italien & Strickler, 1982) and is therefore probably of the high-mannose type, linked through a chitobiose unit to Asn. The sugar composition of the tryptic and staphylococcal protease derived peptides containing this residue is consistent with this interpretation (data not shown).

VSG purified from YNat 1.1 gives a characteristic triplet banding pattern on isoelectric focusing gels (Rosen et al., 1981; Onodera et al., 1981). Each band was electroeluted and sequenced by using preparative flat-bed polyacrylamide gels. All three bands had identical N-terminal sequences through residue 15 (unpublished results). One possible explanation for this behavior is differential sialic acid content (Rautenberg et al., 1980). Qualitative carbohydrate analysis of selected 1.1 VSG peptides indicated the presence of sialic acid. We cannot, however, rule out the possibility that the variation in the IEF migration results from proteolytic cleavage at or near the C-terminus.



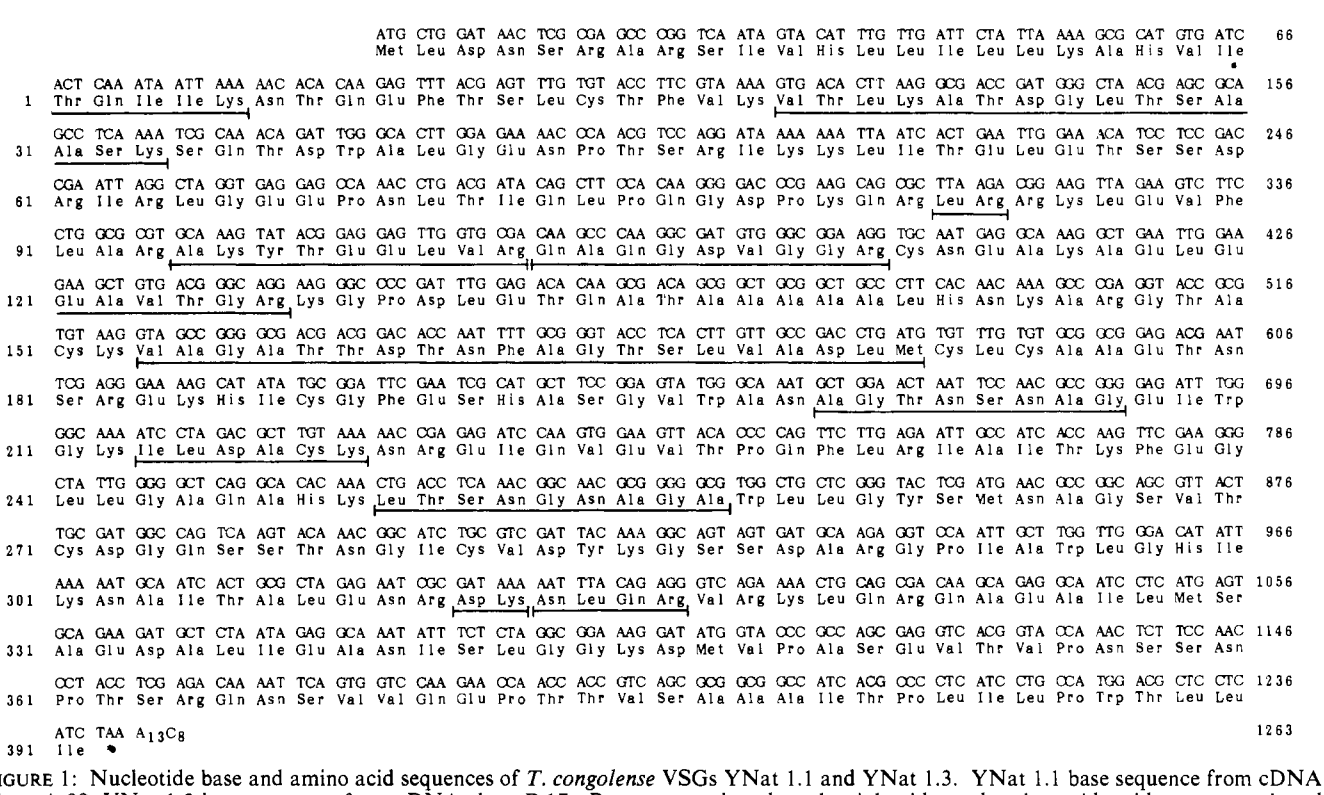




FIGURE 1: Nucleotide base and amino acid sequences of *T. congolense* VSGs YNat 1.1 and YNat 1.3. YNat 1.1 base sequence from cDNA clone A.09; YNat 1.3 base sequence from cDNA clone B.17. Base are numbered on the right side, and amino acid residues are numbered on the left. Number 1 on the left marks the residue for the N-terminus of mature VSG. Numbering for bases initiates at the first base of the Met codon of the respective N-terminal leader peptides. Asterisks signify a stop codon. Amino acid residues underscored with a solid line were confirmed by peptide analysis; Asn residues underscored with a dashed line were blank cycles in peptide sequence analysis and are presumed or known (YNat 1.1, Asn-351) carbohydrate addition sites. The arrow at Ser-372 marks the postulated C-terminal residue of mature YNat 1.1 VSG.

Comparison of the aligned primary amino acid sequence of YNat 1.1 and 1.3 shows that there is some sequence homology but that this is not very striking (Figure 4). There is about 25% identity of amino acid residues. There are, however, homologous blocks of residues in proximity to the cysteine residues, suggesting that our cysteine alignment is justified.

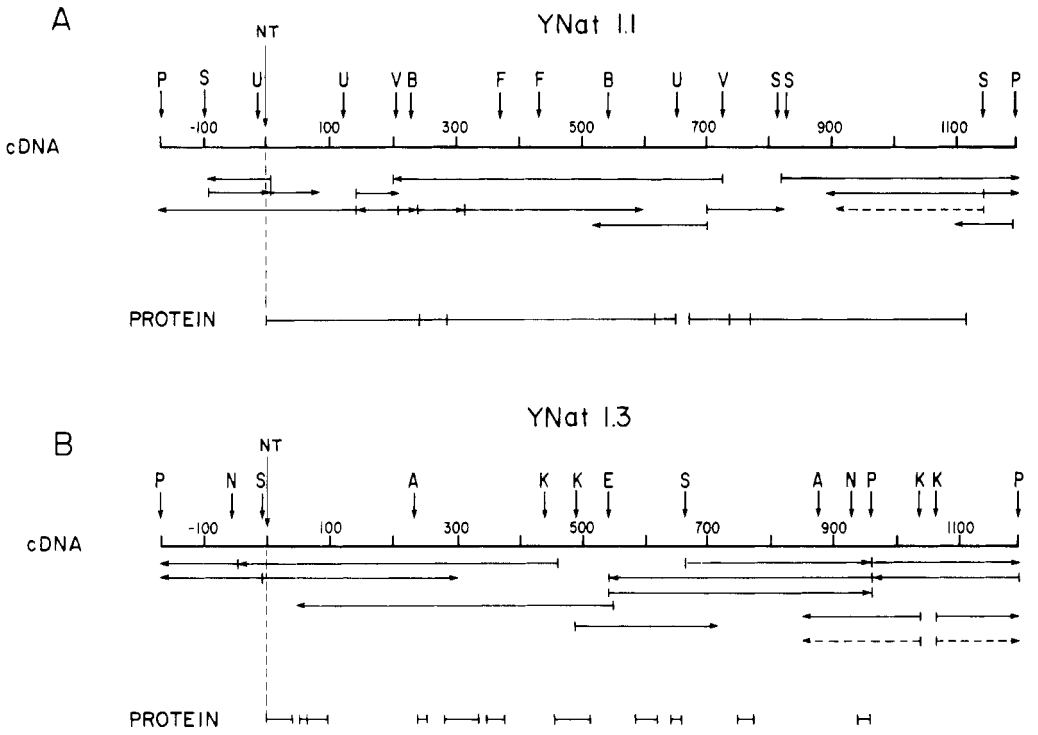



FIGURE 2: Diagram of the amino acid residue and nucleotide sequence strategy adopted for determining the primary structure of the *T. congolense* VSGs YNat 1.1 and 1.3. The cDNA lines show partial restriction maps of respective cDNA clones, and below them, solid lines with arrows indicate the extent and direction of dideoxy sequencing of M13 subclones; broken lines with arrows indicate the extent and direction of Maxam–Gilbert sequencing. The protein lines indicate the extent of overlap of all tryptic, chymotryptic, and acid protease peptides sequenced. Gaps in the peptide sequences are indicated by breaks in the line. Hatch marks on the protein line indicate where peptide information was not sufficient to give overlap information. Sequence information in all gaps and nonoverlapping regions was obtained by reference to the nucleotide base sequences. All nonoverlapping nucleotide sequencing restriction sites were in turn confirmed by peptide sequences. (A) YNat 1.1 cDNA line: restriction map of insert DNA of plasmid A.09. (B) YNat 1.3 cDNA line: restriction map of insert DNA of plasmid B.17. The C-terminal region of YNat 1.3 has been sequenced in four independent cDNA plasmid clones. Numbering of nucleotides: number 1 is the first base of the codon of the N-terminal amino acid of the respective mature VSG. Number −1 marks the third base of the last codon of the putative N-terminal leader peptide. Symbol code: NT = N-terminal amino acid residue of mature VSG; A = *Ava*II, B = *Bsp*I, E = *Eco*RI, F = *Fok*I, K = *Kpn*I, N = *Nru*I, P = *Pst*I, S = *Sau*3A, U = *Pvu*II, V = *Ava*I.

The homologous sequence blocks and single homologous residues are spread throughout the primary sequence but are somewhat more frequent in the C-terminal half of the VSG molecules, especially between residues 180 and 330 (YNat 1.1 numbering). Dot matrix homology analysis reveals a faint line of homology over the same region (180–330) (data not shown).

Comparison of amino acid sequences in *T. brucei* complex VSGs has shown the C-terminal 50 residues exhibit a class or isotypic pattern. Almost all reported sequences have strong homologies in this region, fitting them into one of two classes (Donelson & Rice-Ficht, 1985). The homology seen in the two *T. congolense* VSGs is not sufficient to discern any isotypic resemblance between the two proteins. We have used a dot matrix plotting program to locate similarities in amino acid sequences. The matrix program was set to determine matching amino acid residues in the two proteins compared and to print one dot for every match. The following *T. brucei* complex VSG sequences have been plotted against both YNat 1.3 and YNat 1.1 as well as against each other: AnTat 1.1, IoTat 1.2, ILTat 1.1, 1.2, 1.3, 1.4, and MITat 1.4. No dot matrices have been included, and a summary of our analyses follows.

When the members of the known *T. brucei* complex VSG family are compared, the expected pattern emerges in the C-terminal regions. There is strong homology within isotopes and modest homology between isotypes. In the remainder of the molecule, the degree of homology varies; some VSGs have strong similarity (AnTat 1.1 and ILTat 1.3), and others show little or no discernible similarity (AnTat 1.1 and ILTat 1.1). If, however, the whole set of *T. brucei* complex VSG is considered, there is always some regional or general similarity among its members. In contrast, when dot matrix diagrams are plotted between either of the two *T. congolense* VSGs and each of the *T. brucei* complex VSGs, no discernible homology is found. In other words, even though the degree of similarity varies widely among *T. brucei* complex VSGs, when any given *T. brucei* VSG is compared to all others, modest or strong homology can be seen. The two *T. congolense* VSGs, in contrast, show *no* discernible similarity with any *T. brucei* complex VSG.

We have calculated the secondary structure potential of the *T. congolense* VSGs (Figure 5). These calculations express the potential for forming $\alpha$-helical structures, $\beta$ turns, and $\beta$-stranded or extended forms of the polypeptide backbone. We emphasize that we do not predict the *actual presence* of secondary structures but only the potentials for forming them. When two different proteins contain a region having identical secondary structure potential, the two proteins may or may not express a common secondary structure in that region. If, however, a region of protein has high potential for one type of structure in one region whereas in another protein a comparable region has a different potential, then it is unlikely that these two regions will show a common secondary structure.

Both YNat 1.1 and YNat 1.3 have a high percentage of $\alpha$-helix potential; our programs give strongly predicted $\alpha$-helical regions for approximately 60% of the primary structure. A strongly predicted region is one where the predicted secondary structure potential is at least twice as likely as the next most likely secondary structure (Lalor et al., 1984). As expected, the potential is a conservative estimate since only strongly predicted regions are included, and it is likely that

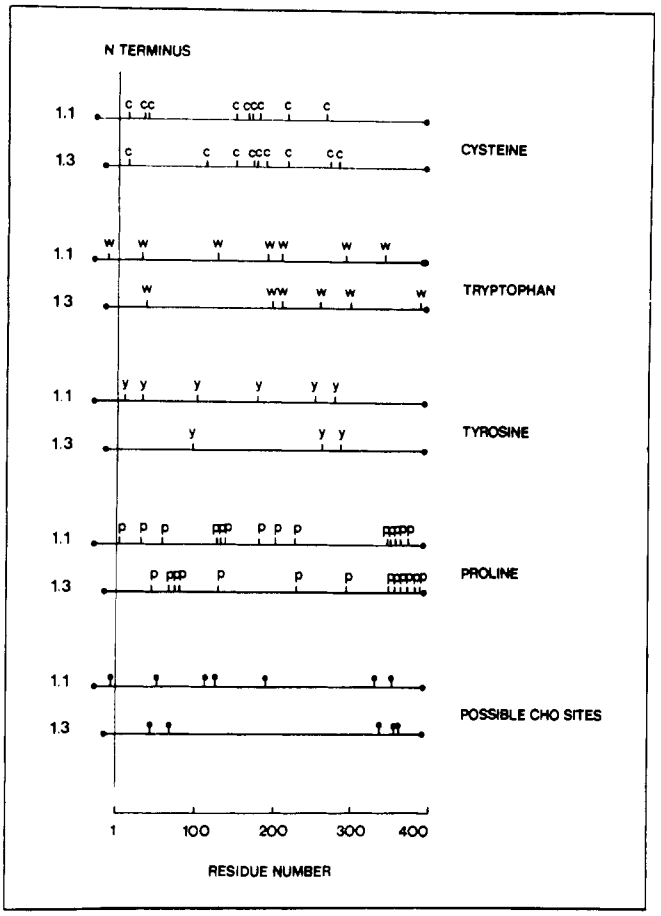



FIGURE 3: Diagram outlining the distribution of cysteine, tryptophan, tyrosine, and proline amino acid residues in the *T. congolense* VSGs YNat 1.1 and YNat 1.3. Potential carbohydrate attachment sites (N-X-S/T) are indicated by the symbol (♦). Amino acid analysis indicated that Asn residues at positions 54, 330, and 351 in YNat 1.1 are modified and therefore probably carbohydrate addition sites. The VSG sequences are aligned by the N-terminal amino acid residues of the mature proteins. The amino-terminal residue of each VSG is marked by "N TERMINUS" in the figure, and its position is numbered 1 on the scale at the bottom of the figure. The letters C, W, Y, and P are single-letter amino acid code designations for cysteine, tryptophan, tyrosine, and proline, respectively.

some less strongly predicted regions will also be $\alpha$ helical. In line with this expectation, the average value for the $\alpha$ helix as calculated from circular dichroism measurements of YNat 1.1 was 65%. It is interesting to note that X-ray crystallographic studies at 6-Å resolution have revealed that the N-terminal 60% of the *T. brucei* complex VSG M.Tat 1.2 contains two long $\alpha$ helices and other segments with helix structures (Freyman et al., 1984).

When the locations of regions of $\alpha$-helical, $\beta$-turn, or $\beta$-strand potential are compared in the two YNat proteins (Figure 5), a pattern emerges that is reminiscent of that observed when a similar comparison is made for the *T. brucei* complex VSGs (Lalor et al., 1984). In both families, there is a similar kind of diversity in the arrangement of breaks in the segments of $\alpha$-helical potential, and in the locations of strong $\beta$-turn and $\beta$-strand potentials. Differences in the extent of $\alpha$-helical potential between these *T. congolense* VSGs are particularly marked in the region from residue 120 to 250 (numbering as in Figure 1). Over this region, the YNat 1.1 protein contains more strongly helix-breaking proline residues (which cause the polypeptide chain to turn through a 110° angle) and more other helix-breaking residues than does the YNat 1.3 protein. Most important, there are regions where the two proteins show strongly divergent potential. In the region centering around residue 178 in YNat 1.1, this protein has strong $\beta$-turn potential, and YNat 1.3 has strong $\alpha$-helical potential. Other regions of contrasting potential center on residues 124 and 218 (YNat 1.1 numbering).

The C-terminal regions of these proteins exhibit similarities in primary structure and secondary structure potential. A cluster of strongly helix-breaking proline residues is contained in the C-terminal region of each VSG (Figure 3). Secondary structure potential in the corresponding regions (residues 340–380) is characteristic of extended, open structures (Figure 5, and unpublished results). Interestingly, some known or possible N-linked carbohydrate addition sites are located in this region (Figure 3). In each protein, the region from residue 305 to residue 340 has strong $\alpha$-helical potential (Figure 5). Additionally, these two VSGs contain similar hydrophobic sequences at the extreme C-termini, reminiscent of the putative membrane-associated hydrophobic tail of the *T. brucei* complex VSGs. Thus, there is evidence for conservation of structure in the C-terminal regions of the YNat VSGs. Such conservation may indicate that these regions form structures required for important conserved functions, e.g., intermolecular VSG–VSG interaction sites, intramolecular helix packing, or domains associated with membrane attachment.

## DISCUSSION

There are stringent conditions imposed on the families of structurally related proteins which mediate antigenic variation. First, a variant protein must have new antigenic determinants, and second, it must lose all old surface determinants present on previous variants. Cross-reactivity is not simply a matter of having exactly the same determinants on two proteins; it can occur with structurally dissimilar determinants and with determinants having only partial resemblance (Richards et al., 1975). In the latter instance, although the $K_a$ of the interaction might be reduced, it could still interfere with antigenic variation. Furthermore, since the cell surface coat presents a repeating array of VSG epitopes to divalent and multivalent host antibodies, avidity effects would increase the effective $K_a$ of antibody–surface coat interactions.

Comparison of the published *T. brucei* complex VSG primary structures has revealed the existence of a C-terminal region of approximately 25 amino acid residues which shows a class or isotypic structure. So far, two classes have been identified. There is considerable homology among members of an isotype, but even between isotypes, there is some homology. Bordering on the N-terminal end of the isotypic region, there is approximately 100 amino acid residues which typically shows less homology (Donelson & Rich-Ficht, 1985). In addition, comparison of the secondary structure potentials in this region shows considerable similarity in all *T. brucei* complex VSGs analyzed so far (Lalor et al., 1984). It has been suggested that these regions both are in contact with the membrane of the trypanosomes and are involved in the interaction which is known to exist between VSG molecules (Strickler & Patton, 1982). Such common structural requirements may account for the conserved secondary structure. The middle region of the molecule containing approximately 200 amino acid residues has many regions of sharply contrasting potential, where, for instance, there is $\alpha$-helical potential in one VSG molecule and $\beta$-turn potential in another (Lalor et al., 1984). This has prompted the idea that this region mediates antigenic variation by allowing folding differences in the polypeptide backbone of VSG so that different caplike regions of the primary structure are exposed to the solvent–surface in different VSG serotypes. Thus, new antigenic determinants would appear, and old ones would dis-

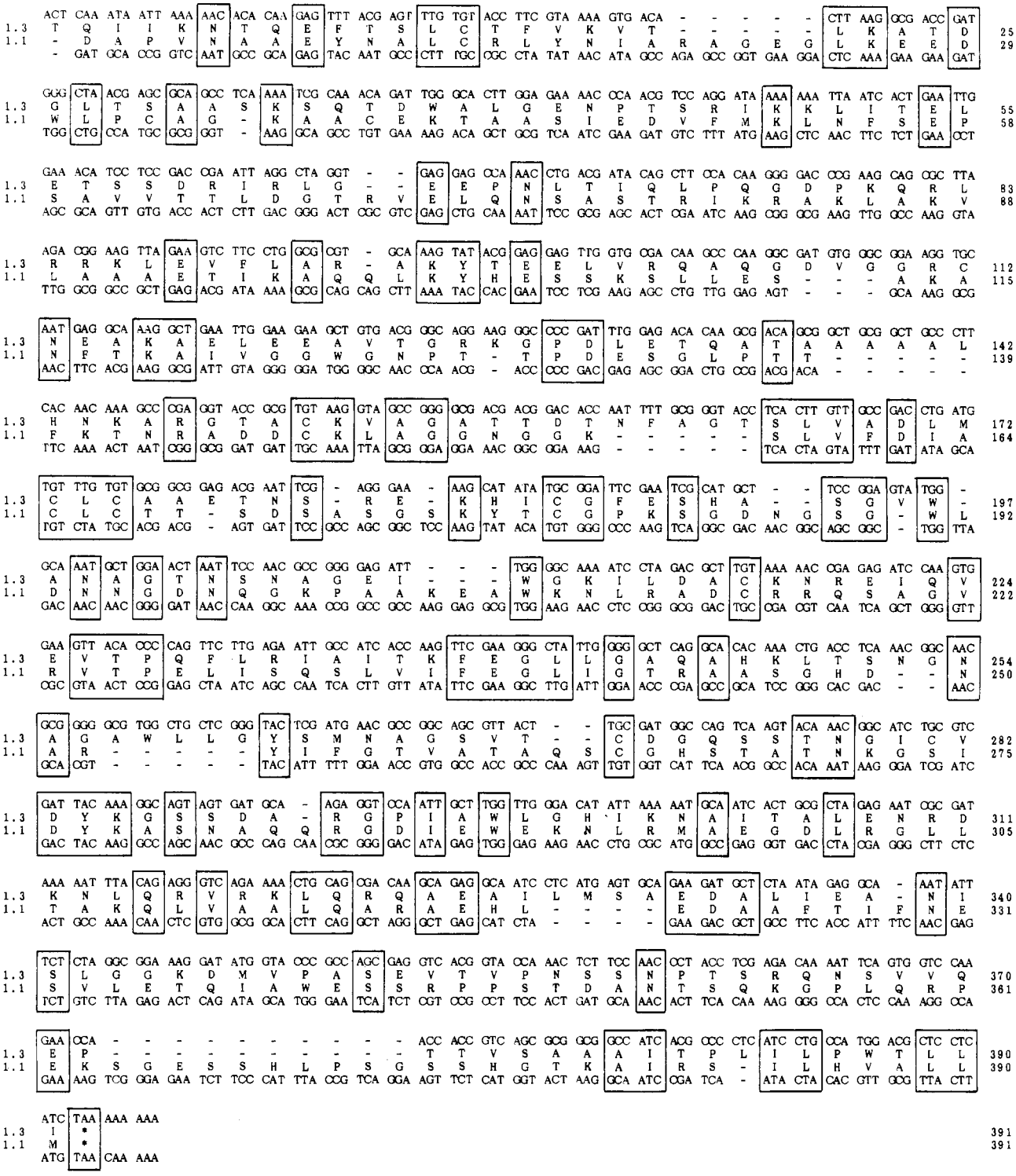


FIGURE 4: Alignment of amino acid and nucleotide sequences of mature YNat 1.1 and YNat 1.3 VSGs. The protein sequences were aligned with emphasis to increase homology about apparently conserved cysteine, tryptophan, and tyrosine residues. Gaps have been introduced to maximize homology. Boxed areas highlight amino acid identities in this alignment. Numbers on the right give the amino acid residue number of the last residue on that line for the respective protein sequence, where number 1 marks the N-terminal residue of the respective mature protein. Top nucleotide and amino acid sequence are of YNat 1.3, marked by 1.3; the lower sequences are of YNat 1.1, marked by 1.1.

appear (Lalor et al., 1984). The following evidence is in favor of this concept. Freymann et al. (1984) have shown in their low-resolution X-ray crystallographic map of one *T. brucei* complex VSG fragment that indeed caplike structures occur at the ends of two $\alpha$-helical rods which are a part of the N-terminal 60% of MITat 1.2. Cohen and her collaborators (Cohen et al., 1984) reported the presence of heptad repeats in *T. brucei* complex VSG primary structures, suggestive of a coiled-coil structure in the $\alpha$-helical stalk of the molecule. Moreover, the breaks in the distribution of heptad repeats occurred in different primary sequence regions in different VSGs, prompting these authors to suggest, as we did, the radical folding differences might make an important contribution in the structural basis of antigenic variation. Additionally, these investigators reported that electron microscopy of two isolated VSG molecules of different serotypes showed differences in the size of caplike structures (Cohen et al., 1984). Lastly, preliminary evidence suggests that radioactive chemical

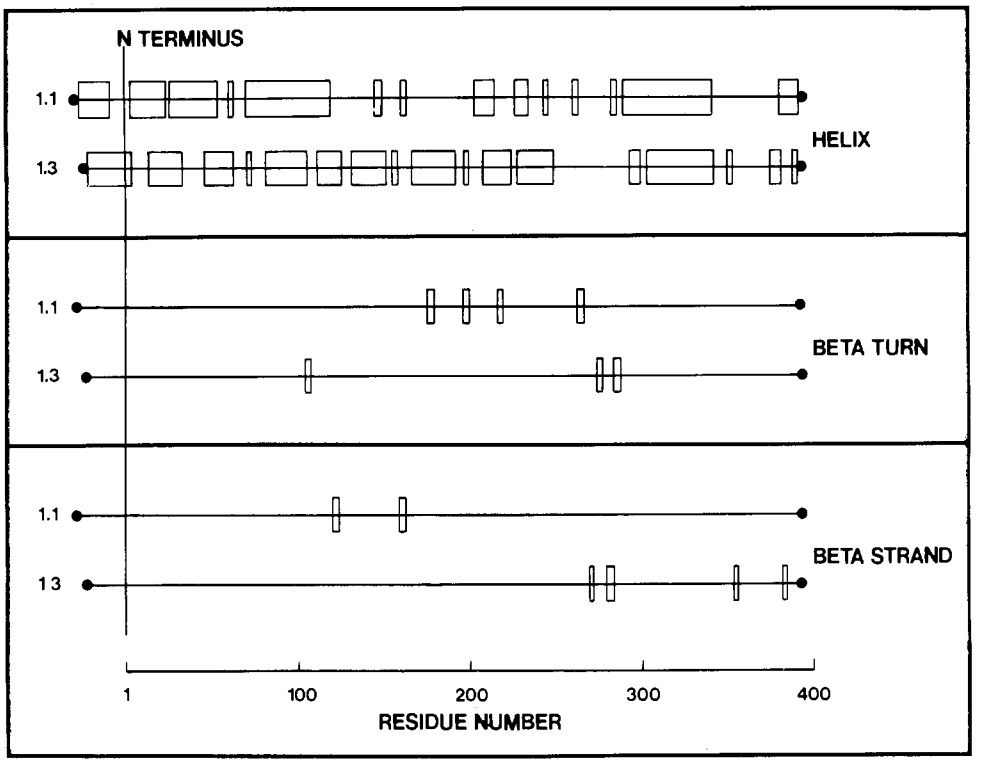



FIGURE 5: Strongly predicted secondary structure potentials of the *T. congolense* VSGs YNat 1.1 and 1.3, as calculated with the DELPHI program (Lalor et al., 1984). In a strongly predicted region, designated by open boxes, the indicated secondary structure potential is at least twice as the next most likely secondary structure. $\beta$-Turn regions were mapped only when at least four consecutive $\beta$ turns calls occurred. Both VSGs are strong in $\alpha$-helical potential but show differences in the distribution of strongly predicted $\alpha$-helical segments. Strongly predicted $\beta$-turn and $\beta$-strand potentials vary widely in their distribution in the two proteins.

agents which modify surface lysine or tyrosine residues on intact, live trypanosomes label different regions of the primary structure in different serotypes. This labeling has been shown to occur at or close to regions where $\alpha$-helical potential regions become regions of open structure potentials (manuscript submitted for publication). These studies support our idea but do not constitute definitive proof. High-resolution X-ray crystallographic studies of VSGs having different folding potential will provide the rigorous test of the multiple folding pattern hypothesis.

The question now arises whether other organisms which exhibit antigenic variation in a single surface protein, such as the nannomonad *T. congolense* or the spirochete *Borrelia hermsii*, show similar phenotypic mechanisms. This paper analyzes this question with regard to *T. congolense*. There are some morphological, physical, and physiological differences between *T. brucei* complex and *T. congolense* trypanosomes, and the two organisms were placed into a different subgenera on the basis of these differences. This suggests that there may be some evolutionary distance between trypanosomes of the *T. brucei* complex and *T. congolense*.

The results of our study show that certain of the organizational features found in the *T. brucei* complex VSGs are present in *T. congolense* VSGs. The *T. congolense* VSGs not only have a high $\alpha$-helical potential but from the CD study we know that in YNat 1.1 this potential is expressed. The middle regions of the YNat molecules show the same pattern of broken $\alpha$-helical potential with the breaks in different regions of the primary structure. The YNat glycoproteins also contain sequences with coiled-coil related, heptadic periodicities. Strongly predicted $\alpha$-helical segments of the YNat 1.1 protein sequence which exhibit heptad repeat potential are (numbering as in Figure 1) as follows: 4–28, 30–54, 69–120, 228–244, and 292–340. Similar potential in the strongly predicted $\alpha$-helical segments of YNat 1.3 sequence is found in 12–33, 47–63, 112–126, 132–158, 167–192, 208–227, and 294–342. It is interesting to note that both proteins exhibit coiled-coil potential in the N-terminal regions (within residues 1–60) and a strong potential in the C-terminal one-third of the molecules (290–342). This suggests the possibility that the N- and C-terminal regions may associate in a coiled-coil helical bundle to form a stemlike structure in analogy to the *T. brucei* complex VSGs (Cohen et al., 1984; Freyman et al., 1984). Also, the distributions of remaining coiled-coil potential in the two variants are different, particularly in the middle region. Further, the middle regions contain sharply contrasting primary structure potential, suggesting the same kind of pattern that is found in the *T. brucei* complex VSGs (Lalor et al., 1984).

The comparison of the primary structure homology shows considerable differences. The dot-matrix plots demonstrated (data not shown) that in nearly every instance there is homology between the members of the *T. brucei* complex VSG family. Sometimes homology is extensive and sometimes it is modest, but with one noted exception relatedness is always apparent. A modest degree of scattered homology (approximately 25% overall) also exists between the two *T. congolense* VSGs, and this falls in the same range which is observed between some members of the *T. brucei* complex VSG family. However, when each of the two *T. congolense* VSGs is compared in dot-matrix plots to every sequenced member of the *T. brucei* complex VSG family, there is no homology found at all even when the conditions of the comparison are sensitive to distant homologies.

Examination of the last 20 amino acids of the YNat 1.1 and YNat 1.3 variants reveals that the sequence of each variant contains tri- and tetrapeptides which also appear as consensus sequence elements in the last 17–23 amino acid residues of *T. brucei* complex VSGs. For instance, the YNat 1.3 sequence includes the tetrapeptide VSAA; the tripeptide SAA is a consensus tripeptide in isotype I of the *T. brucei* complex VSGs, and VSAA is found 8/9 times in that group (Donelson

& Rice-Ficht, 1985). The sequence TPLXL defines a consensus element among isotope II of the *T. brucei* complex VSGs (Donelson & Rice-Ficht, 1985) and is found in the same YNat 1.3 sequence as TPLIL. The YNat 1.1 C-terminal sequence VALLM is closely related to the VALLF sequence, which is found with high frequency at the C-terminus of the members of isotype I of *T. brucei* complex VSGs (Donelson & Rice-Ficht, 1985). Because of shortness of the sequences and the limited data base, the significance of short homologous peptides to the question of the evolutionary relationship between *T. congolense* and *T. brucei* complex VSGs is not clear.

The lack of homology between *T. brucei* complex and *T. congolense* VSGs is striking. These two related hemoflagellates may use variants of the same ur-protein as the antigenic variation locus. It can be argued that rapid gene evolution has produced so many mutations that these VSGs no longer resemble each other. A second possibility is that each of the two parasitic hemoflagellates originally has adopted a different gene family to code for its respective VSG polygene family. The similar secondary structure in the face of a different primary structure may then be the result of the common evolutionary pressure leading to antigenic variation. According to this view, independent adaptations, using different surface proteins as sources of parasite-covering variant-specific glycoproteins, have occurred. However, the limited sequence data base does not allow us to distinguish between these two possibilities.

As noted above, the last 20 or so residues of YNat 1.1 and 1.3, as predicted from the respective cDNA clones, are similar to the hydrophobic tail sequences of *T. brucei* complex VSGs. The tail sequence is cleaved from most, if not all, mature *T. brucei* complex VSGs, and a glycophospholipid structure is added to the new C-terminal amino acid through an amide linkage (Ferguson et al., 1985). Given the similarities at the extreme C-termini of the two sets of VSGs, it likely that similar posttranslational modifications may occur with the *T. congolense* VSGs. Such glycophospholipid structures are thought to mediate membrane attachment of cell surface molecules and are found in various eukaryotes (Tse et al., 1985).

We see no common isotype structure in the C-terminal region of the two *T. congolense* VSGs, although there is more homology in the C-terminal region than elsewhere in the two molecules. However, the analysis of two structures is not sufficient to draw any conclusions about class structure. It is interesting to note that Majiwa et al. (1985) found that the VSG cDNA probe from *T. congolense* ILNat 2.1 did not hybridize with genomic DNA of strains from geographically separated *T. congolense* serodemes, suggesting a possible absence of a conserved isotypic C-terminal region.

That the YNat cDNA clones appear to contain little or no 3′ untranslated region (UTR) raises a number of questions. Assuming that the cDNA clones accurately reflect the structure of the respective mRNA molecules, the lack of a substantial 3′ UTR represents a marked difference from other eukaryotic mRNAs in general and, in particular, from the *T. brucei* complex VSG mRNAs. In that case, the 3′ UTRs, are approximately 75 bp in length, though variable, and contain considerable sequence conservations, including two consensus sequences (Borst & Cross, 1982). It was suggested (Borst & Cross, 1982) that the conserved sequences could serve an important function, such as in recombinational events in VSG gene switching. Thus, the lack of a 3′ UTR in the *T. congolense* VSG mRNAs may indicate a difference in the nature and location of nucleic acid structural elements involved in *T. congolense* VSG duplication/transposition events. Our studies on the genetic rearrangements in the *T. congolense* VSG system will be the subject of another publication (manuscript in preparation).

The location and nature of the poly(A) addition signal sequences for the *T. brucei* complex mRNAs have not yet been determined with certainty, though the sequence AAAATTPyT has been proposed (Hasan et al., 1984). The apparent lack of a 3′ UTR in the YNat VSG messages may imply that the poly(A) addition signal sequences are located in the coding region of the VSGs; however, in other more complicated schemes, the signal sequences would map to portions of the messenger precursors which are processed off at the addition of the poly(A) tail.

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**Registry No.** DNA (*Trypanosoma congolense* variant surface glycoprotein YNat 1.1 messenger RNA complementary), 106160-76-3; glycoprotein YNat 1.1 (*Trypanosoma congolense* variant surface precursor protein moiety reduced), 106160-78-5; glycoprotein YNat 1.1 (*Trypanosoma congolense* variant surface protein moiety reduced), 106160-79-6; DNA (*Trypanosoma congolense* variant surface glycoprotein YNat 1.3 messenger RNA complementary), 106160-77-4; glycoprotein YNat 1.3 (*Trypanosoma congolense* variant surface precursor protein moiety reduced), 106160-80-9; glycoprotein YNat 1.3 (*Trypanosoma congolense* variant surface protein moiety reduced), 106160-81-0.

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# The $M_r$ 93 000 Polypeptide of the Postsynaptic Glycine Receptor Complex Is a Peripheral Membrane Protein†

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ABSTRACT: The glycine receptor of mammalian spinal cord is an oligomeric membrane protein that, after affinity purification on aminostrychnine–agarose or immobilized antibody, contains three polypeptides of $M_r$ 48 000, 58 000, and 93 000. Here, the association and the properties of the polypeptides of the rat glycine receptor were investigated. Upon phase partitioning in the nonionic detergent Triton X-114, the three receptor polypeptides behaved as a hydrophilic protein complex exhibiting phospholipid binding. Sucrose gradient centrifugation or gel filtration in the presence of dithiothreitol and Triton X-100 separated the $M_r$ 93 000 polypeptide from the $M_r$ 48 000 and 58 000 polypeptides, which harbor the antagonist binding site of the glycine receptor. Alkaline or dimethylmaleic acid anhydride treatment of crude synaptic membrane fractions resulted in extraction of the $M_r$ 93 000 polypeptide. Lectin binding was observed for the $M_r$ 48 000 and 58 000 glycine receptor subunits but not the $M_r$ 93 000 polypeptide. These results indicate that the $M_r$ 93 000 polypeptide is a peripheral membrane protein that is located at the cytoplasmic face of the postsynaptic glycine receptor complex.

Glycine is a major inhibitory neurotransmitter in spinal cord and other regions of the central nervous system (Werman et al., 1967; Curtis et al., 1968; Aprison & Daly, 1978). Binding of this amino acid to the postsynaptic glycine receptor (GlyR)[1] increases the chloride conductance of the neuronal membrane and thus produces a hyperpolarization, i.e., inhibition of the postsynaptic neuron. The convulsive alkaloid strychnine an-

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[1] Abbreviations: DMMA, dimethylmaleic acid anhydride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GlyR, glycine receptor; HRP, horseradish peroxidase; IgG, immunoglobulin G; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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