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MOLECULAR CHARACTERIZATION AND EXPRESSION OF A N-ACETYLNEURAMINATE LYASE GENE FROM *TRICHOMONAS VAGINALIS*

A Thesis Submitted to the

School of Graduate Studies

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In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

School of Medicine

By

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ABSTRACT

By screening a *Trichomonas vaginalis* λgt11 cDNA library with anti-serum raised against a purified preparation of *T. vaginalis* cell-detaching factor (CDF), two clones were identified. Molecular analysis of the cDNA sequences indicated that the clones were distinct and could potentially represent two unique *T. vaginalis* genes. Further characterization of the CDF anti-serum used in the initial library screening indicated that the serum possessed reactivity to CDF and a variety of other *T. vaginalis* proteins. The multiple specificities of the anti-serum are likely to have contributed to the identification of the two distinct cDNA clones. While the relationship between the weaker of the two immunoreactive cDNA sequences (CDF-2) and the CDF protein was unclear, the results of Northern hybridization and sequence analysis suggested that the strongly reactive CDF-1 clone did not contain sequences representing a CDF gene. Since the CDF-1 cDNA clone contained an almost complete coding sequence and because the encoded polypeptide exhibited significant protein sequence similarity to bacterial N-acetylneuraminate (Neu5Ac) lyases, this *T. vaginalis* sequence was further analyzed.

Using the cDNA sequence and cloned segments of overlapping genomic DNA, the complete coding and flanking regions of this *T. vaginalis* gene, designated Tv*nanA*, were determined. Tv*nanA* is a single copy gene that lacks introns and contains a 954 bp open-reading frame that is predicted to encode a 318 amino acid polypeptide with a calculated molecular mass of 35 kDa. The gene is transcribed as a single 1.1 kb polyadenylated mRNA with short 5' (17-18 nt) and 3' (28 nt) untranslated regions. A sequence resembling the *T.*

vaginalis initiator element was identified in the region surrounding the transcriptional initiation site, however this Inr-like element did not direct accurate gene transcription. The Tv*nanA* encoded polypeptide shows 35% identity and 53% similarity to the Neu5Ac lyase of *Escherichia coli*, and 73% identity to a predicted Neu5Ac lyase of *Haemophilus influenzae*. The homology includes significant similarity within a region corresponding to the putative active site pocket of the *E. coli* enzyme. A complete copy of the Tv*nanA* gene was assembled and the gene product was expressed in *E. coli* as a glutathione S-transferase fusion protein. In the absence of detergents required for efficient solubilization, the *T. vaginalis* gene product exhibited Neu5Ac lyase activity that was 30 times greater than background activity in controls. Lyase activity was increased a further 5-fold by deletion of the N-terminal hydrophobic region of the *T. vaginalis* protein. While fusion proteins exhibited Neu5Ac lyase activity, they did not appear to be capable of producing cell-detaching activity when incubated in the presence of eukaryotic cell monolayers. Preliminary experiments to determine the size and location of the *T. vaginalis* Neu5Ac lyase suggests that the enzyme is present as an intracellular 35 kDa species.

While questions remain concerning the regulation of the Tv*nanA* gene and the role of the N-terminal region in the encoded polypeptide, this is the first reported characterization of a *T. vaginalis* gene product involved in sialic acid metabolism. The presence of this enzyme combined with the reports of a *T. vaginalis* neuraminidase suggests that the parasite may utilize host sialic acid as a nutrient source.

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LIST OF ABBREVIATIONS

A	adenosine
Amp	ampicillin
AMV	avian myeloblastosis virus
AP	adhesin protein
A_{xxx}	absorbance at xxx nm
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CDF	cell detaching factor
CPE	cytopathic effects
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FSB	frozen stock buffer for chemically competent *E. coli*
G	guanine
GST	glutathione S-transferase

Inr	initiator
IPTG	isoproyl β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria broth
M-MLV	Moloney murine leukemia virus
MWCO	molecular weight cut-off
nanA	N-acetylneuraminate lyase gene of *E. coli*
nanT	sialic acid permease gene of *E. coli*
N	any of the four nucleotides
NBT	nitroblue tetrazolium
NCBI	National Center for Biological Information
Neu5Ac lyase	N-acetylneuraminate lyase
nt	nucleotide
ORF	open-reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	phage dilution buffer
Py	pyrimidine
SDS	sodium dodecyl sulphate
sd	standard deviation

STD	sexually transmitted disease
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
TBST	Tris buffered saline with Tween 20
TdT	terminal deoxynucleotidyltransferase
TF	transcription factor
TLCK	N-α-p-tosyl-L-lysine chloromethyl ketone
T	thymidine
Tv*nanA*	N-acetylneuraminate lyase gene of *T. vaginalis*
TYI	Diamond's TYI-S-33 medium
UTR	untranslated region
U	units of enzyme activity
VECs	vaginal epithelial cells
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1: *TRICHOMONAS VAGINALIS* - GENERAL INTRODUCTION

PHYLOGENY AND STRUCTURE

Trichomonas vaginalis (phylum Parabasalidea; family Trichomonadida; subfamily Trichomonadinae) is an aero-tolerant anaerobic protozoan first described by Donné in 1836. Since trichomonads are amitochondrial, it has been suggested that they emerged early among eukaryotic lineages and before the advent of mitochondrial endosymbiosis. The placement of *T. vaginalis* amongst the early branches of the eukaryotic tree has been established by sequence analysis of the organism's large (23S-like) and small (16S-like) subunit ribosomal RNAs (Baroin *et al.* 1988; Gunderson *et al.* 1995; Katiyar *et al.* 1995; Viscogliosi *et al.* 1993). Further confirmation of the parasite's eukaryotic lineage has been made by sequence analysis of homologs to ancient genes that are considered universal among higher eukaryotes and yeast. Riley *et al.* (1993) and Riley and Krieger (1995) have identified *T. vaginalis* homologs to the CDC2/28 and cyclin-dependent kinase genes. Both of these genes, critical in the control of eukaryotic cell division, belong to gene families that are believed to have evolved at the same time nuclear envelopes and mitotic spindles first appeared, but prior to the advent of mitochondria. These finding combined with the rRNA phylogenetic evidence, firmly place *T. vaginalis* amongst the earliest eukaryotes.

Based on existing literature, *T. vaginalis* lacks a cyst stage and exists solely in trophozoite form (Kulda *et al.* 1986). The parasite generally ranges in size from 10-30 μm (Moldwin 1992); however both its size and morphological shape have been shown to vary depending upon the conditions under which the organisms are maintained. Grown axenically

in vitro, *T. vaginalis* tends to be ovoid or "pear-shaped", while organisms grown in the presence of vaginal epithelial cells (VECs) appear to transform to an amoeboid shape (Arroyo *et al.* 1993). The *T. vaginalis* genome consists of approximately 2.5 x 10^7 basepairs of unique sequence and has a G+C content of 36% (Wang and Wang 1985). The parasite is considered to have four major morphological characteristics: (i) an anteriorly located nucleus that has been reported to contain six telocentric chromosomes (Kulda *et al.* 1986); (ii) four anterior flagella; (iii) an undulating membrane and, (iv) a long axostyle that bisects the cell longitudinally and protrudes through the posterior end of the organism. The combined movements of the flagella and axostyle impart the jerky, non-directional movement characteristic of the organism. While *T. vaginalis* possesses a typical eukaryotic endoplasmic reticulum and Golgi complex, the organism also contains a unique membrane-bound organelle termed the hydrogenosome. Hydrogenosomes, which are the sites of pyruvate fermentation, are notably different from mitochondria in that they are devoid of genetic material and contain enzymes such as hydrogenase and pyruvate:ferredoxin oxidoreductase, that are not present in mitochondria. In efforts to elucidate the origin of and the relationships between the hydrogenosome and the more typical eukaryotic organelles, many hydrogenosomal enzymes and genes have been characterized. Currently, two possible explanations exist as to the biogenesis of the hydrogenosome: (i) that mitochondria and hydrogenosomes originated from a common progenitor organelle or, (ii) that hydrogenosomes arose through the endosymbiosis of an ancient eukaryote with hydrogenase-containing anaerobic bacteria (Johnson *et al.* 1993; Lahti and Johnson 1991).

EPIDEMIOLOGY / CLINICAL FEATURES / RISK FACTORS

Of the 3 genera (Trichomonas, Pentatrichomonas and Tetratrichomonas) in the subfamily Trichomonadinae, *T. vaginalis* is the only species considered to be a true human pathogen. The organism is site specific, infecting the urogenital tract, although respiratory infections in newborns, believed to be acquired during delivery from infected mothers, have been reported (Hiemstra *et al.* 1984; McLaren *et al.* 1983). *T. vaginalis* is the causative agent of the sexually transmitted disease (STD) trichomonosis. According to figures compiled by the World Health Organization (1995), there were estimated to be 170 million new cases of trichomonosis worldwide with 8 million new cases in North America in 1995. These estimates give trichomonosis the distinction of being the most prevalent STD in both industrialized and developing countries.

In women, the clinical features of trichomonosis have been found to be wide-ranging, with the onset of menses exacerbating symptoms in some women. It is estimated that 25-50% of infected women are asymptomatic. In 50-75% of women presenting symptoms the chief complaint is of vaginal discharge and/or vulvovaginal irritation (Cotch *et al.* 1991; Rein 1990). The malodorous, frothy, green vaginal discharge, considered one of the hallmarks of the "classical" presentation of trichomonosis, is actually apparent in less than half of symptomatic women, however the "strawberry cervix" or colpitis macularis, caused by capillary dilatation and punctate haemorrhage, is detected by colposcopy in approximately 90% of cases (Wolner-Hanssen *et al.* 1989). Trichomonosis in men is usually an asymptomatic infection with spontaneous resolution. The asymptomatic infection does however provide a carrier state for transmission and cases persisting for up to four months

have been documented (Krieger *et al.* 1993a). *T. vaginalis* urethral infections in men have been significantly associated with nonchlamydial, nongonococcal urethritis (Krieger *et al.* 1993b; Krieger 1995). In this type of urethritis, the chief complaint noted is mild to moderately severe discharge.

T. vaginalis infection has recently been implicated as a risk factor in both human immunodeficiency virus type 1 (HIV-1) transmission and in adverse pregnancy outcomes. Studies involving HIV-1 sero-negative female prostitutes in Kinshasa, Zaire have, by statistical analysis, shown that trichomonosis is among one of the independently associated risk factors for HIV-1 seroconversion (Laga *et al.* 1993, 1994). The increased risk of HIV transmission in the presence of trichomonosis is believed to be due to an accumulation of lymphocytes and macrophages at the site of infection, which could provide pools of HIV-susceptible and HIV-infected cells. The colpitis macularis associated with trichomonosis may also allow a portal of entry for the virus. Vaginitis and pregnancy studies from the National Institutes of Health have estimated that 12.6% of pregnant women have trichomonosis (Cotch *et al.* 1991). *T. vaginalis* carriage at mid-gestation has been associated with low birth weight, an increased incidence of postpartum endometritis and perhaps most importantly, preterm delivery, one of the most common causes of neonatal morbidity in the United States (Cotch *et al.* 1991; McGregor *et al.* 1995; Minkoff *et al.* 1984; Read and Klebanoff 1993). Recent studies by McGregor *et al.* (1995) demonstrated that 28% of women with both bacterial vaginosis and trichomonosis have preterm deliveries. Although drug treatment was shown to reduce this risk, it did not eliminate the possibility of delivery prior to 37 weeks gestation.

DIAGNOSIS AND TREATMENT

The identification and treatment of *T. vaginalis* infections could not only reduce the forementioned risks, but could significantly defray their associated health care costs. In the past, the gold standard for diagnosis of trichomonosis has been microscopic wet mount coupled with sample cultivation, a technique that provides a 95% level of sensitivity (Krieger *et al.* 1988). Unfortunately, this diagnostic combination has been hampered by two shortcomings: (i) that the number of organisms required for wet mount detection is quite high (10^4 organisms/mL of vaginal fluid) and, (ii) that several days of incubation may be required for culture positivity, presenting a window of opportunity for continued disease transmission. Molecular techniques may provide the most sensitive and specific assays for diagnosis of *T. vaginalis* infections. Dot blots (Rubino *et al.* 1991), *in situ* hybridization (Muresu *et al.* 1994), nucleic acid capture assays (Briselden and Hillier 1994; Chapin-Robertson 1993) and polymerase chain reactions (PCR) (Rappelli *et al.* 1995; Riley *et al.* 1992) have been tested in diagnostic settings. These techniques have shown promise since they are: (i) sensitive; detecting from 1 x10^4 organisms (Briselden and Hillier 1994; Chapin-Robertson 1993) to as few as 5-100 organisms by PCR (Rappelli *et al.* 1995; Riley *et al.* 1992); (ii) rapid; the nucleic acid capture system is automated and can be completed in less than one hour; and (iii) specific; no cross-reactivity to bacteria, flagellated protozoa, genital pathogens or human DNA have been detected by dot blot, *in situ* hybridization or PCR analysis. Each of these techniques is also capable of detecting *T. vaginalis* specimens from different geographic locations.

Treatment of trichomonosis is primarily with the 5-nitroimidazole drug metronidazole,

which has a selective effect on anaerobic microorganisms. After diffusion into the cell, metronidazole is activated in the *T. vaginalis* hydrogenosome by the coupled reaction of the enzymes pyruvate:ferredoxin oxidoreductase and ferredoxin. Activation of the drug occurs by the transfer of a single electron from ferredoxin to the nitro group of metronidazole resulting in the production of cytotoxic free-radical intermediates. These intermediates react with parasite DNA and cause strand breakage, primarily between adenosine and thymidine residues (Edwards 1993; Heine and McGregor 1993). Typically, the treatment regimen has been a single 2 gram oral dose or 250 mg three times daily for 7 days, each resulting in a cure rate of 82-88% (Hager *et al.* 1980). There have however been well documented reports of metronidazole resistant trichomonosis (Grossman and Galask 1990; Voolmann and Boreham 1993) and concerns have arisen over the increase in the number of resistant strains reported. *T. vaginalis* appears to have developed both aerobic and anaerobic forms of metronidazole resistance. *T. vaginalis* strains exhibiting aerobic resistance are normally isolated from patients refractory to metronidazole treatment. *In vitro*, these isolates appear resistant to the drug but are susceptible when grown under anaerobic conditions, thereby indicating the existence of functional hydrogenosomal enzymes in the parasite (Tachezy *et al.* 1993). It is believed that aerobic resistance is a result of impaired *T. vaginalis* oxidases that have a lower affinity for O_2. Such defective oxidases would permit increased intracellular O_2 levels that would subsequently interfere with drug activation (Yarlett *et al.* 1986).

Anaerobic resistance has been detected *in vitro* using metronidazole susceptibility assays. Under anaerobic conditions, the minimal lethal concentration of metronidazole reported for susceptible organisms ranges from 0.8 - 25 μg/mL (Nix *et al.* 1995). *T.*

vaginalis isolates possessing anaerobic resistance however, require minimal lethal concentrations of metronidazole as high as 1,000 μg/mL and can replicate in the presence of 100 μg/mL of the drug (Kulda *et al.* 1993). Two hydrogenosomal enzymes, critical in the reductive activation of metronidazole, may be involved in this form of resistance. *T. vaginalis* strains exhibiting anaerobic resistance show no pyruvate:ferredoxin oxidoreductase activity (Kulda *et al.* 1993) and reduced intracellular ferredoxin levels (Quon *et al.* 1992). This decline in ferredoxin levels has been found to correspond to a decrease in the levels of ferredoxin mRNA transcription. In one characterized metronidazole resistant *T. vaginalis* strain, a point mutation was identified upstream of the ferredoxin transcriptional start site, that appeared to affect binding of a 23 kDa protein to the parasite DNA (Quon *et al.* 1992). The involvement of this point mutation in the downstream regulation of ferredoxin transcription remains unclear however, as other resistant *T. vaginalis* strains did not appear to carry the same point mutation.

PATHOGENESIS

Although *T. vaginalis* is the causative agent of the most prevalent STD worldwide, its pathogenic mechanisms remain largely undefined. Research has focused primarily on the production of extracellular factors, including numerous cysteine proteinases, and on the initial events required for infection.

Cysteine Proteinases and Other Extracellular Virulence Factors

T. vaginalis has high levels of proteinase activity, the majority of which is attributed to cysteine proteinases, although several metallo-proteinases have also been identified (Bózner and Demes 1991; Coombs and North 1983; Neale and Alderete 1990). These *T. vaginalis* cysteine proteinases appear to be both intracellular and secreted into culture media, with recent data indicating that secreted proteinases are released from lysosomes. Scott *et al.* (1995a) have shown that treatment of *T. vaginalis* with either amines or monensin, compounds known to alter vacuolar pH thereby affecting delivery to and secretion from lysosomes, stimulated the release of proteinase activity from the organism. Furthermore, proteinase activity in the parasite has been found to accumulate prior to its release, indicating slow lysosomal secretion as opposed to the more rapid and typical pathway offered by the Golgi complex. The clinical significance of *T. vaginalis* proteinases is supported by the presence of serum and vaginal anti-proteinase antibodies and, by detectable proteinase activity in vaginal washes. Alderete *et al.* (1991a, 1991b) have shown that of 20 patients analyzed, approximately 1/3 had soluble proteinases in vaginal washes and that 50/50 women infected with *T. vaginalis* possessed proteinase-specific serum antibodies. This immune response did appear to be short-lived however, as serum antibodies were not detected after metronidazole treatment. There have also been reports of proteinase-specific antibodies in the vaginal washes of 86% of women with trichomonosis (Bózner *et al.* 1992). Similarly, Garber and Lemchuk-Favel (1994) demonstrated the presence of a specific 60 kDa *T. vaginalis* proteinase in vaginal washes of 3/3 women with active infections. Although this 60 kDa proteinase was detected at the site of infection, its presence did not correlate with the clinical

presentation of the disease.

Several potential functions have been described for *T. vaginalis* proteinases. It appears that one or more of the cysteine proteinases is required to facilitate binding of parasite adhesin molecules to the surface of host cells. Arroyo and Alderete (1989) demonstrated that organisms treated with N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), a cysteine proteinase inhibitor, exhibited a drastic reduction in their capacity to adhere to epithelial cells. Adherence was restored when TLCK-treated organisms were exposed to papain or a proteinase active *T. vaginalis* extract, or when organisms were washed and incubated in fresh growth media. As they have been shown to be essential for erythrocyte lysis, there is also the potential for the cysteine proteinases to be required for nutrient acquisition. Dailey *et al.* (1990) demonstrated that TLCK-treated parasites had up to a 90% reduction in haemolytic activity as compared to untreated organisms. *T. vaginalis* erythrocyte lysis, which can provide the organism with a source of lipids and iron, has been shown to result from direct contact between parasite and erythrocyte (Fiori *et al.* 1993; Lehker *et al.* 1990). Fiori *et al.* (1993) identified five *T. vaginalis* adhesins involved in binding to host cells, two of which were specific for erythrocyte attachment. *T. vaginalis* haemolysis is believed to occur via a three-step process that involves: (i) parasite recognition and attachment to erythrocytes; (ii) release of a pore-forming cytolysin by the organism and, (iii) parasite detachment and erythrocyte lysis. Whether the *T. vaginalis* cysteine proteinases aid in the parasite's binding to erythrocytes, similar to their putative role in epithelial cell adhesion, or whether they are directly involved in erythrocyte lysis remains undetermined at this time.

The most recent and interesting of the proposed roles for the cysteine proteinases is in the evasion of the immune response. Several distinct cysteine proteinases with the ability to degrade human IgG, IgM and IgA antibodies have been identified in *T. vaginalis* cell lysates and culture supernatants (Provenzano and Alderete 1995). These findings may have clinical relevance as the authors identified these immunoglobulin degrading proteinases in the vaginal washes from 4/20 women with *T. vaginalis* infections. Additionally, Alderete *et al.* (1995b) have demonstrated that cysteine proteinases can mediate evasion of the alternative complement pathway. This group has shown that, while *T. vaginalis* grown in iron-supplemented medium are resistant to complement lysis, organisms treated with TLCK are readily killed by complement. In this situation, *T. vaginalis* cysteine proteinases are believed to degrade C3 bound to the surface of the parasite. By molecular techniques, several other *T. vaginalis* cysteine proteinases have been identified, yet their functions remain undefined (Garber and Lemchuk-Favel 1989; Garber *et al.* 1993; Mallinson *et al.* 1994). There has been speculation that proteinases may also serve a role in disease establishment. This possibility has arisen due to the identification of an acid-stable, secreted proteinase that could potentially remain functional in the acidic vaginal environment (Garber *et al.* 1993; McGrory *et al.* 1994).

Cytopathic Effects on Cell Monolayers

To address whether the vaginitis caused by *T. vaginalis* infection was the result of mechanical injuries sustained by the cell during contact with the parasite or was instead due to the secretion of cytotoxic factors, Hogue (1943) grew *T. vaginalis* in the presence of tissue

cultures from human fetal material and chick embryos. Despite the early limitations in cell culture technology, Hogue reported that the cytopathic effects caused by *T. vaginalis* were the result of a secreted, heat-labile cytotoxic substance. Since this initial study, cell culture techniques and cell lines have become well established, allowing investigators to analyze, under defined conditions, the interaction between parasite and the eukaryotic cell monolayer.

Cytoadhesion and Contact-dependent Cytotoxicity

The first evidence implicating direct contact between *T. vaginalis* and eukaryotic cells in the production of cytopathic effects (CPE) came in the early 1960's when Christian *et al.* (1963) reported that *T. vaginalis* replicated and destroyed HeLa cells in culture. Since that time, various groups have reported that *T. vaginalis*, upon incubation with eukaryotic monolayers, formed masses of organisms or "clumps" which adhered to cells and caused destruction in the areas of contact (Alderete and Pearlman 1984; Farris and Honigberg 1970; Heath 1981; Krieger *et al.* 1985; Nielsen and Nielsen 1975; Rasmussen *et al.* 1986). Using kinetic analysis of target cell killing, Krieger *et al.* (1985) demonstrated that the fraction of target cells killed was directly proportional to the probability of cells coming into direct contact with the parasite. This work also indicated that a single parasite had the potential to be lethal for a target cell. Contact-dependent cytotoxicity appears to consist of two components: (i) initial parasite adherence to the cell and, (ii) post-adherent cytolytic events.

Cytoadherence of *T. vaginalis* has been studied most extensively by Alderete and co-workers. Early work by this group demonstrated that *T. vaginalis* isolates can adhere to both HeLa cells and VECs in a time, temperature and pH dependent fashion, with optimal

adherence occurring at 37°C and at a pH of 6.0 (Alderete and Garza 1985, 1988; Alderete *et al.* 1988; Arroyo *et al.* 1992). Four *T. vaginalis* adhesin proteins (AP) have been implicated in cytoadherence and are designated AP65, AP51, AP33 and AP23 according to molecular mass. Specific antibodies raised to each adhesin inhibit parasite binding, but do not cross-react, indicating an independent role for each molecule in the adhesion process (Arroyo *et al.* 1992). The *T. vaginalis* adhesins have several unique properties. First, as described previously, the adhesins require the presence of one or more parasite cysteine proteinases to facilitate attachment to host cells (Arroyo and Alderete 1989). Since adhesin precursor molecules have not been detected, it is believed that the adhesins may be protected by a "masking" protein that must be degraded before the adhesins can become functional. Surface expression of adhesins is also higher in fresh *T. vaginalis* isolates compared to long-term maintained laboratory strains (Arroyo *et al.* 1992). These findings corroborate the earlier work of Rasmussen *et al.* (1986) who demonstrated that fresh *T. vaginalis* isolates were more cytotoxic to epithelial cells *in vitro* than those axenically maintained in the laboratory. Finally, adhesin protein synthesis appears to be regulated at several different levels, and by several different signals. Lehker *et al.* (1991) showed that surface expression of adhesins is increased with high iron concentration and that this regulation was associated with the level of gene transcription. Increased synthesis of both the AP65 and AP33 adhesins has also been demonstrated after adhesin binding to VECs (Arroyo *et al.* 1993). This increased surface expression of the adhesins may promote higher affinity binding of the parasite to the host cell and therefore provide the organism with an advantage for establishment in the continuously bathed vaginal environment.

Recently, two cDNA clones were isolated using a monoclonal antibody raised against *T. vaginalis* AP65 (Alderete *et al.* 1995a). In ligand binding assays, recombinant proteins expressed by these sequences bound to HeLa cell surfaces. Furthermore, these recombinant adhesins inhibited the binding of native *T. vaginalis* AP65 to HeLa cells in a concentration-dependent manner. Since these *T. vaginalis* cDNA clones encode proteins with 38% amino acid identity to malic enzyme, a ubiquitous eukaryotic protein, it has been suggested that the cell surface location of the AP65 adhesin may not only play a key role in parasite binding, but also in the evasion of the immune response by a form of molecular mimicry (Alderete *et al.* 1995a).

Cytoadherence may also be mediated by "lectin-like" adhesins on the surface of *T. vaginalis,* that could interact with specific carbohydrate moieties on the target cell surface. Adherence facilitated by lectin binding has been demonstrated for two other amitochondrial protozoa. *Giardia lamblia* expresses a surface lectin termed taglin that binds specifically to mannose 6-phosphate (Farthing *et al.* 1986; Ward *et al.* 1990). *Entamoeba histolytica* adherence to host cells is mediated by a surface lectin which binds galactose and N-acetylgalactosamine (Adler *et al.* 1995; McCoy *et al.* 1994). Roussel *et al.* (1991) and Bonilha *et al.* (1995) have suggested that *T. vaginalis* cytopathology may be dependent upon specific lectin binding however, Alderete and Garza (1985) were unable to inhibit binding of organisms to HeLa cells using several different simple sugars and glycoproteins. The discrepancies between these studies may be due to the various cell lines used, and the assays employed in determining cytotoxicity and cytoadherence. Alderete and Garza (1985) used radiolabelled parasites in ligand binding assays with HeLa cell monolayers to assess lectin-

mediated adherence. As HeLa cells possess receptors for *T. vaginalis* protein adhesins, cytoadherence mediated by these molecules may mask that produced by lectin binding. Using McCoy cells and visual assessment of monolayer CPE, Roussel *et al.* (1991) demonstrated that *T. vaginalis* specific lectin binding could be inhibited by N-acetylglucosamine and mannose. The assays employed in this study however, may not have been specific for adherence considering that *T. vaginalis* secreted cytotoxins could enhance CPE. Finally, Bonilha *et al.* (1995) evaluated adherence using visual enumeration of bound and stained parasites to Chinese hamster ovary (CHO) cells and glycosylation deficient mutants that expressed one major type of N- or O-linked carbohydrate. *T. vaginalis* bound to mutant cell lines with terminal mannose, galactose or N-acetylglucosamine residues. This cytoadherence appeared specific as the addition of exogenous sugar to the culture medium inhibited binding. The authors could not demonstrate similar interference when assays were performed with cultured epithelial cells. These last results appear to emphasize the importance of the cell line chosen for evaluating parasite adherence.

Although it is clear that parasite adherence leads to host cell death (Alderete and Pearlman 1984; Krieger *et al.* 1985), the events which occur post-attachment and result in cytotoxicity are not well defined. Upon adherence to VECs (Arroyo *et al.* 1993), rabbit kidney tubule epithelial cells (RK-13) (Heath 1981) or Madin Darby canine kidney (MDCK) cells (González-Robles *et al.* 1995), *T. vaginalis* morphologically transforms from a round, ovoid shape to an amoeboid form. While this morphological change can occur within 5 minutes after binding to VECs, its importance in pathogenicity remains unknown. Both proteinase content and secretion appear indistinguishable between the two morphological

forms of the parasite (Scott *et al.* 1995b), yet the possibility remains that some proteinases are differentially regulated as a result of the transformation. Transformation to an amoeboid form may simply provide *T. vaginalis* a larger surface area for adherence, or it may signal the parasite to secrete a chemoattractant factor. This latter possibility comes from the observation that, following the adherence of a single organism to a host cell, there is a rapid recruitment of parasites to the cell surface producing what has been termed a "swarming" effect (Arroyo *et al.* 1993; González-Robles *et al.* 1995). Between adjacent parasites in this swarm, there appears to be extensive and multiple membrane interdigitations (Arroyo *et al.* 1993; González-Robles *et al.* 1995). The mechanism involved in cell destruction appears to involve modifications to the host cell plasma membrane, that includes the opening of tight cell membrane junctions, membrane blebbing and ultimately, membrane leakage (González-Robles *et al.* 1995). The precise mechanism of membrane damage requires further study, but may involve *T. vaginalis* secretion of proteinases and pore-forming proteins similar to the cytotoxic mechanism employed by *E. histolytica* (Martínez-Palomo 1993).

Cytopathic Effects Produced by Contact-independent Mechansims

While substantial evidence supports a contact-dependent mechanism of cytotoxicity, most investigators have not ruled out the possibility that *T. vaginalis* produces other factors that could induce a cytopathic effect (Alderete and Pearlman 1984; González-Robles *et al.* 1995). This hypothesis has been substantiated primarily by clinical findings of severe and diffuse vaginitis during *T. vaginalis* infections, despite the localization of the organism (Nielsen and Nielsen 1975). Not until some forty years after Hogue's (1943) initial report of

a secreted *T. vaginalis* cytotoxin were Pindak *et al.* (1986) able to reproducibly demonstrate a cell-detaching factor (CDF) in cell-free *T. vaginalis* filtrates. CDF was initially produced by allowing parasites to incubate with confluent McCoy cell monolayers. The resulting culture supernatants were collected, clarified, adjusted to pH 7.3, and filter sterilized to yield CDF-containing filtrate. CDF caused detachment and clumping of susceptible cell monolayers and could inhibit freshly seeded cells from forming monolayers (Pindak *et al.* 1986). As cells exposed to CDF remained viable, the factor was considered to be cytopathic, but not cytotoxic to host cells (Lushbaugh *et al.* 1989; Pindak *et al.* 1986).

Since its initial identification, several properties have been associated with CDF (Garber *et al.* 1989; Lushbaugh *et al.* 1989; Pindak *et al.* 1986). All *T. vaginalis* isolates tested to date produce CDF while non-pathogenic Trichomonads such as *Pentatrichomonas hominis* do not. Production of CDF is solely dependent on the viability of the parasite, and not of the eukaryotic cell monolayer. Although CDF is optimally produced by allowing parasite replication in the presence of cell monolayers, it has been detected in axenic cultures of *T. vaginalis* (Garber *et al.* 1989; Lushbaugh *et al.* 1989). It has also been established that not all cell lines are susceptible to CDF activity. HeLa, McCoy, HEp-2, CHO, baby hamster kidney and human foreskin fibroblast cell lines all appear sensitive to CDF, while Vero, MDCK, trypsin-sensitive RK-13 and trypsin-insensitive murine macrophage-like (WEHI-3) cell lines remain unaffected by the presence of CDF. Three factors appear critical for CDF production: (i) the duration of *T. vaginalis* growth prior to filtrate preparation; (ii) the size of the initial parasite inoculum and, (iii) the pH of the filtrate at the time of harvest (Garber *et al.* 1989). This last item is of critical importance since CDF has been shown not only to

be heat labile but also acid labile and inactive at pH < 5.0. It has been suggested that the inability to detect CDF in the past was a direct result of the molecule's acid lability. Following protein purification and SDS-PAGE analysis, the major protein species in active CDF preparations is a 200 kDa molecule, that is sensitive to proteinase K digestion and periodate treatment (Garber *et al.* 1989). CDF also appears immunogenic, and can be detected in immunoblots probed with high-titer *T. vaginalis* human anti-serum (Garber *et al.* 1989). Finally, a correlation has been found between the *in vitro* production of CDF and the clinical presentation of trichomonosis, therefore suggesting CDF to be a marker of *T. vaginalis* disease (Garber and Lemchuk-Favel 1990).

T. vaginalis CDF shows interesting similarity to the cysteine proteinase histolysin of *E. histolytica*. This invasive parasite causes ulceration of the intestinal wall, and its trophozoite form can produce cytotoxic effects on cell culture monolayers *in vitro*. The cytotoxicity of *E. histolytica* appears to be mediated by mechanisms similar to those proposed for *T. vaginalis*, namely: (i) contact-dependent cytolysis involving surface lectin binding and pore-forming proteins (Adler *et al.* 1995; Leippe *et al.* 1991; Young *et al.* 1982), and (ii) contact-independent cytotoxicity produced by the secretion of histolysin (Keene *et al.* 1986; Luaces and Barrett 1988). Histolysin, which is active at neutral pH, shows the same functional activity as CDF. Monolayers of baby hamster kidney cells or human skin fibroblasts incubated with histolysin exhibit cell rounding and detachment, however cells remain viable. Histolysin degrades cell anchoring proteins and has demonstrated activity against cartilage proteoglycan and kidney glomerular basement-membrane collagen (Luaces and Barrett 1988). A correlation has also been established between the clinical severity of

amoebiasis and the level of proteinase/histolysin activity exhibited by *E. histolytica* trophozoites (Keene *et al.* 1986; McKerrow *et al.* 1993). While functional similarity exists between histolysin and CDF, it has not been established if CDF is a cysteine proteinase. The molecules also differ drastically in size. Histolysin has been identified as a 26-29 kDa protein (Luaces and Barrett 1988), while *T. vaginalis* CDF appears to be a 200 kDa molecule.

Finally, there is a distinct possibility that CDF may be regulated by female steroid hormones, most notably estrogens. The idea of mammalian hormones inducing biological responses in microorganisms is not without precedent. Estrogens inhibit the transformation of the mycelial form of the dimorphic fungus, *Paracoccidioides brasiliensis*, to its pathogenic yeast form (Restrepo *et al.* 1984; Salazar *et al.* 1988). Human luteinizing hormone and chorionic gonadotrophin similarly induce the transition of *Candida albicans* from blastospore to mycelial form (Caticha *et al.* 1992). Two observations suggest that estrogen may play a role in *T. vaginalis* pathogenesis: (i) trichomonosis has been reported to be exacerbated during menses, a time of increased estrogenic activity and, (ii) specific receptors for 17-β-estradiol have been identified in *T. vaginalis* (Ford *et al.* 1987). Recently, Garber *et al.* (1991) demonstrated that β-estradiol, at molar concentrations of 10^{-7} to 10^{-8}, had a significant effect on CDF activity. Parasites grown in the presence of hormone had reduced CDF activity, yet when the hormone was added back to active filtrates, CDF activity was not affected. This finding suggests that estrogen regulates CDF expression, however the level at which this regulation occurs remains undefined.

RATIONALE / OBJECTIVES

Little is known about the virulence factors that *T. vaginalis* employs during the establishment and maintenance of disease. CDF may be important in pathogenesis since: (i) CDF has been shown to be produced in only Trichomonad species pathogenic to humans and, (ii) CDF production has been found to correlate with clinical presentation of disease. The original objective of this study therefore was to characterize *T. vaginalis* CDF. This characterization was to be accomplished by isolating cDNA sequences following immunological screening of a *T. vaginalis* cDNA expression library. cDNA clones would then be used to isolate a genomic copy of CDF, including sequences flanking the gene. This work would lead to information about CDF's structure and functional domains. Additionally, if protein similarity existed to other known cytotoxins, it would help to elucidate CDF's mode of action. Identification of the 5' and 3' sequences flanking the gene would also establish the genomic organization of CDF and would provide a background for future studies involving the regulation of CDF expression.

CHAPTER 2: MATERIALS AND METHODS

PARASITES

A single, well characterized *T. vaginalis* clinical isolate (#202), obtained from the vaginal secretions of a woman with symptomatic trichomonosis, was used exclusively throughout this work. Based upon a scoring scheme that evaluates the clinical presentation of disease, this isolate has been classified as moderate (Garber and Lemchuk-Favel, 1990). Additionally, the isolate has been shown to produce nodule formation in the subcutaneous mouse assay as well as the production of extracellular proteases and a cell-detaching factor (Garber and Lemchuk-Favel 1990, 1994). Parasite cultures were grown in Diamond's TYI-S-33 medium (TYI), pH 6.2 (Diamond *et al.* 1978) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco/BRL, Burlington, Ontario), 100 U/mL penicillin, 100 µg/mL streptomycin (Penicillin-Streptomycin solution, Gibco/BRL) and 2.5 µg/mL Amphotericin B (Fungizone, Gibco/BRL). Cultures were incubated at 37°C in a 5% CO_2 atmosphere and passed every 2-3 days. Axenic stocks were prepared by the addition of 10% FBS and 10% dimethyl sulfoxide (DMSO) to log phase parasite cultures that were then mixed, dispensed into aliquots and stored frozen at -70°C.

CELL CULTURE

McCoy cells (ATCC CRL 1696) were grown in CMGA medium (Garber *et al.* 1987) and incubated at 37°C in a 5% CO_2 atmosphere. Cells used for filtrate production were grown to confluence in 75 cm^2 tissue culture flasks and washed 3 times with phosphate-

buffered saline solution (PBS: 6.8 M KH_2PO_4, 3.7 M KCl, 73 mM NaCl, 657 mM Na_2HPO_4, pH 7.2) prior to the addition of parasite suspensions. For cytotoxicity assays, confluent cell monolayers were washed with PBS, trypsinized, and resuspended in pre-warmed CMGA medium. Cell were counted by trypan blue exclusion, using a hemacytometer, and diluted in CMGA medium to the appropriate concentration required for seeding.

PRODUCTION OF *T. VAGINALIS* FILTRATE AND CYTOTOXICITY ASSAYS

For filtrate production, parasites were grown to log phase in TYI medium, pelleted by centrifugation at 1,000 rpm for 10 minutes (Sorvall GLC-1 or a Heraeus OmnifugeRT model centrifuge), and then washed three times with PBS. Organisms were resuspended in CMGA/TYI medium (2:1 v/v ratio, pH 6.8) containing either 10% FBS or serum-free. Parasites were counted by trypan blue exclusion, and diluted to a density of 1×10^5 viable parasites/mL. 20 mL aliquots of the parasite suspension were added to confluent McCoy cell monolayers, and flasks were allowed to incubate for 22-24 hrs at 37°C, in 5% CO_2. Following incubation, medium was collected and centrifuged for 10 minutes at 1,000 rpm to produce cell-free supernatants. Supernatant pH was determined and, if less than pH 6.0, was adjusted with 1 N NaOH to a final pH of 6.5. Supernatants were sterilized by passage through 0.22 µm filters and the filtrates dispensed into aliquots and frozen at -20°C. Protein concentration of filtrates was determined with the Bio-Rad Protein assay kit (Bio-Rad, Mississauga, Ontario) using bovine serum albumin (BSA) as a standard.

Cytotoxicity assays were performed using either confluent cell monolayers or by co-incubation of McCoy cells with filtrates and/or protein samples. To produce confluent cell

monolayers, 48 well plates were seeded with 1 mL of 5 x 10^4 McCoy cells in CMGA medium, and allowed to incubate for 48 hrs at 37°C, in 5% CO_2. Monolayers were washed twice with PBS and 300 µL of the test sample were added to each well. For co-incubation assays, 500 µL of 1.5 - 2 x 10^5 McCoy cells/mL in CMGA medium were added to 48 well plates containing 300 µL of neat or diluted test samples. All samples used in cytotoxicity assays were diluted in serum-free CMGA/TYI medium and were syringe filter sterilized (0.22 µm Millex-GV_4 filter units, Millipore, Mississauga, Ontario). Cytotoxicity plates were incubated for 24 hrs at 37°C, in 5% CO_2 and were then assessed for monolayer confluence using a staining procedure initially described by Alderete and Pearlman (1984). Briefly, medium was aspirated from wells and adherent cells were gently washed twice with PBS. Monolayers were fixed by the addition of 300 µL of 2% formaldehyde in PBS, stained with 150 µL of crystal violet solution (5:2 ethanol:formaldehyde (v/v) containing 0.13% crystal violet) and washed several times with distilled H_2O. Stained monolayers were air dried and solubilized with 150 µL of 1% sodium dodecyl sulfate (SDS) in 50% ethanol. Sample solutions were then transferred to 96 well plates and the A_{595} measured in a Bio-Rad Model 3550 Microplate Reader.

ISOLATION OF *T. VAGINALIS* NUCLEIC ACIDS

Total Parasite RNA

Total RNA was isolated from 1 x 10^8 log phase parasites by either high salt SDS-Proteinase K extraction (Heebner and Albach 1982) or by acid guanidinium thiocyanate extraction (Chomczynski and Sacchi 1987). For both methods, parasites were grown in TYI

medium, pelleted by centrifugation at 1,000 rpm for 10 minutes (Sorvall GLC-1 or Heraeus OmnifugeRT centrifuge) and washed once with PBS. High salt SDS-Proteinase K extraction involved resuspension of the parasite pellet in high salt extraction buffer (HSEB: 50 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 500 mM NaCl) containing 1% polyvinyl sulfate and 1% spermine-tetrahydrochloride. Immediately after suspension, an equal volume of pre-warmed 2X NENS buffer (100 mM sodium acetate, pH 5.1, 200 mM NaCl, 20 mM EDTA, 1% SDS, 400 µg/mL Proteinase K) was added and the mixture was incubated for 30 minutes at 37°C. This solution was phenol extracted once (phenol:chloroform:isoamyl alcohol 25:24:1), chloroform extracted (chloroform:isoamyl alcohol 24:1) and the nucleic acids were precipitated by the addition of 2.5 volumes of absolute ethanol. Following centrifugation, nucleic acids were resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O, an equal volume of 5 M lithium chloride was added, and the solution was incubated at 4°C for 15 minutes to allow precipitation of high molecular weight RNA. The RNA precipitate was pelleted, resuspended in DEPC-H_2O, ethanol precipitated overnight at -20°C and then washed with 70% ethanol and resuspended in DEPC-H_2O. RNA was quantitated by absorbance at A_{260} using a Beckman DU-8B spectrophotometer.

For acid guanidinium thiocyanate extraction, the parasite pellet was resuspended in 1 mL of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl and 0.1M 2-β-mercaptoethanol) to which was added 0.1 mL of 2 M sodium acetate, pH 4.0; 1 mL of water-saturated phenol and 0.2 mL of chloroform:isoamyl alcohol (49:1). This final suspension was shaken vigorously for 10 seconds, cooled on ice for 15 minutes and then centrifuged for 20 minutes at 13,000 rpm, 4°C (Beckman J2-21M

centrifuge, JA20 rotor). RNA in the aqueous phase was precipitated with isopropanol for approximately 3 hours at -20°C, pelleted and re-precipitated from 300 µL of denaturing solution with 1 mL of isopropanol. The RNA was lithium chloride precipitated, ethanol precipitated, resuspended in DEPC-H_2O and quantitation by absorbance at A_{260}.

Polyadenylated RNA

Polyadenylated RNA was purified by oligo-(dT) cellulose column chromatography (Gibco/BRL). Briefly, total *T. vaginalis* RNA was precipitated and resuspended in 1 mM EDTA, pH 7.0 at a concentration of between 1-2 mg/mL. This solution was heated at 70°C for 1 minute and immediately chilled on ice. An equal volume of 2X binding buffer (0.01 M Tris-HCl, pH 7.5, 1 M NaCl, 2 mM EDTA, 1% SDS) was added to the RNA and the sample was applied to an oligo-(dT) cellulose column previously equilibrated with binding buffer (0.01 M Tris-HCl, pH 7.5, 0.5 M NaCl, 1 M EDTA, 0.5% SDS). The flow through sample was passed over the column three times to ensure binding. The column was washed with binding buffer and polyadenylated RNA was eluted with 0.01 M Tris-HCl, pH 7.5, 10 mM EDTA and 0.1% SDS. Eluted fractions were analyzed by absorbance at A_{260} and fractions containing RNA were pooled and precipitated with ethanol. The final RNA pellet was resuspended in DEPC-H_2O.

High Molecular Weight Genomic DNA

High molecular weight DNA was isolated from 1 x 10^8 log phase parasites by the method described by Wang and Wang (1985). Parasites were harvested by brief

centrifugation, washed twice in cold PBS and the cell pellet was lysed at room temperature in ten volumes of 4 M guanidinium thiocyanate solution (Chirgwin *et al.* 1979) (4 M guanidinium thiocyanate, 0.5% Sarkosyl, 25 mM sodium citrate, pH 7.0, 0.1 M 2-β-mercaptoethanol, final pH 7.0). The lysate was vortexed for 10 seconds and cesium chloride was added to a final concentration of 0.4 g/mL. The mixture was layered on a cushion of 5.7 M CsCl/ 0.1 M EDTA, pH 7.0 and centrifuged for 16 hours at 34,000 rpm, 20°C (RC70 Sorvall Ultracentrifuge, TH695 rotor). Fractions were collected, diluted two-fold with TE buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA), and extracted repeatedly with chloroform:isoamyl alcohol (49:1) until a clear interface was achieved. Ethanol was then added to all fractions for nucleic acid precipitation. Pellets were resuspended in TE buffer and samples were treated with RNAase A (200 µg/mL) for 30 minutes at 37°C. Aliquots from each fraction were analyzed by electrophoresis on 0.4% agarose gels. Those samples found to contain high molecular weight DNA (>44 kb) were pooled, and the DNA was re-precipitated, suspended in TE buffer and quantitated by absorbance at A_{260}.

BACTERIOPHAGE METHODOLOGY

Preparation of Plating Cells

Unless otherwise stated, *Escherichia coli* strain Y1090 was used to titer phage and screen plaques. Bacteria were prepared for plating by streaking cells onto TYN-ampicillin (Amp) plates (TYN: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.2% maltose, 50 µg/mL ampicillin with 1.5% agar added for plates). Fresh single colonies were inoculated into 10 mL of TYN-Amp broth and cells were grown

overnight at 37°C with shaking. One mL of overnight culture was inoculated into 49 mL of TYN-Amp broth and the culture was incubated at 37°C with shaking until an A_{600} of approximately 0.5 was reached. The culture was cooled on ice, and cells were pelleted by centrifugation at 3,000 rpm, 4°C for 10 minutes (Heraeus OmnifugeRT model centrifuge). Pelleted cells were resuspended in 15 mL of cold 10 mM $MgSO_4$, diluted to an A_{600} of 0.5 and then stored at 4°C and used within 3 days.

Bacteriophage Titration

For titration of the λgt11 library or recombinant phage stocks, duplicate 10-fold serial dilutions of samples were prepared in Phage Dilution Buffer (PDB: 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.01% gelatin). 100 µL of diluted phage was mixed with 100 µL of bacteria and the phage were allowed to adsorb to the cells for 30 minutes at 37°C. This phage/bacteria suspension was added to TYN top agar (TYN without ampicillin and with 0.7% agar), mixed, and the suspension poured onto TYN-Amp plates that were then incubated overnight at 42°C. To purify individual recombinant phage, plaques were isolated in agar plugs using a Pasteur pipette. Plugs were placed in a 1.5 mL microfuge tube containing 0.5 mL of PDB, a drop of chloroform was added, and the mixture was rotated for 2-3 hours at room temperature, to allow bacteriophage particles to diffuse from the agar plugs. Phage from individual plaques were stored at 4°C.

Plate and Liquid Lysates

Purification of bacteriophage was achieved by producing plate or liquid lysates. For

plate lysates, 600 μL of bacteria (final A_{600} ~1.0) were mixed with a dilution of phage stock that would produce uniform lysis of the bacterial lawn (usually between 1-2 x 10^5 PFU). The suspension was incubated at 37°C for 20 minutes and was then added to 7 mL TYN top agarose. This mixture was poured onto 150 mm TYN-Amp plates that were incubated for 6-8 hours at 42°C. Following complete bacterial lysis, plates were overlaid with 10 mL of PDB and incubated overnight at 4°C with gentle rocking. PDB was collected and the top agarose was scraped from the plates and added to the harvested solution. Plates were washed with an additional 5 mL of PDB that was added to the pooled sample and chloroform was added (0.3% of the total volume). The suspension was briefly vortexed and centrifuged at 3,000 rpm, 4°C for 20 minutes (Heraeus OmnifugeRT centrifuge). Several drops of chloroform were added to the collected supernatants and phage titers were determined. Plate lysate stocks were stored at 4°C.

Liquid lysates were produced by initially adding 100 μL of PDB to the agar plug of a single plaque. This suspension was incubated overnight at 4°C and then added to 500 μL of an overnight culture of *E. coli* Y1090. The bacteria/phage suspension was incubated for 20 minutes at 37°C, to allow for phage to adsorb to the bacteria. The suspension was added to 100 mL of pre-warmed TYN-Amp broth and the culture was shaken at 37°C until lysis occurred (usually after ~5 hours). Upon lysis, cultures were centrifuged at 7,000 rpm, 4°C for 10 minutes (RC2-B Sorvall centrifuge, JA-S rotor) and the supernatant was transferred to a sterile tube and chloroform was added. The titer of phage in the liquid lysate was determined and the stocks were stored at 4°C.

Bacteriophage Purification

To either plate or liquid lysates, 1 μg/mL DNAase I and 5 μg/mL RNAase A were added and the suspension was incubated for 1 hour at 37°C. Sodium chloride was then added to a final concentration of 1 M, and the lysate was incubated for 1 hour on ice. Bacterial debris was pelleted by centrifugation at 7,000 rpm, 4°C for 20 minutes (RC2-B Sorvall centrifuge, JA-S rotor). Polyethylene glycol (PEG) 8000 (final concentration 10% w/v) was added to the lysate supernatant and allowed to dissolve. Phage was left to precipitate overnight at 4°C and was then pelleted by centrifugation at 7,000 rpm, 4 °C for 20 minutes (RC2-B Sorvall centrifuge, JA-S rotor). The phage pellet was gently resuspended in PDB (10 mL/100 mL of initial lysate), and extracted with an equal volume of chloroform. The aqueous phase was centrifuged at 30,000 rpm, 4°C for 3 hours (RC70 Sorvall Ultracentrifuge, TH-641 rotor). Phage pellets were resuspended in 1 mL of PDB and stored at 4°C.

Extraction of Phage DNA

Proteinase K (final concentration 50 μg/mL), EDTA (final concentration 5 mM) and SDS (final concentration 0.5%) were added to purified phage resuspended in PDB. The suspension was mixed, incubated for 1 hour at 56°C, allowed to cool to room temperature and extracted twice with phenol and once with chloroform. Phage DNA was precipitated from the aqueous phase with ethanol, washed with 70% ethanol, air dried and resuspended in an appropriate volume of TE buffer.

PLASMID METHODOLOGY

Small Scale Isolation of Plasmid DNA from *E. coli* (Mini-prep)

Isolation of plasmid DNA was by a modified alkaline extraction method originally described by Birnboim and Doly (1979). Bacterial cells were grown overnight at 37°C with shaking in Luria-ampicillin broth (LB-Amp: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 50 μg/μL ampicillin). Cells were pelleted, resuspended in 200 μL of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 4 mg/mL lysozyme) and incubated at room temperature for 5 minutes. Bacteria were lysed by addition of 400 μL of freshly prepared Solution II (0.2 N NaOH, 1% SDS), and samples were incubation for 5 minutes at room temperature and then neutralized with 300 μL of 7.5 M ammonium acetate. This suspension was thoroughly mixed, placed on ice for 10 minutes, and centrifuged at 13,000 rpm for 10 minutes (Biofuge A Microcentrifuge). Plasmid DNA was precipitated from the supernatant by the addition of 500 μL of isopropanol, and pelleted by centrifugation at 13,000 rpm for 15 minutes. DNA was washed with 70% ethanol and then resuspended in 100 μL of TE buffer.

Large Scale Isolation of Plasmid DNA from *E. coli* (Maxi-prep)

Initially plasmid DNA was isolated using an alkaline extraction method similar to that described above. Briefly, 5 mL of a LB-Amp broth culture were inoculated with a single colony and incubated overnight at 37°C with shaking. The following day, 200 μL of this culture were inoculated into 25 mL of LB-Amp broth, bacteria were grown for 3 hours and the entire culture was then added to 1 L of LB-Amp broth and incubated overnight at 37°C

with shaking. Cells were harvested by centrifugation at 6,000 rpm, 4°C for 10 minutes (Beckman J2-21M model centrifuge, JA-10 rotor) and resuspended in 6 mL (per 500 mL of pellet) of Solution I (containing 20 mg/mL lysozyme). This suspension was transferred to polyallomer centrifuge tubes and incubated for 10 minutes at room temperature. 12 mL of Solution II were added for lysis, the suspension was mixed, incubated 10 minutes at room temperature and then neutralized by the addition of 9 mL of 7.5 M ammonium acetate. After thorough mixing, the suspension was placed on ice for 20 minutes and then centrifuged for 25 minutes at 20,000 rpm, 4°C (Beckman Model L3-50 Ultracentrifuge, SW28 rotor). DNA was precipitated from supernatants with isopropanol, and pelleted by centrifugation for 30 minutes 10,000 rpm, 4°C (Beckman Model J2-21 Centrifuge, JA-20 rotor). DNA was resuspended in TE buffer and plasmid DNA was purified on CsCl gradients as described by Sambrook *et al.* (1989) using a RC70 Sorvall Ultracentrifuge with a ViT65 rotor.

In later experiments, large scale plasmid DNA isolations were performed with either Qiagen plasmid DNA isolation kits (Q-500 or Q-100 tips, Qiagen Inc., Chatsworth, CA) or Nucleobond AX kits (AX-100 cartridges, Vector Biosystems, Toronto, Ont.) using the instructions provided by the manufacturers. Purified plasmid DNA was quantitated by absorbance at A_{260}.

Bacterial Cracking Procedure for Rapid Estimation of Plasmid Size

For rapid screening of nested deletion clones, a cracking procedure modified from that described by Sambrook *et al.* (1989) was used. Individual colonies were picked from transformation plates, smeared along the inside wall of a 0.5 mL microfuge tube and

subsequently streaked onto an LB-Amp plate (LB-Amp broth with 1.5% agar). To tubes containing the bacterial smear, 50 µL of 10 mM EDTA, pH 8.0 was added and the bacteria were resuspended by vortexing. Freshly made cracking buffer (50 µL of 0.2 M NaOH, 0.5% SDS, 0.58 M sucrose) was added and the tubes were vortexed, incubated at 70°C for 5 minutes, and allowed to cool to room temperature. 1.5 µL of 4 M KCl were added to samples that were then mixed and placed on ice for 5 minutes. Samples were centrifuged at 13,000 rpm for 5 minutes (Biofuge A Microfuge) and 2 µL of 0.4% bromophenol blue were added to the supernatants. 25 µL of each sample were used for agarose gel electrophoresis.

T. VAGINALIS λgt11 cDNA LIBRARY CONSTRUCTION AND SCREENING

Library Construction

cDNA was synthesized from 3 µg of polyadenylated RNA template using the Gibco/BRL cDNA synthesis system and the protocol supplied by the manufacturer. cDNA fragments were filled in with T4 DNA polymerase and then ligated to *EcoR* I adaptors (Amersham, Oakville, Ontario). The "adapted" cDNA was purified by Sepharose CL-4B column chromatography, phosphorylated using T4 polynucleotide kinase and ligated to dephosphorylated λgt11 arms (Amersham cDNA cloning system for λgt11). Ligation mixes were incubated with bacteriophage packaging components and the recombinant phage were titered on *E. coli* Y1088. Initial library titration was by the same method as previously described except that TYN top agar was supplemented with 1 mM isoproyl β-D-thiogalactopyranoside (IPTG) and 0.64 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to identify recombinant phage. The library was amplified on *E. coli*

Y1090 by the method described by Sambrook *et al.* (1989) with library aliquots dispensed and stored at -70°C. Library integrity was evaluated by analyzing the size of cDNA inserts isolated from 12 randomly selected phage clones.

Immunological Screening of the λgt11 cDNA Library

Immunologically screening was carried out using the Promega ProtoBlot λgt11 immunoscreening system (Fisher Scientific, Nepean, Ontario) and rabbit anti-serum raised against a purified preparation of *T. vaginalis* CDF. For plaque lifts, *E. coli* Y1090 cells were infected with 1 x 10^4 PFU of λgt11. Infected cells were added to TYN top agar and the agar suspension was poured onto TYN-Amp plates that were then incubated for 3½ hours at 42°C to allow for phage replication. Plates were overlaid with nitrocellulose filters (Hybond-C Nitrocellulose Gridded Discs, Amersham) impregnated with 10 mM IPTG and incubated for an additional 3½ - 4 hours at 37°C to induce fusion protein synthesis. Filters were removed from plates, washed twice in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and stored damp at 4°C overnight. Plates were stored at 4°C until the results of screening were known.

Screening was performed with *T. vaginalis* CDF anti-serum that had been previously adsorbed for 30 minutes at room temperature with 1 mg/mL *E. coli* extract (Promega) to reduce background reactivity, and then diluted 1:6000 in TBST. Filters were blocked for non-specific binding by incubation in TBST containing 20% FBS for 30 minutes at room temperature and then incubated with the pre-adsorbed serum for 30 minutes. Following three 10 minute washes in TBST, filters were incubated for 30 minutes in a 1:7500 dilution of anti-

rabbit (Fc) alkaline phosphatase-conjugated secondary antibody (Promega), washing was repeated and the filters were incubated in NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate). Plaques that produced a positive signal were picked and purified by two additional rounds of immunological screening. Large scale bacteriophage purification was then carried out.

PREPARATION OF COMPETENT *E. COLI* AND TRANSFORMATIONS

Unless stated otherwise, *E. coli* DH5αF' was utilized as the host bacterial strain for all recombinant plasmids.

Preparation and Transformation of Chemically Competent *E. coli*

Bacteria were made competent by the protocol initially described by Hanahan (1983). Four to five bacterial colonies were picked from a SOB plate (2% tryptone, 0.5% yeast extract, 8 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, with 1.5% agar) and inoculated into 1 mL of SOB broth. The suspension was vortexed and added to 100 mL of SOB broth. The culture was incubated at 37°C with shaking until an A_{600} of between 0.3-0.4 was reached. Cells were then transferred to 50 mL polypropylene tubes that were incubated on ice for 10 minutes and then centrifuged at 3,000 rpm, 4°C for 10 minutes (Heraeus OmnifugeRT centrifuge). Bacterial pellets were resuspended in 20 mL of ice-cold FSB (10 mM potassium acetate, pH 7.5, 3 mM hexaminecobalt chloride, 45 mM $MnCl_2$-$4H_2O$, 10 mM $CaCl_2$-$2H_2O$, 100 mM KCl, 10% glycerol). Cell suspensions were stored on ice for 10 minutes, centrifuged again and the pellets resuspended in 4 mL of ice-cold FSB. 140 µL of DMSO were added

to the cell suspension and the mixture was stored on ice for 15 minutes. Another 140 μL of DMSO were added and 200 μL aliquots were dispensed into chilled cryovial tubes, snap-frozen in liquid nitrogen and stored at -70°C.

For transformations, competent cells were thawed and transferred to an ice bath for 10 minutes. DNA (10 μL) was added to cells (200 μL) and the sample was mixed gently and stored on ice for 30 minutes. Cells were heat-shocked at 42°C for 90 seconds and then returned to ice for 2 minutes. 500 μL of SOC broth (SOB broth supplemented with 20 mM glucose) were added and the tubes were shaken for 1 hour at 37°C. Aliquots of the transformation mix were plated on selective media and plates were incubated overnight at 37°C.

Preparation and Transformation of Electroporation Competent *E. coli*

From a fresh LB plate, several colonies were picked and inoculated into 10 mL of LB broth. The culture was grown overnight at 37°C with shaking and used to inoculate 1 L of LB broth. Cells were grown at 37°C with shaking until an A_{600} of between 0.5-0.8 was achieved. The culture was chilled on ice for 15-30 minutes and cells were pelleted by centrifugation at 7,000 rpm, 4°C for 10 minutes (Beckman J2-21M centrifuge, JA-10 rotor). The bacterial pellet was washed once with 1 L of cold, sterile H_2O, and then a second time with 500 mL of cold, sterile H_2O. Cells were resuspended in 20 mL of cold 15% glycerol, pelleted by centrifugation at 3,000 rpm, 4°C for 10 minutes (Heraeus OmnifugeRT centrifuge) and resuspended in a final volume of 3 mL of cold 15% glycerol. 100 μL aliquots of the bacterial suspension were snap-frozen in liquid nitrogen and stored at -70°C.

Electroporation of DNA into competent bacterial cells was performed with the BRL Cell-Porator Electroporation System (Gibco/BRL) using a field strength of 16.6 kV/cm. Briefly, 10 ng of DNA (usually 1-3 μL of a ligation mix) were gently mixed with 20 μL of freshly thawed, electroporation competent cells. The mixture was placed between the electrode bosses of microelectroporation chambers (0.15 cm-gap chambers, Gibco/BRL) and electroporation was carried out using the following settings: 400 V, 330 μF, low ohm setting and fast charge rate on the cell-porator; and 4,000 ohm resistance on the voltage booster. Immediately after electroporation, cells were recovered, added to 1 mL of pre-warmed SOC broth and allowed to grow for 1 hour at 37°C with shaking. Aliquots of the transformation mix were plated on selective media and plates were incubated overnight at 37°C.

OLIGONUCLEOTIDES

All oligonucleotides were either synthesized and purified at the University of Ottawa Biotechnology Research Institute by Mrs. Nancy Delcellier or Mr. André Bergeron, or were a generous gift provided by Dr. K. Dimock, made during his sabbatical at the National Institutes of Health, Bethesda, Maryland. Sequences for all oligonucleotides are listed in the Appendix I.

RNA ANALYSIS

Glyoxal Agarose Gel Electrophoresis (McMaster and Carmicheal 1977)

RNA samples in DEPC-H_2O (5 μL) were added to 15 μL of sample buffer (0.01 M NaH_2PO_4/Na_2HPO_4, pH 7.0, 0.1% SDS, 50% DMSO, 6% deionized glyoxal). Samples were

heated at 60°C for 15 minutes, immediately cooled on ice and then loaded onto 1% agarose gels containing 10 mM sodium phosphate buffer, pH 7.0. Gels were run with re-circulation of the reservoir buffer in either a LKB 2013 Miniphor submarine electrophoresis unit (LKB/Pharmacia, Baie d'Urfé, Quebec) (50 volts for 1½-2 hours) or a LKB 2012 Maxiphor submarine electrophoresis unit (LKB/Pharmacia) (100 volts for 3-4 hours). After electrophoresis, gels were stained for 30 minutes in reservoir buffer containing 30 μg/mL acridine orange and then destained in sodium phosphate buffer, pH 7.0 for 30 minutes. RNA was visualized and gels were photographed using short-wave ultraviolet light.

Northern Blotting and Hybridization

After glyoxal gel electrophoresis, RNA was transferred to Zeta-probe membrane (Bio-Rad) using the capillary transfer, alkaline blotting protocol described by the manufacturer. This procedure involved overnight transfer of RNA using 10 mM NaOH as transfer buffer and the removal of glyoxal adducts after transfer by baking under vacuum at 80°C for 1 hour. Membranes were soaked in 2X SSC (0.3 M NaCl, 0.03M sodium citrate dihydrate) and pre-hybridized for approximately 1½ hours at 65°C in 10% w/v Dextran sulphate, 1% SDS and 1 M NaCl using an Autoblot Micro Hybridization Oven (Bellco Glass Inc, Vineland, NJ). Hybridizations were performed overnight at 65°C in the same buffer supplemented with probe solution (0.1 mL of 5 mg/mL sheared salmon sperm DNA, 0.9 mL of H_2O and approximately 1×10^6 cpm/mL of labelled DNA per 10 mL of hybridization buffer). Probe solution was heated for 10 minutes at 95-100°C and chilled on ice for 15 minutes prior to its addition to the hybridization solution. Following hybridization, each membrane was washed twice for

7 minutes at room temperature in 2X SSC, twice for 30 minutes at 60°C in 2X SSC/1% SDS, once for 30 minutes at 60°C in 0.1X SSC/0.1% SDS and twice for 30 minutes at room temperature in 0.1X SSC. Washed membranes were wrapped and exposed to X-ray film (Cronex 4, Picker International, Brampton, Ontario) at -70°C using cassettes with intensifying screens.

RNA Primer Extension Analysis

Primer extension was performed using a synthetic negative sense oligonucleotide (TvPE) that was complementary to a region of the *T. vaginalis nan*A cDNA near the 5' end of the predicted open reading frame. Briefly, primer end-labelled with [γ-^{32}P] ATP and polynucleotide kinase (5×10^5- 1×10^6 cpm) was co-precipitated overnight with total *T. vaginalis* RNA and the nucleic acid pellet was resuspended in 30 µL of hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl, 80% formamide). Samples were heated for 10 minutes at 90°C, and were annealed overnight at 30°C. Nucleic acids were precipitated with ethanol and pellets were dissolved in 20 µL of elongation buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM of each dNTP, 20 U Rnasin (Promega), 0.05 µg/µL Actinomycin D (Boehringer-Mannheim, Laval, Quebec)). The primer was extended by the addition of 40 U of AMV reverse transcriptase (Pharmacia) and incubation for 90 minutes at 42°C. Reactions were stopped by the addition of 25 mM EDTA and samples were treated with DNAase-free pancreatic RNAase A (1 µg) for 30 minutes at 37°C. Following phenol extraction, nucleic acids were ethanol precipitated, resuspended in formamide loading buffer (20 mM EDTA, pH 8.0, 95% formamide, 0.05% bromophenol

blue, 0.05% xylene cyanol FF), and analyzed on 8% polyacrylamide sequencing gels containing 8 M urea.

DNA ANALYSIS

DNA Agarose Gel Electrophoresis

DNA samples were mixed with loading buffer (5% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and electrophoresis was performed in 0.4 - 3% agarose gels using Tris-acetate buffer (TAE: 40 mM Tris-acetate, 2 mM EDTA) or Tris-borate buffer (TBE: 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) and LKB electrophoresis units (see Glyoxal Agarose Gel Electrophoresis section for appropriate voltages and times). For high molecular weight DNA, low percentage gels (0.4%) were cast onto a bed of 1% agarose and electrophoresis was performed overnight at 30 volts and 4°C.

Isolation and Purification of DNA Fragments From Agarose Gels

To ensure efficient purification, low-melting point agarose/TAE gels were employed whenever DNA fragments were to be isolated. Gels were stained with ethidium bromide (0.5 μg/mL) and the DNA fragment was located using long-wave ultraviolet light. The band of interest was cut from the gel and placed in a tared microfuge tube. The DNA was purified with either the GeneClean II Kit (BioCan Scientific, Mississauga, Ontario) or the Wizard PCR Preps DNA Purification System (Promega) using the directions provided by the manufacturers.

Southern Blotting and Hybridization

After agarose gel electrophoresis, DNA was transferred to Zeta-probe membrane (Bio-Rad) using the capillary transfer, alkaline blotting protocol described by the manufacturer. Prior to transfer, DNA was depurinated by soaking the gel in 0.25 M HCl for 10-15 minutes with gentle rocking. The acid solution was decanted and the gel was rinsed several times in H_2O. Overnight transfer was performed using 0.4 M NaOH. Membranes were washed in 2X SSC and then baked under vacuum at 80°C for 1 hour. Hybridizations and washes were done using either the Zeta-probe formamide protocol, as recommended by the manufacturer (Bio-Rad), or using the hybridization protocol previously described (see RNA Analysis- Northern Blotting and Hybridization).

RESTRICTION AND MODIFICATION OF DNA

Enzymes

Unless otherwise indicated, all restriction endonucleases and modifying DNA enzymes were supplied by the following manufacturers: Gibco/BRL, Promega, New England BioLabs (Mississauga, Ontario), Boehringer-Mannheim or Pharmacia.

Restriction Digests

Typically 2-5 units of an enzyme were used per μg of DNA in a total reaction volume of 20 μL. All digestions were performed for approximately 1 hour in the buffers and at the temperatures recommended by the supplier. When required, restriction digests were stopped by heat inactivation or by phenol-chloroform extraction and ethanol precipitation.

Ligations

Restriction fragments, PCR products or cDNA were mixed with linearized plasmid or bacteriophage vector arms, generally in a 3:1 molar ratio of vector to insert. Ligations were performed in 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP with approximately 3 Weiss units of T4 DNA ligase per 20 μL reaction. Reactions mixtures were incubated overnight at 16°C.

Dephosphorylation

Whenever applicable, linearized vector was dephosphorylated to reduce background during subcloning. Up to 1 μg of DNA was dephosphorylated using 0.5-1 units of calf intestinal phosphatase (Promega) in 50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine in a final volume of 20 μL. Reaction mixtures were incubated for 1 hour at 37°C and the enzyme was inactivated by the addition of EDTA to 10 mM and heating for 10 minutes at 75°C. Samples were extracted once with phenol, twice with chloroform and DNA was precipitated with ethanol.

DNA SEQUENCING

Plasmid DNA Isolation For Sequencing (mini-preps) and DNA Denaturation

The isolation of plasmid DNA for sequencing was similar to the mini-prep technique described previously but with several slight modifications. Briefly, bacteria were pelleted from 2 mL of overnight culture, resuspended in 100 μL of Solution I (without lysozyme but containing 100 ng/μL of RNAase A) and incubated for 15 minutes at room temperature.

Cells were lysed by the addition of 200 µL of Solution II, the lysates were mixed, incubated at room temperature for 5 minutes and then were neutralized by addition of 150 µL of potassium acetate solution (Stock: 60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid and 28.5 mL H_2O). After thorough mixing, the suspensions were incubated on ice for 15 minutes. Chromosomal DNA was pelleted by centrifugation for 10 minutes at 13,000 rpm, (Biofuge A microcentrifuge) and the supernatant was extracted with 400 µL chloroform. Following overnight precipitation with ethanol (1 mL), nucleic acids were washed with 70% ethanol, air dried and then resuspended in 60 µL TE buffer.

Plasmid DNA (~2.5 µg/reaction or 10 µL of sequencing mini-prep) and 50 ng of oligonucleotide primer were co-incubated for 8 minutes at room temperature in 0.2 N NaOH. The solution was neutralized by the addition of sodium acetate, pH 5.2 (final concentration 0.6 M) and DNA was precipitated overnight with ethanol. The nucleic acid pellet was washed with ethanol, dried and used in sequencing reactions.

Sequencing Reactions and Gels

Sequencing reactions were performed by the dideoxy-chain termination procedure (Sanger *et al.* 1977) using Sequenase version 2.0 enzyme (USB/Amersham) and the protocol recommended by the manufacturer. Sequencing reactions were separated by electrophoresis on 6 or 8% polyacrylamide gels containing 8 M urea using an IBI sequencing apparatus, Model STS-45. For electrophoresis, 1X TBE was used as upper chamber buffer, while the lower chamber contained a 1.5X TBE solution. Gels were pre-warmed and electrophoresis was carried out using 40 watts constant power and maximal settings of 2500 volts and 40

mAmps (Pharmacia LKB ECPS 3000/1500 power supply). After electrophoresis, gels were lifted onto 3 MM Whatman paper, dried under vacuum at 80°C for 1 hour (Model 583 Gel Dryer, Bio-Rad) and then exposed to X-ray film (Cronex 4).

Nested Deletion Constructs for Sequencing

To sequence the *T. vaginalis* genomic clone (pTvEco4.5) and the CDF-2 cDNA clone, a series of nested deletion constructs were produced using exonuclease III (Exo III) directional digestion (Henikoff 1984)(Promega Erase-a-Base System). Briefly, plasmids (10 μg) were digested with restriction endonucleases to produce a linear molecule with a 4-base 3' overhang (resistant to Exo III digestion) protecting the sequencing primer binding site and a 5' overhang or blunt-end (Exo III sensitive) adjacent to the insert. Plasmid DNA digests were incubated with Exo III at 35°C and aliquots were removed at 30 second intervals. Samples were incubated with S1 nuclease for 30 minutes at room temperature to remove the remaining single-stranded tails produced by the Exo III digestion. Klenow DNA polymerase was used to repair single stranded termini and the plasmid DNAs were re-circularized by blunt-end ligation and were transformed into electro-competent *E. coli*. Colonies from each Exo III time point were picked, screened for plasmid size using cracking gels and the plasmid DNA from selected clones was sequenced using either SP6 or T7 primers. For the CDF-2 cDNA, plasmids to be sequenced with the SP6 primer were constructed using DNA digested with *Sma* I and *Sac* I, while T7 deletions were produced using plasmids digested with *Sph* I and *Xba* I. Nested deletion constructs for the genomic clone pTvEco4.5 were produced using plasmid DNA digested with *Sph* I and *Xho* I for T7 sequencing, and DNA digested with

BamH I and *Sac* I for SP6 sequencing.

Sequence Analysis

DNA sequences were analyzed using the IBI Pustell sequence analysis program in order to identify restriction endonuclease sites and open-reading frames, to deduce amino acid sequences and to calculate molecular masses. Database searches were performed using NCBI BLAST programs (Altschul *et al.* 1990; Gish and States 1993). The PSORT version 6.3 program (Nakai and Kanehisa 1992) was used in the analysis of protein sorting signals and the Predator program was used to predict protein secondary structure. These programs were accessed through the European Molecular Biology Laboratory world-wide web site (www.embl-heidelberg.de).

PREPARATION OF RADIOLABELLED PROBES AND BACTERIAL COLONY LIFTS

Nick Translation

Approximately 1 μg of DNA was nick translated using the Gibco/BRL nick translation system. Briefly, reaction mixtures (45 μL) were prepared containing DNA, 20 μM each of dATP, dGTP and dTTP, 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM β-2-mercaptoethanol and 125 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham). Two units of DNA polymerase I/DNase I (Gibco/BRL) were added and the reaction was incubated at 15°C for 1 hour and then stopped by the addition of 27 mM EDTA. Unincorporated nucleotides were separated from labelled DNA by passage through Sephadex G-50 columns (Pharmacia). Fractions containing probe were pooled, counted in a LKB 1214 RackBeta Liquid

Scintillation counter and then stored at -20°C.

Random Prime Labelling

DNA was labelled with the Prime-a-Gene System (Promega) which is based upon the method of Feinberg and Vogelstein (1983) and utilizes random hexanucleotides for priming DNA synthesis. Briefly, 25-50 ng of DNA were heated for 2 minutes at 95-100°C, rapidly chilled on ice and then added to a reaction mix containing 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM DTT, 0.2 M HEPES, pH 6.6, 400 µg/mL BSA, 20 µM dATP, dGTP, dTTP, 100 µCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) and 5.2 A_{260} units/mL random hexadeoxyribonucleotides. Five units of the Klenow fragment of DNA polymerase were added and the reaction incubated at room temperature for 1 hour and then terminated by heating for 2 minutes at 95-100°C. EDTA was added to 20 mM, and the reaction mix was passed through a Sephadex G-50 column in order to remove unincorporated nucleotides. Fractions containing probe were pooled, counted and stored at -20°C.

5' End-Labelling of Oligonucleotides Primers

Approximately 200 ng of oligonucleotide primer, in a reaction buffer containing 10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, and 50 mM potassium acetate, were incubated with 200 µCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and 20 units of T4 polynucleotide kinase (Pharmacia) for 30 minutes at 37°C. Reactions were stopped by heating at 70°C for 5 minutes and the reaction mix passed through a Sephadex G-25 column to removed unincorporated ATP from labelled primer. Column fractions were collected,

counted and fractions containing labelled primer were pooled. 1 µL of glycogen was added (20 µg/µL stock, Boehringer-Mannheim), and the primer was precipitated with ethanol.

Colony Lifts and Hybridizations

Bacterial colonies were lifted onto Colony/Plaque Screen hybridization transfer membrane discs (NEN Research Products, DuPont). Discs were processed and hybridizations were performed using the protocols recommended by the manufacturer.

INR ELEMENT ACTIVITY

Construction of Inr containing clones

To test the ability of the *T. vaginalis nan*A Inr-like element to initiate accurate transcription, two plasmids were constructed, one contained the *T. vaginalis nan*A Inr-like element and the other contained a sequence previously shown to be incapable of Inr activity (Javahery *et al.* 1994). Briefly, plasmid p2032 (Smale and Baltimore 1989), a generous gift from Dr. S. Smale (Howard Hughes Medical Institute Research Laboratories, UCLA) was digested with *Sac* I and *BamH* I and ligated to annealed oligonucleotides that had been designed to contain the Inr sequence in question, flanked by *Sac* I and *BamH* I sequences. Prior to ligation, 0.5-1 µg of oligonucleotides, in a 1:1 molar ratio, were annealed in a total volume of 20 µL by heating for 3 minutes at 100°C and then allowing the oligonucleotide mix to slowly cool to 35°C. Ligations, using a vector to oligonucleotide ratio of 1:10, were performed overnight at 15°C using 2-3 Weiss units of T4 DNA ligase. Ligation reactions were used to transform electro-competent bacterial cells, transformation mixes were plated

and transformed colonies were picked. Plasmid DNA was isolated from transformants and sequenced using a SP6 primer to ensure that the correct oligonucleotide sequence had been inserted.

Inr Activity - *In Vitro* Transcription

To determine the ability of the Inr sequence to accurately initiate transcription, plasmids were transcribed *in vitro* using HeLa cell nuclear extracts. Briefly, 300 ng of supercoiled template DNA was transcribed in a final volume of 50 µL containing 250 µM of each ribonucleoside triphosphate, 6.25 mM $MgCl_2$ and 20 µg of HeLaScribe nuclear extract in 1X HeLa nuclear extract buffer (Promega). Following incubation at 30°C for 60 minutes, reactions were terminated by the addition of 350 µL stop mix (0.3 M Tris-HCl, pH 7.4, 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, 3 µg/mL tRNA). Reactions were extracted once with phenol, once with chloroform and nucleic acids were precipitated with ethanol overnight at -20°C. RNA transcripts were pelleted, washed with 70% ethanol and used as templates in primer extension reactions with a [γ^{32}P]ATP end-labelled SP6 primer. The protocol for primer extension was identical to that described previously (see RNA Analysis section) except that hybridization was performed at 37°C.

CONSTRUCTION OF GST FUSION PROTEIN CLONES

Using the genomic clone pTvEco4.5 as a template for PCR amplification, two glutathione S-transferase (GST) fusion protein clones were constructed in the expression vector pGEX-KG (Guan and Dixon 1991), generously gift from Dr. J. Waring (Molecular

Genetics, Children's Hospital of Eastern Ontario). For PCR amplification, pTvEco4.5 was linearized with *Xho* I, extracted with phenol and chloroform and precipitated with ethanol. One to ten ng of DNA in 100 µL of 25 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM dNTPs and 1µM of each oligonucleotide primer, were used as a template for PCR amplification with 5 units of Taq DNA polymerase (Gibco/BRL) in a Hybaid Thermal Reactor (Interscience, Markham, Ontario). Amplifications were performed using 30 cycles of : 1 minute at 94°C, 1 minute at 37°C and 1 minute at 72°C, followed by a final extension of 10 minutes at 72°C. PCR products were analyzed by electrophoresis on 2.5% agarose gels in TBE buffer. If the product was of the correct size, reaction mixes were extracted with phenol/chloroform, and the DNA was precipitated with ethanol and resuspended in a small volume of TE buffer. Purified PCR products were subcloned into the pCRII vector using the InVitrogen TA cloning kit (InVitrogen, San Diego, CA) and the instructions provided by the manufacturer.

Two different PCR products were generated, one contained the 5' end of the *T. vaginalis nan*A gene with an *EcoR* I site designed to be directly upstream of the translational start codon (primer set: TvLyase-1/CDF-1R4; expected product 401 bp) and the other contained the 3' end of the *T. vaginalis nan*A gene with a *Hind* III site designed to be adjacent to the translational stop codon (primer set: TvLyase-2/KM1-3B; expected product 376 bp). The two PCR products were cloned into pCRII, checked for orientation by restriction endonuclease mapping and sequenced to verify that amplification did not generate point mutations. Plasmids containing the PCR products (pCRIITvLy1 and pCRIITvLy2), to pCDF-1 (which contained the original *T. vaginalis nan*A cDNA insert) and pGEM-7Zf(+)

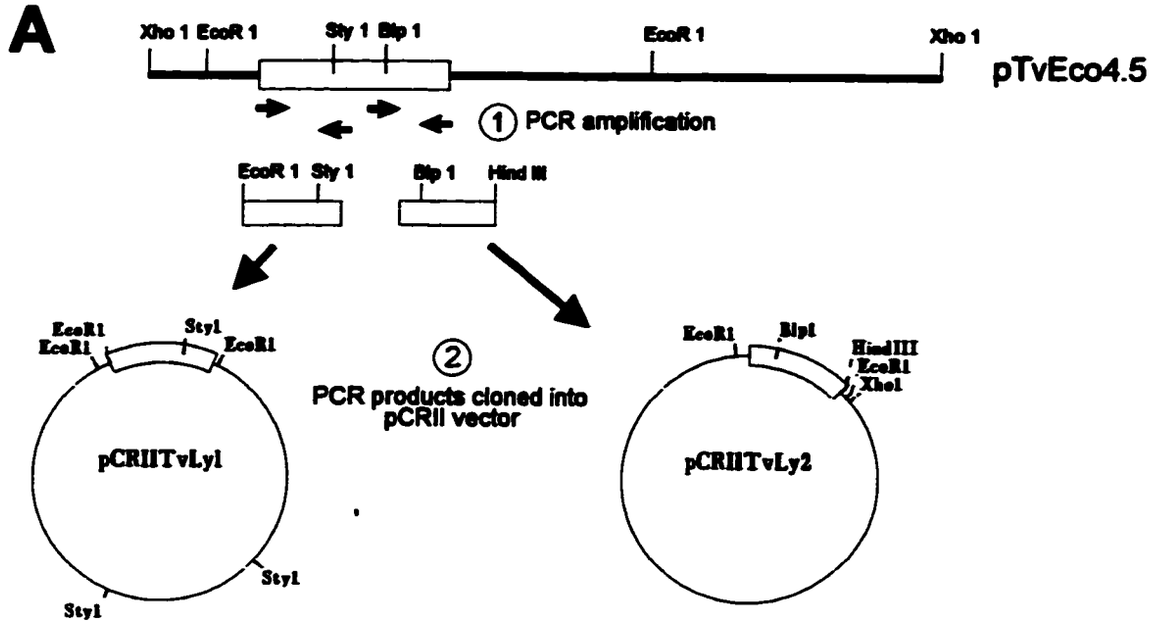
(Promega) were then used in a series of subcloning steps to produce pGEM-TvLyase (Figure 1). This plasmid contained the full-length *T. vaginalis nan*A gene in an *EcoR* I/ *Hind* III coding cassette. This cassette was subcloned into the GST expression vector pGEX-KG to yield pTvNeu5AcLy, a construct that would express a full-length GST fusion protein.

Construction of the deletion fusion protein clone involved PCR amplification of the 5' end of the *T. vaginalis nan*A gene using the CDF-1R4 primer and a primer (TvLyase Δsig) designed to create an *EcoR* I site 72 nucleotides inside the 5' end of the coding region of the gene, thereby deleting 24 amino acids from the N-terminus of the protein. pGEM-TvLyase linearized with *EcoR* I was used as a template for PCR amplification and amplifications were performed using the same conditions described above. The 328 bp PCR fragment was extracted with phenol and chloroform, precipitated with ethanol and digested with *EcoR* I and *Sty* I to produce a 218 bp DNA fragment. This fragment was cloned into the *EcoR* I / *Sty* I sites of pGEM-TvLyase to yield pGEM-TvLyΔsig which contained an *EcoR* I / *Hind* III coding cassette of the *nan*A gene with the first 72 nt of the gene deleted. This cassette was subcloned into pGEX-KG to create pTvΔaa24Neu5AcLy, a construct that would express a deleted fusion protein.

EXPRESSION AND PURIFICATION OF GST FUSION PROTEINS

Both large and small scale fusion protein purifications were performed using the protocol described by Frangioni and Neel (1993). For small scale preparations of fusion proteins, fresh colonies were picked and inoculated into 2 mL of LBAmp broth to produce a slight turbidity. The culture was allowed to grow for 4 hrs at 37°C with shaking and then

Figure 1. Schematic of the cloning strategy used in the construction of pGEM-TvLyase (pGEMTvLy). Panel A. Plasmid pTvEco4.5, which contains a genomic *EcoR* I fragment including the entire coding region of the *T. vaginalis nanA* (Tv*nanA*) gene (open box), was linearized with *Xho* I and used as a template for PCR amplification. Two products were amplified: one contained the 5' coding region of the *nanA* gene with an *EcoR* I site upstream of the translational start codon; the second contained the 3' coding region of the gene with a *Hind* III site downstream of the translational stop codon. The PCR products were cloned into the pCRII vector to generate pCRIITvLy1 and pCRIITvLy2. Panel B. pCRIITvLy2 and pCDF-1, a clone that contained a cDNA insert lacking 17 nucleotides from the 5' end of the Tv*nanA* gene (filled box), were digested with *Blp* I and *Xho* I to generate a 280 bp fragment containing the 3' end of the *nanA* gene (pCRIITvLy2) and a *Blp* I/ *Xho* I linearized vector (pCDF-1). Ligation of these DNA fragments yielded plasmid pTvLy2A. pTvLy2A was digested with *EcoR* I and *Hind* III and the 950 bp DNA fragment containing the partial *nanA* gene was ligated to *EcoR* I/ *Hind* III linearized pGEM-7Zf(+) to produce pTvLy2B. Panel C. pCRIITvLy1 and pTvLy2B were digested with *EcoR* I and *Sty* I to generate a 280 bp fragment containing the 5' end of the *nanA* gene (pCRIITvLy1) and an *EcoR* I/ *Sty* I linearized vector (pTvLy2B). Ligation of these two DNA fragments created pGEM-TvLyase (pGEMTvLy), a plasmid that contained the full-length coding region of the *T. vaginalis nanA* gene within an *EcoR* I/ *Hind* III fragment.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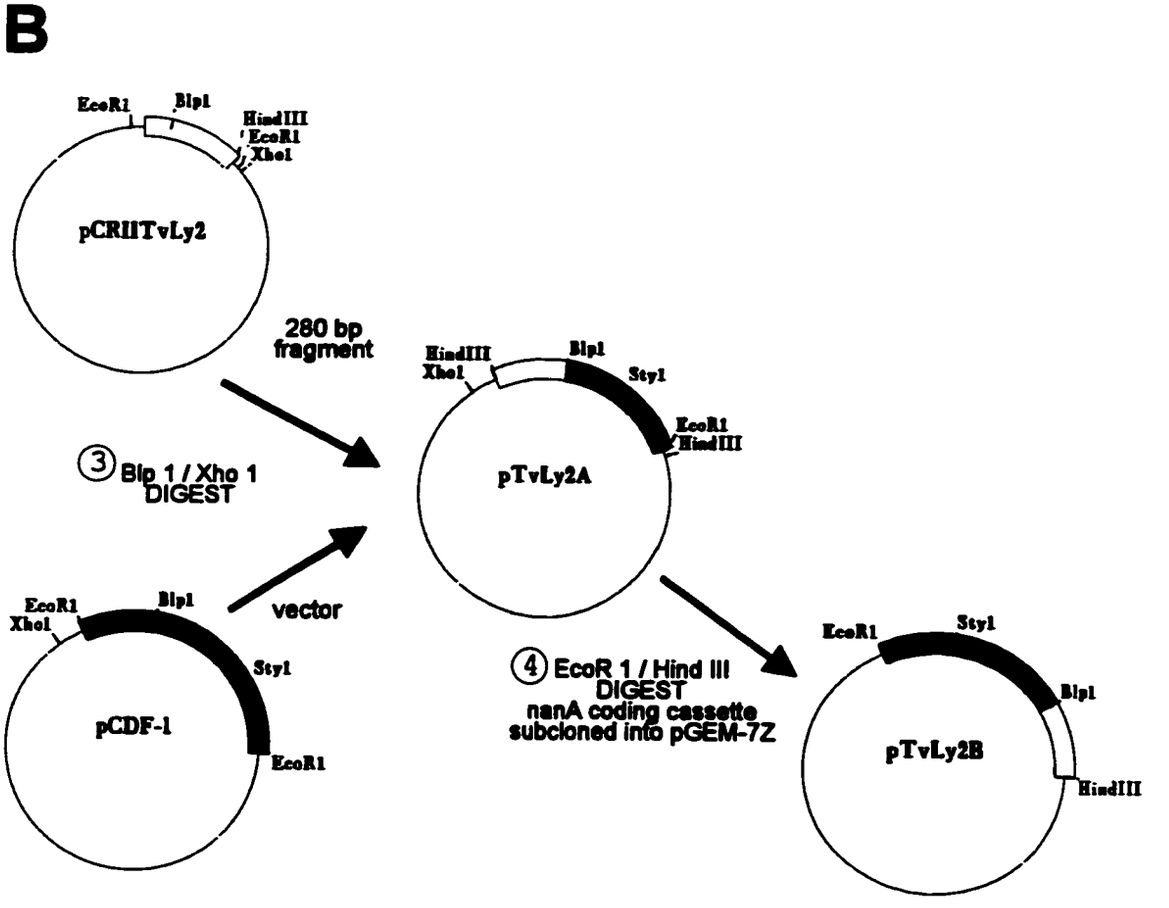
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pTvLy2A
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XhoI
BipI
StyI
pCDF-1
EcoR1
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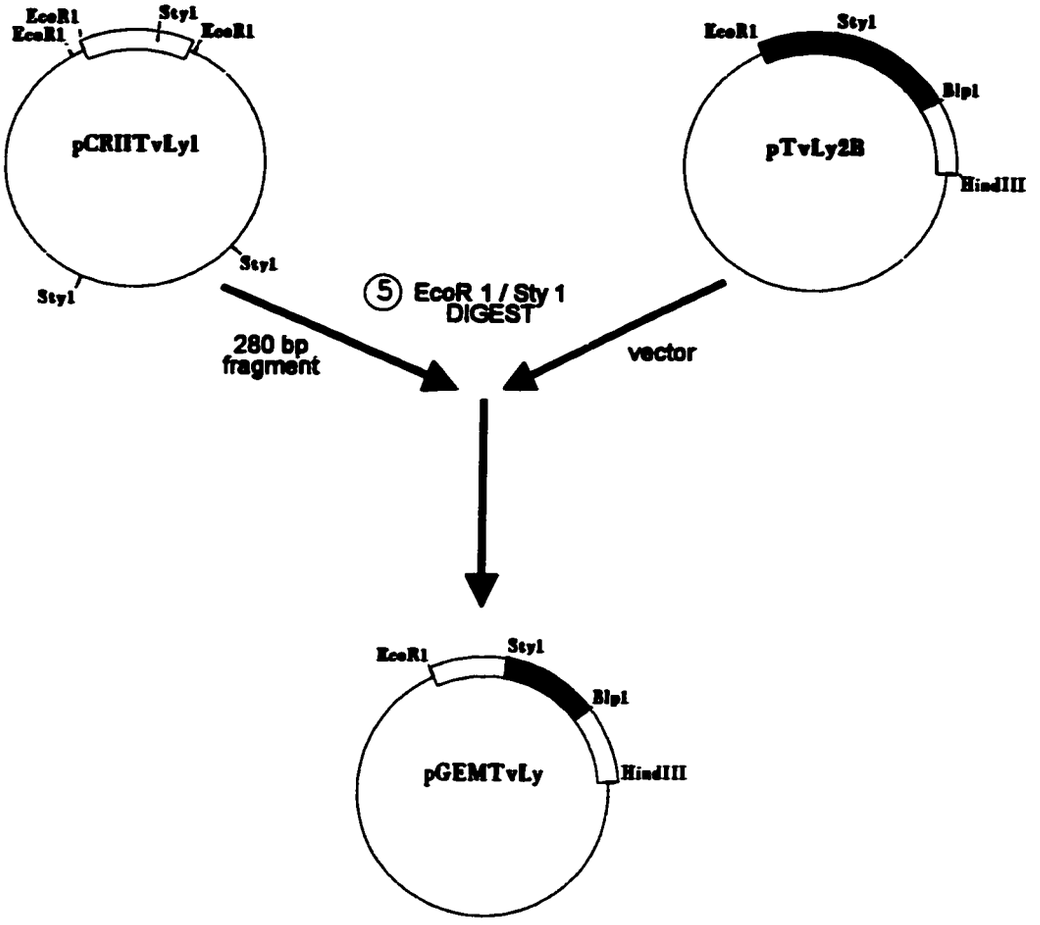
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DIGEST
280 bp
fragment
vector
EcoR1
StyI
BlpI
pGEMTvLy
HindIII

bacteria were induced by the addition of IPTG to a final concentration of 0.1 mM. Cells were grown an additional 2½ hrs at 37°C with shaking and were then pelleted and washed once with 500 µL of ice-cold STE (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 100 µg/mL lysozyme. Cells were incubated on ice for 15 minutes and dithiothreitol (DTT) was added to a final concentration of 5 mM. For efficient solubilization, Sarkosyl was added to a final concentration determined for each construct, and samples were sonicated for 12 seconds with a continuous pulse at 70% maximum power using a microtip probe and a Cell Disruptor 350 Sonifier (Branson Sonic Power Company). Sonicated samples were centrifuged for 5 minutes at 13,000 rpm in a microcentrifuge and both the supernatant and pellet (resuspended in 300 µL of STE) were analyzed for fusion protein expression and for N-acetylneuraminate lyase activity.

For large scale preparation of fusion proteins, a single fresh bacterial colony was inoculated into 20 mL of LBAmp broth. This culture was incubated overnight at 37°C with shaking and 5 mL were used to inoculate 500 mL of LBAmp broth. This culture was incubated at 37°C with shaking until an A_{600} of approximately 0.5-0.6 was achieved, at which time IPTG was added to a final concentration of 0.1 mM. Bacteria were grown for an additional 3½ hours at 37°C with shaking, and then harvested by centrifugation at 8,000 rpm, 4°C for 10 minutes (Beckman J2-21M centrifuge, JA-10 rotor). The pellet was washed once with 75 mL of ice-cold STE and stored overnight at -20°C. Bacterial pellets were resuspended in 19 mL STE containing 100 µg/mL lysozyme (for each 250 mL of original culture), the cell suspension was incubated on ice for 15 minutes and DTT (final concentration 5 mM) and Sarkosyl (concentration optimized for each construct) were added

to the suspension. Samples were gently mixed, sonicated on ice for 30-45 seconds and the lysates were clarified by centrifugation at 10,000 rpm, 4°C for 10 minutes (Beckman J2-21M centrifuge, JA-20 rotor). Triton X-100 was added to the supernatants (concentrations optimized for each construct), the lysates were vortexed for 5 seconds and 1 mL of a 50% slurry of glutathione Sepharose 4B beads (Pharmacia) was added. This mixture was incubated at 4°C for 30 minutes with gentle agitation. Beads were washed five times with 1X PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and fusion proteins were eluted by a 30 minute incubation at room temperature in 1 mL of glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0). Beads were pelleted and the supernatants were collected and dialyzed overnight at 4°C against 1X PBS using a Pierce 10,000 MWCO Slide-A-Lyzer dialysis cassette (Chromatographic Specialties, Brockville, Ontario). Dialyzed supernatants were concentrated using a Centricon-30 concentrator (30,000 MWCO, Amicon, Oakville, Ontario) and protein concentrations were determined with the Bio-Rad protein assay system using bovine serum albumin as a standard. For large scale preparation of the GST protein, the same procedure was used except that lysozyme was not required for bacterial lysis and Sarkosyl was not necessary for solubilization. For analysis of fusion protein products, 2X denaturing sample buffer (62.5 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 10% β-mercaptoethanol) was added to samples. Samples were heat at 100°C for 5 minutes and aliquots were analyzed by SDS-PAGE.

PRODUCTION OF GST FUSION PROTEIN ANTI-SERUM

Male New Zealand white rabbits (2.5-3 kg) were immunized with full-length *T. vaginalis* GST fusion protein resuspended in Freund's Complete Adjuvant (Sigma). Briefly, affinity purified pTvNeu5AcLy fusion protein (140 µg total protein) was separated by electrophoresis on a preparative SDS-PAGE gel containing 11% polyacrylamide. The gel was rinsed 3-4 times in deionized, distilled water (ddH_2O) and was stained for 10 minutes with a 0.05% solution of Coomassie Brilliant Blue R-250. The gel was destained in ddH_2O and the fusion protein band was cut from the gel. The polyacrylamide gel piece was fragmented by repeated passage through a 21 gauge needle attached to a 3cc syringe and the slurry was mixed with adjuvant and given to rabbits via an intramuscular injection. One month after primary immunization, rabbits were boosted with a similar preparation of full-length fusion protein resuspended in Freund's Incomplete Adjuvant (Sigma). Test bleeds were performed two weeks after boost and antibody titers were established by a modified ELISA assay using fusion protein coated plates (Desjardins *et al.* 1992). When satisfactory antibody titers were achieved, rabbits were exsanguinated and the serum was collected and stored at -70°C. This protocol for immunization (protocol number OGH-43) was reviewed and approved by the University of Ottawa Animal Care Committee.

N-ACETYLNEURAMINATE LYASE ASSAYS

N-acetylneuraminate lyase (Neu5Ac lyase) activity was assayed colorimetrically by measuring the amount of N-acetyl-D-mannosamine formed from the cleavage of N-acetylneuraminic acid (Sialic acid A-9290, Sigma). Briefly, 50 µL of N-acetylneuraminic acid

(20 μmoles/mL), 50 μL of 0.1 M KPO_4 buffer, pH 7.2 and 100 μL of sample or STE buffer were incubated at 37°C for 10 minutes and then heated at 100°C for 2 minutes. The samples were diluted with water, centrifuged and the supernatants were assayed for N-acetyl-D-mannosamine by a modified Morgan-Elson colour reaction (Brunetti *et al.* 1962, Reissig *et al.* 1955). The Morgan-Elson reaction involved the addition of 40 μL of 0.8 M potassium tetraborate, pH 9.0 to 200 μL of each supernatant. Samples were mixed, heated to 100°C for 12 minutes and were cooled on ice. 1.2 mL of diluted ρ-Dimethylaminobenzaldehyde solution (for DMAB stock solution see Appendix II) were added and the solutions were mixed and incubated at 37°C for 10 minutes. The absorbance of each sample at A_{585} was then determined. Concentrations of amino-sugar were calculated from a standard curve generated by assaying known concentrations of N-acetyl-D-mannosamine (ICN, Montreal, Quebec) and plotting the concentration versus the A_{585}.

To assay for *T. vaginalis* Neu5Ac lyase activity, 3-4 x 10^8 log phase parasites were washed twice with PBS and the cell pellet was resuspended in a final volume of 1 mL of PBS. Parasites were sonicated on ice for 15 seconds, the lysates were clarified by centrifugation for 5 minutes at 13,000 rpm and the supernatants were used for protein determinations and lyase assays. *T. vaginalis* cell-free filtrates were concentrated with Centriprep 10 concentrators (Amicon) prior to use in lyase enzyme assays.

PROTEIN ANALYSIS

Polyacrylamide Gel Electrophoresis (Laemmli 1970)

Proteins were analyzed by SDS-PAGE. Separating gels consisted of 0.375 M Tris-

HCl, pH 8.8, 0.1% SDS and either 7.5% acrylamide and 0.2% bis-acrylamide (7.5% gels), 10% acrylamide and 0.26% bis-acrylamide (10% gels) or 11% acrylamide and 0.29% bis-acrylamide (11% gels). Stacking gels contained 4% acrylamide, 0.11% bis-acrylamide, 0.125 M Tris-HCl, pH 6.8 and 0.1% SDS. Electrophoresis was carried out at 100 volts in electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) using the Bio-Rad Mini-Protean II Electrophoresis cell system. Following electrophoresis, gels containing fusion proteins were stained with Coomassie Brilliant Blue (40% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue) and then destained with 30% methanol and 10% acetic acid. Gels containing immunoprecipitation samples were fixed in 30% methanol and 10% acetic acid for 30-60 minutes and then incubated in Amplify (Amersham) for 1 hour. Fixed and amplified gels were dried under vacuum and exposure to X-ray film.

In Vivo Radiolabelling of *T. vaginalis* Proteins in Cells and Cell Filtrates

Log phase *T. vaginalis* were pelleted by centrifugation, washed three times in PBS, and resuspended at a final concentration of 1 x 10^5 parasites/mL in either 20 mL of CMGA/TYI medium or in 10 mL of TYI medium. For labelling of parasite cell proteins, 10 µCi of L-[^{35}S]-methionine (1186 Ci/mmol, NEN, DuPont) were added to parasites resuspended in TYI medium and cultures were incubated for 24 hours at 37°C, in 5% CO_2. Parasites were pelleted and the radiolabelled cells were washed once with PBS and stored at -70°C. TYI spent medium was collected and the pH of the medium was determined. Spent medium with a pH of <6.0 was adjusted with 1 N NaOH to a final pH of 6.5 and the medium was then filter sterilized, dispensed into aliquots and frozen at -70°C.

For the identification of radiolabelled proteins in filtrates, parasites resuspended in CMGA/TYI medium containing 200 μCi of L-[^{35}S]-methionine were added to washed, confluent McCoy cell monolayers. Cultures were incubated for 22-24 hours at 37°C, in 5% CO_2. Filtrates were harvested, centrifuged to remove cells and debris, and the pH of the filtrate was adjusted to 6.5, where required. Filtrates were filter sterilized (0.22 μm filters) and stored at -70°C. Medium control filtrate was produced in the same manner except that parasites were not added. Where necessary, filtrates and supernatants were concentrated using a Centriprep 10 concentrator (10,000 MWCO, Amicon) or proteins were precipitated by the addition of 10% (w/v) trichloroacetic acid.

T. vaginalis Cell Lysates

Pellets of radiolabelled parasites were resuspended in 300 μL immunoprecipitation lysis buffer (20 mM Tris, pH 9.0, 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10% glycerol, 1% aprotinin, 2 mM phenylmethyl sulfonyl fluoride (PMSF), 1% NP-40). Samples were vortexed, incubated on ice for 5 minutes and then insoluble cell debris and nuclei were removed by centrifugation at 13,000 rpm for 1 minute. Supernatants were stored at -70°C.

Immunoprecipitations

To 300 μL of clarified *T. vaginalis* cell lysates, 30 μL of polyclonal rabbit CDF anti-serum and 170 μL of 58.8 mg/mL protein A Sepharose CL-4B beads (Sigma) (~10 mg beads/sample) were added. For culture supernatants and filtrates, 1 mL of sample was mixed with 100 μL of CDF anti-serum and 560 μL of protein A Sepharose CL-4B beads (~33 mg

beads/sample). All samples were allowed to mix overnight at 4°C and then beads were washed five times in immunoprecipitation wash buffer (100 mM Tris, pH 9.0, 500 mM LiCl). Pelleted beads were resuspended in 30 µL of sample buffer (60.5 mM Tris-HCl, pH 6.8, 2% SDS, 10 % glycerol, 2% β-mercaptoethanol, 0.05% bromophenol blue) and heated at 95°C for 5 minutes to dissociate protein-antibody complexes from beads. Beads were removed by a brief centrifugation and samples were either used immediately in SDS-PAGE or were stored at -70°C.

Western Blotting and Immuno-detection

Proteins were transferred from gels onto Hybond-C Nitrocellulose membranes (Amersham). Prior to electroblotting, the membrane and gel were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for at least 15 minutes. The transblotting cassette was assembled (Bio-Rad Mini Trans-Blot cell system) according to the instructions provided by the manufacturer, and transfer was performed at 100 V for 1 hour at room temperature. For determination of molecular size, pre-stained high range protein molecular weight standards (Gibco/BRL or Bio-Rad) were used as standards.

Immunodetection of proteins transferred to the membranes was by the procedure described in the section entitled, "Immunological Screening of cDNA Libraries", but with two modifications made to the protocol. Immunoblot membranes were blocked by incubation in TBST containing 2% BSA for 30 minutes at 37°C. Additionally, GST fusion protein anti-serum was adsorbed with 100 µg of purified GST protein for 1 hour at room temperature prior to use. The CDF anti-serum did not require pre-adsorption.

CHAPTER 3: CHARACTERIZATION OF *T. VAGINALIS* cDNA CLONES

INTRODUCTION

CDF was originally prepared by Garber *et al.* (1989) using a four-step protein purification procedure. Proteins were precipitated from *T. vaginalis* cell-free filtrates with ethanol and then fractionated with ammonium sulfate. The majority of CDF activity was found in the 65% ammonium sulfate supernatant, and this fraction was sequentially applied to ion-exchange (DEAE Sephacel) and gel filtration (Sephacryl S-300) columns. Following chromatography, fractions containing CDF activity were pooled. This preparation exhibited a 5-fold increase in specific activity over that of the initial cell-free filtrate (Garber *et al.* 1989) and a single protein species of approximately 200 kDa was identified following SDS-PAGE and silver staining.

SDS-PAGE of parasite lysates and cell-free filtrates (Alderete 1983; Garber *et al.* 1986; Garber *et al.* 1989) indicates that CDF and other secreted *T. vaginalis* proteins comprise only a small percentage of the total number of parasite proteins. The amount of CDF secreted by *T. vaginalis* appears to be small, and the protein cannot be detected in SDS-PAGE silver stained gels prior to its purification. Despite the low levels of CDF in filtrates, it has been found to be immunogenic, and can be detected in filtrates by immunoblot analysis using high-titer human serum reactive to *T. vaginalis* (Garber *et al.* 1989). The serum of rabbits immunized with a purified CDF fraction also shows reactivity with CDF in cell-free *T. vaginalis* filtrates using both Western blots and ELISA assays (Garber *et al.* unpublished data).

The initial objective of this study was to characterize the *T. vaginalis* CDF gene, as it was considered to be a marker of virulence. In order to achieve this objective, two items were essential: (i) a *T. vaginalis* library, and (ii) a CDF specific probe for screening. The availability of the polyclonal CDF anti-serum prompted the decision to employ immunological screening of a *T. vaginalis* cDNA expression library to obtain clones that encoded CDF protein sequences.

The bacteriophage vector λgt11 (Young and Davis 1983a, 1983b) was chosen for the construction of the *T. vaginalis* cDNA library. This vector accommodates DNA fragments of up to 7 kilobases (kb), and has two unique features that make it amenable to screening. First, the vector allows recombinant phage to be identified by colour selection. λgt11 carries the *E. coli lacZ* gene with a unique *EcoR* I site located at the 3' end of the gene, preceding the translational stop codon. DNA fragments cloned into this site disrupt the gene and subsequently, upon IPTG induction, an inactive β-galactosidase protein is produced. When plated with chromogenic substrate, recombinant phage plaques containing inactive enzyme are clear while wild-type phage with active β-galactosidase generate blue plaques. The second key feature of the λgt11 system is that recombinant phage, upon induction, produce fusion proteins that contain β-galactosidase at the amino terminus and a foreign polypeptide at the carboxy terminus. These fusion proteins, which may display antigenic epitopes, can be transferred to solid support and then screened with antibodies directed against the protein of interest. Immunological screening, in this fashion, has been successful in the isolation of well over 200 eukaryotic genes.

This chapter details the identification of two *T. vaginalis* cDNA clones by

immunological screening. The likelihood that the immunoreactive clones encode the CDF protein is discussed in lieu of the results obtained from Northern blot and sequence analysis. Additional characterization of the polyclonal CDF anti-serum used in library screening is also described.

RESULTS

(i) Identification of cDNA clones by immunological screening of the λgt11 library

A *T. vaginalis* clinical isolate, designated #202, was used for the construction of the cDNA library. Total *T. vaginalis* RNA was extracted using a high salt SDS-DEPC technique (Albach *et al.* 1984; Heebner and Albach 1982), and polyadenylated RNA was subsequently isolated by oligo-dT cellulose column chromatography. Polyadenylated RNA (mRNA) was used to synthesize cDNA by a modified procedure of Gubler and Hoffman (1983) employing M-MLV reverse transcriptase. From approximately 3 μg of mRNA, 1.44 μg of cDNA were synthesized, and ligated to *EcoR* I adaptors (see Appendix I). Using a Sepharose CL-4B column, which allows for the selection of DNA ≥350 bp in size, "adapted" cDNA molecules were purified from free adaptors, and ligated to *EcoR* I digested λgt11 vector arms. Linear concatemeric molecules were then packaged *in vitro* to produce infectious phage particles.

The *T. vaginalis* cDNA library contained 9.6×10^5 PFU/mL and at least 75% of plaques were identified as recombinant. Library integrity was evaluated by determining the sizes of the cDNA inserts in twelve randomly picked recombinant phage. This evaluation was done to ensure that the Sepharose CL-4B size selection of cDNA had been efficient, and that the recombinant library had not been generated from small cDNAs arising from degraded

RNA templates or aborted products of cDNA synthesis. DNA was extracted from each phage clone, digested with *EcoR* I to release the cDNA insert, and samples were analyzed by agarose gel electrophoresis. Of the twelve recombinant clones, only two were found to contain cDNA inserts of < 200 bp. The remaining ten clones contained inserts ranging in size from 460-3,150 bp with an average cDNA insert size of 1,344 bp. Using *E. coli* Y1088, the library was subsequently amplified to a titer of 3.3×10^9 PFU/mL.

E. coli Y1090 was chosen as the bacterial host for immunological screening. This strain is *lon* protease deficient and carries a lac repressor on the pMC9 plasmid. For screening, phage were initially plaqued on *E. coli* Y1090 at 42°C without IPTG, to prevent lysogenization and the production of potentially toxic fusion proteins. Once plaque formation was apparent, plates were incubated at 37°C and overlaid with IPTG-saturated filters to induce fusion protein synthesis. The number of plaques (N) required for screening was calculated to be 7×10^5 PFU using the equation: $N = \ln(1-P)/\ln(1-1/n)$ (Clarke and Carbon 1976), with a desired probability (P) of 99.9% and a fractional proportion of CDF transcript among the mRNA population (1/n) of 1/100,000. Library screening was performed using a 1:6000 dilution of pre-adsorbed polyclonal CDF anti-serum and an alkaline phosphatase conjugated secondary antibody for chromogenic detection. Two immunoreactive clones were identified; CDF-1, which was strongly reactive, and CDF-2, which showed moderate immunoreactivity. Agarose gel electrophoresis of *EcoR* I digested DNA from each clone indicated that CDF-1 contained a cDNA insert of approximately 1 kb, while the CDF-2 clone contained an insert of approximately 3 kb. cDNA inserts were subcloned into the *EcoR* I site of pGEM-7Zf(+) and these plasmid were used in further analyses.

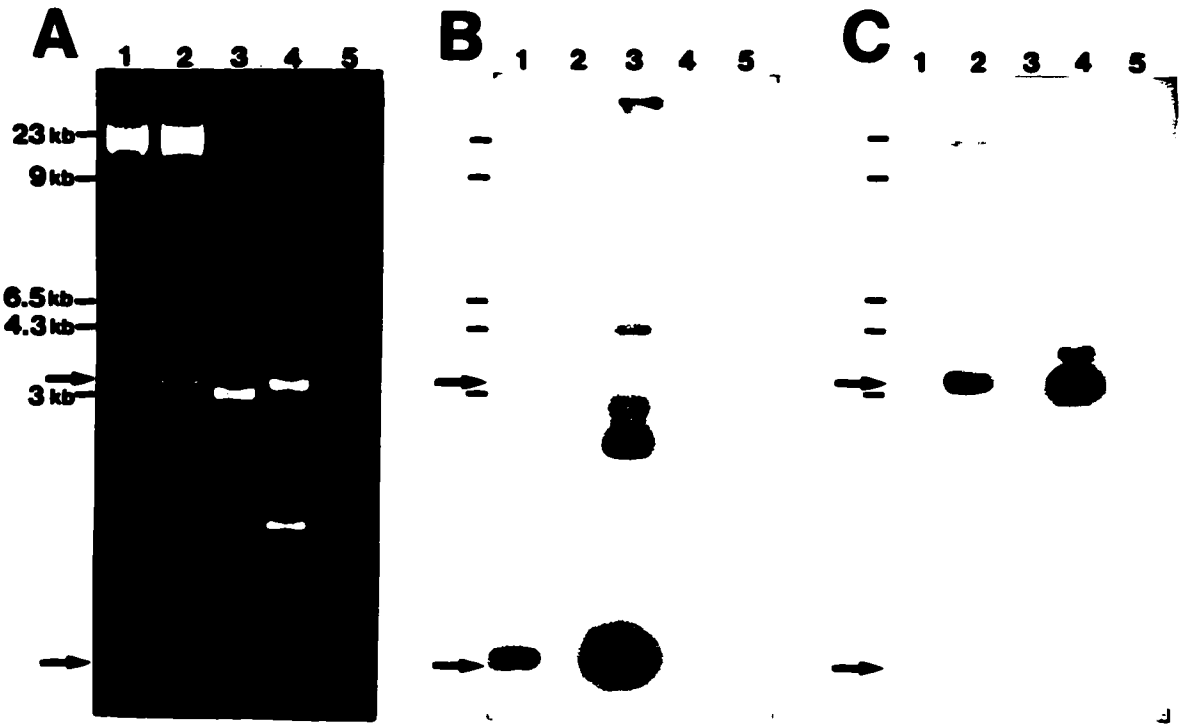
(ii) Southern blot analysis and restriction endonuclease mapping of CDF cDNA clones

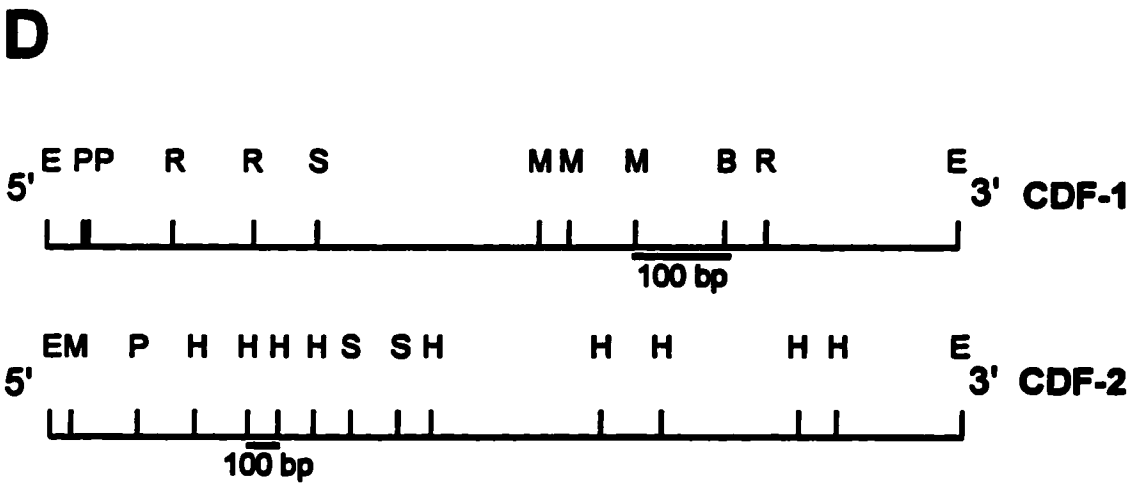
To determine if the cDNA clones identified by immunological screening contained overlapping sequences, Southern blot hybridization and restriction endonuclease mapping were employed. For Southern blot analysis, agarose gel electrophoresis was performed using phage and plasmid DNA digested with *EcoR* I or, in the case of the CDF-2 plasmid, *EcoR* I and *Sca* I to release the cDNA inserts (Figure 2, panel A). Gels were transferred to nylon membranes and blots were probed with nick-translated CDF-1 cDNA (Figure 2, panel B) or nick-translated CDF-2 cDNA (Figure 2, panel C). No cross-hybridization was apparent between the two cDNA clones as indicated by the absence of a signal in panel B, lanes 2 & 4 and in panel C, lanes 1 & 3. Restriction endonuclease mapping was also performed to confirm the distinct nature of the cDNA clones. Plasmids containing CDF-1 and CDF-2 cDNA inserts were digested with a variety of restriction endonucleases, the cleavage patterns were analyzed by agarose gel electrophoresis and a restriction map was generated for each cDNA insert (Figure 2, panel D). By both sets of analyses, the cDNA clones appeared to be distinct from one another and therefore were believed to represent two unique *T. vaginalis* genes.

(iii) Molecular characterization of the *T. vaginalis* cDNA clones

As two distinct cDNA clones were identified with the CDF anti-serum, it was necessary to verify whether either of these clones could encode the CDF protein. To provide additional information about the coding potential of the two cDNA sequences, Northern blot hybridizations and sequence analysis were performed.

Figure 2. Southern blot analysis and restriction endonuclease maps of the *T. vaginalis* CDF cDNA clones. Panel A. Ethidium bromide stained 0.8% agarose gel of phage and plasmid cDNA clones; lane 1, *EcoR* I digest of the CDF-1 phage clone; lane 2, *EcoR* I digest of the CDF-2 phage clone; lane 3, *EcoR* I digest of the CDF-1 plasmid clone; lane 4, *EcoR* I / *Sca* I double digest of the CDF-2 plasmid clone; lane 5, undigested pGEM7Zf(+). Arrows indicate the positions of the CDF-1 (1 kb) and CDF-2 (3 kb) cDNA inserts. Molecular weight markers were fragments of λ phage DNA digested with *Hind* III and are shown on the left. Panel B. Blot of the gel shown in panel A probed with a nick-translated ^{32}P-labelled CDF-1 cDNA insert. Panel C. Blot of the gel shown in panel A probed with a nick-translated ^{32}P-labelled CDF-2 cDNA insert. Panel D. Restriction endonuclease maps of the CDF cDNA inserts. E, *EcoR* I; H, *Hind* III; P, *Pst* I; S, *Sty* I; B, *Blp* I; M, *Mbo* I; and R, *Rsa* I.


A
1 2 3 4 5
23 kb
9 kb
6.5 kb
4.3 kb
3 kb
B
1 2 3 4 5
C
1 2 3 4 5


D
5'
E PP R R S M M M B R
E
3'
CDF-1
100 bp
5'
EM P H H H H S S H H H H H
E
3'
CDF-2
100 bp

To determine the molecular size of the transcripts represented by the cDNA clones, glyoxal agarose gel electrophoresis was performed using total *T. vaginalis* RNA or, as a negative control, *T. vaginalis* RNA depleted of polyadenylated RNA species. Gels were transferred to nylon membranes and probed with ^{32}P-labelled cDNA inserts. Despite numerous attempts and regardless of the type of probe, membrane or hybridization protocol employed, detection of a CDF-2 specific transcript was unsuccessful. On blots probed with a ^{32}P-labelled CDF-1 cDNA insert, a single polyadenylated transcript of 1.1 kb was detected (Figure 3, lane 1).

Using synthetic oligonucleotide primers and nested deletions, the cDNA inserts of both clones were sequenced in their entirety by the dideoxy-chain termination method. Sequence analysis of the CDF-2 clone identified a 3060 bp open-reading frame (ORF) contained within the 3062 bp insert. This ORF did not contain a translational start codon, and although a long tract of adenosine residues was present at the 3' end of the cDNA, no translational stop codon was apparent. When used in a non-redundant database search, the CDF-2 nucleotide sequence showed no similarity to other sequences available in the database. Likewise, the translated sequence of the CDF-2 ORF displayed only weak homology (20% amino acid similarity and no identity) with the human nucleolar phosphoprotein p130 (Pai *et al.* 1995). This similarity was primarily to a region of the p130 protein that contains repetitive serine-rich stretches interspersed with acidic residues. An interesting feature of the polypeptide that may be encoded by CDF-2 is its repetitive nature (Figure 4). The sequence EEKPKL is repeated a total of 37 times in the polypeptide, generally in one of two major motifs: EEKPKL$^{G}_{S}$LNL$^{G}_{P}$KSPS or EEKPKLQLGGIKL. An identical 79 amino acid tandem

Figure 3. Northern blot analysis identifying the transcript associated with the CDF-1 cDNA clone. 20 μg of *T. vaginalis* total RNA (lane 1) and 10 μg of *T. vaginalis* RNA depleted of polyadenylated RNA species (lane 2) were separated on a 1% glyoxal agarose gel, and RNA was transferred to nylon membrane. The blot was probed with a randomly-primed ^{32}P-labelled CDF-1 cDNA insert. The positions of 0.24-9.5 kb RNA species (Gibco/BRL RNA ladder) are shown to the left of the figure.

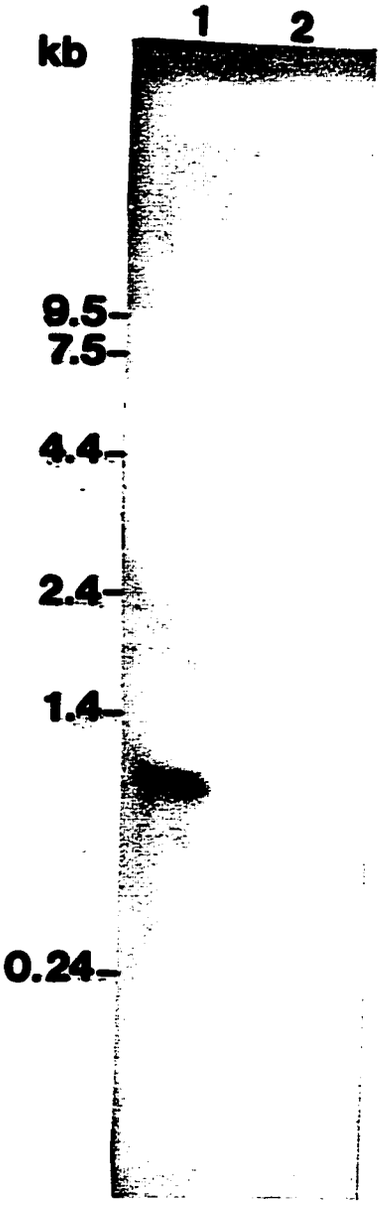
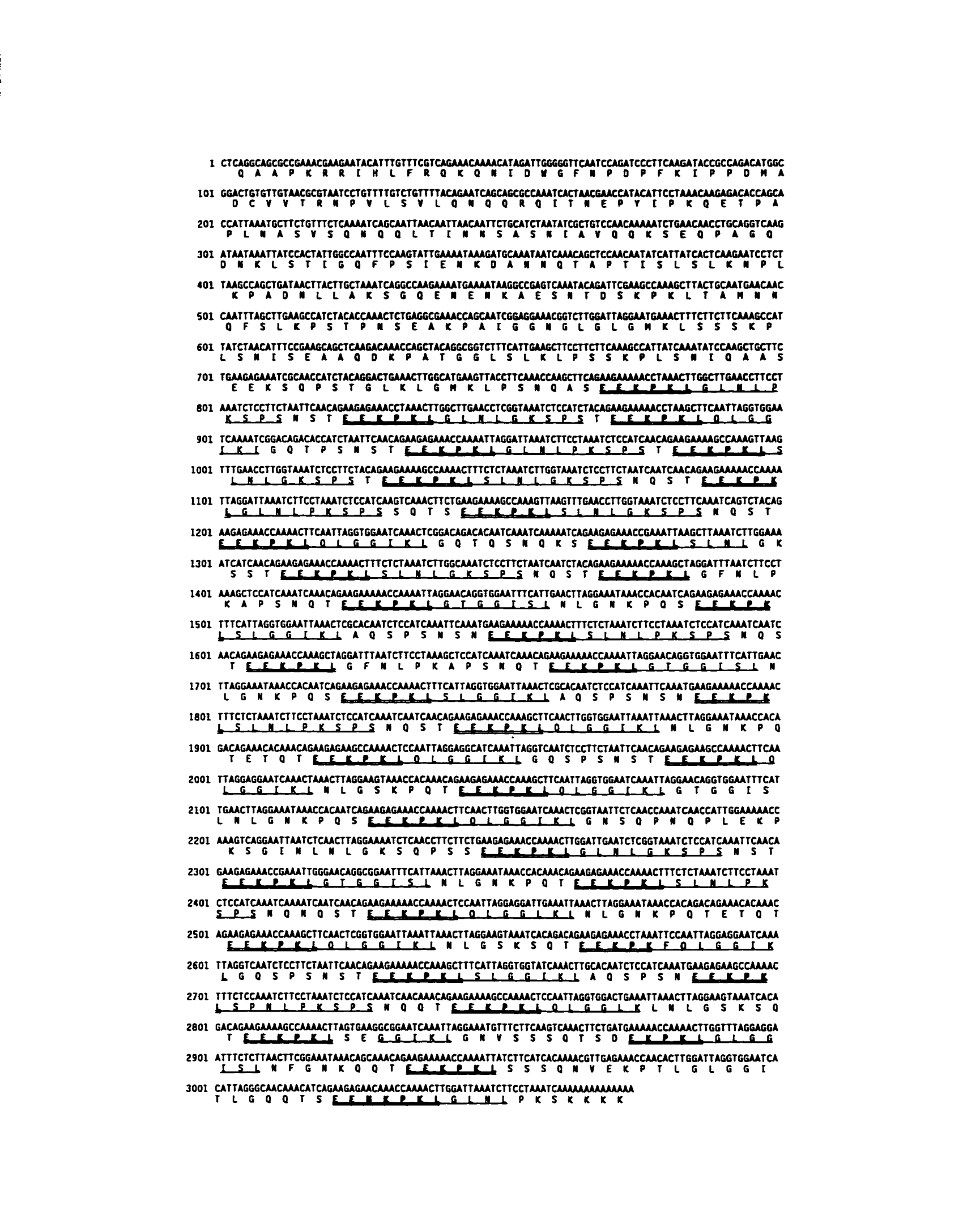

1
2
kb
9.5
7.5
4.4
2.4
1.4
0.24

Figure 4. Nucleotide sequence and the deduced amino acid sequence of the *T. vaginalis* CDF-2 cDNA insert. The two major repeated motifs within the predicted polypeptide are underlined, and the common hexapeptide EEKPKL is bold-faced and double underlined. As no translational stop codon was apparent, the adenosine tract at the 3' end of the cDNA sequence appears to encode a lysine-rich stretch of the protein. This nucleotide sequence is available in the EMBL, GenBank™ and DDJB data bases under the accession number U44915.



repeat is also found between nucleotides 1344-1871.

The CDF-1 cDNA insert (977 bp) contained a 936 bp ORF with a translational stop codon present 28 nt upstream of a poly(A) tract, however no translational start codon was apparent. A similar protein database search using the CDF-1 ORF indicated that the encoded polypeptide had substantial homology to the N-acetylneuraminate lyase of *E. coli* (Kawakami *et al.* 1986a; Ohta *et al.* 1985) and *Haemophilus influenzae* (Fleischmann *et al.* 1995). The CDF-1 polypeptide sequence exhibited 35% amino acid identity and 53% similarity with that of the *E. coli* lyase and 73% identity with the *H. influenzae* lyase. The complete sequence of this *T. vaginalis* gene and its homology to the bacterial lyases will be presented in the chapters that follow.

(iv) Characterization of the polyclonal CDF anti-serum

As the polyclonal CDF anti-serum used in screening the *T. vaginalis* cDNA library identified two distinct clones, it was necessary to re-evaluate the specificity of the serum for CDF. The CDF anti-serum had been used exclusively in Western blot analysis of *T. vaginalis* cell-free filtrates, that contained only secreted parasite proteins, therefore it was uncertain whether or not the serum contained antibodies that might react with other intracellular *T. vaginalis* proteins. To address this possibility, the anti-serum and *T. vaginalis* filtrates and lysates were used in radioimmunoprecipitations and Western blotting.

Radioimmunoprecipitations were performed with lyastes and filtrates of ^{35}S-methionine labelled *T. vaginalis* using a 1:16 dilution of CDF anti-serum. Samples were subjected to SDS-PAGE, and gels were processed for fluorography and exposed to X-ray

film. As indicated in Figure 5, a number of *T. vaginalis* proteins were recognized and precipitated by the CDF anti-serum. A large protein, which was believed to correspond to CDF, was identified in cell-free filtrates (lane 2) and culture supernatants (lane 3) as a doublet of slightly greater than 218 kDa. CDF was also detected, though weakly, in parasite lysates (lane 1) as a single protein species of similar molecular mass. It is unclear why, by radioimmunoprecipitation, the secreted CDF protein appears as a doublet, however the presence of reactive high molecular weight protein species in medium control filtrate (lane 4), may provide a possible explanation for this finding. Aside from CDF, several other secreted and intracellular *T. vaginalis* proteins were detected with the anti-serum. Secreted proteins detected by CDF anti-serum (lane 2) included a protein of 40 kDa and a number of high molecular weight species. Whether these larger protein species constituted unique *T. vaginalis* proteins or were degradation products of CDF remains unknown. Intracellular *T. vaginalis* proteins that reacted with the anti-serum (lane 1) included a 70 kDa species and several smaller proteins ranging in size from 30-45 kDa.

Western blot analysis, using the CDF anti-serum at a 1:100 dilution, also revealed numerous *T. vaginalis* proteins that reacted with the serum (Figure 6). As expected, the CDF protein was readily detected in both cell lysates (lane 1) and filtrates (lane 2) as a protein species of approximately 218 kDa. The absence of the protein in medium control filtrate (lane 3), re-enforced the fact that CDF was a parasite specific protein. The anti-serum also recognized other intracellular and secreted *T. vaginalis* proteins. Among the intracellular species detected (lane 1) were a predominant protein of 70 kDa, that appeared to correspond to the intracellular protein detected by immunoprecipitation, at least four small proteins

Figure 5. *T. vaginalis* proteins identified by radioimmunoprecipitation with the CDF anti-serum. *T. vaginalis* was radiolabelled with ^{35}S-methionine and resultant parasite lysates, cell-free filtrates and culture supernatants were incubated with a 1:16 dilution of polyclonal CDF anti-serum. Following immunoprecipitation, samples were analyzed by SDS-PAGE on 7.5% polyacrylamide gels, fluorography was performed and dried gels were exposed to X-ray film. Lane 1, *T. vaginalis* cell lysate; lane 2, *T. vaginalis* cell-free filtrate; lane 3, *T. vaginalis* culture supernatant; lane 4, medium control filtrate. The positions of high range prestained protein molecular weight standards (Gibco/BRL) are shown to the left of the figure.

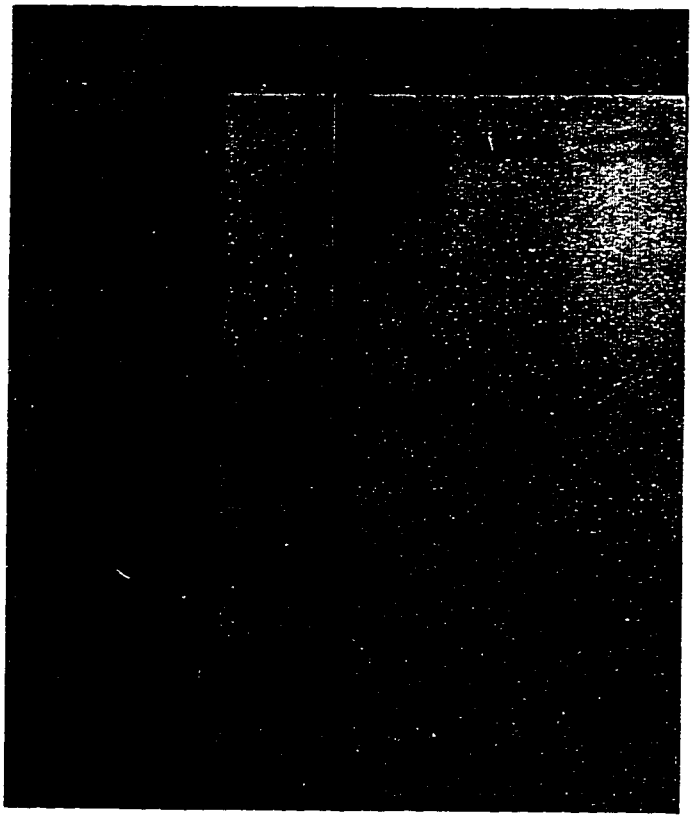
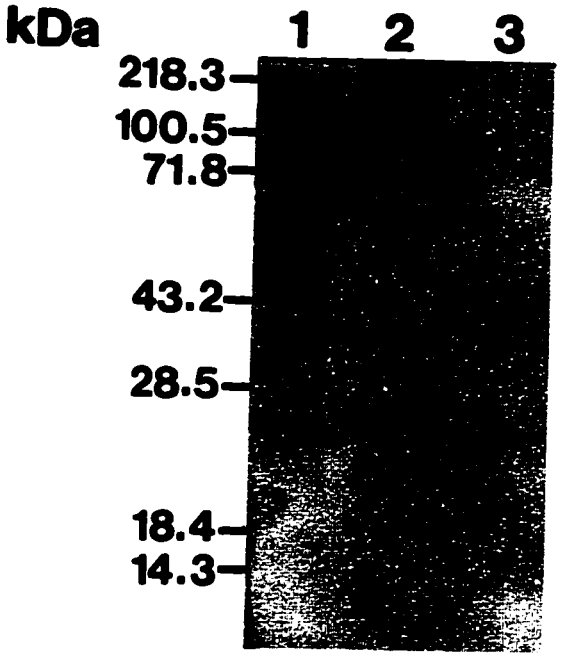


Figure 6. *T. vaginalis* proteins identified by Western blot analysis with the CDF anti-serum. *T. vaginalis* proteins were separated by SDS-PAGE on 10% polyacrylamide gels and proteins were transferred to nitrocellulose. Membranes were incubated with a 1:100 dilution of polyclonal CDF anti-serum and immunologically reactive proteins were detected with an alkaline phosphatase-conjugated secondary antibody and NBT/BCIP. Lane 1, *T. vaginalis* cell lysate (30 μg total protein); lane 2, *T. vaginalis* cell-free filtrate (5 μg total protein); lane 3, medium control filtrate (5 μg total protein). The positions of high range prestained protein molecular weight standards (Gibco/BRL) are shown to the left of the figure.


kDa
1
2
3
218.3
100.5
71.8
43.2
28.5
18.4
14.3

between 25-45 kDa and several large proteins of over 100 kDa. Secreted proteins (lane 2) included a species of 35-40 kDa and several high molecular weight proteins which may or may not have been the products of CDF degradation by proteinases.

DISCUSSION

The original objective of this study was to characterize the *T. vaginalis* CDF gene by obtaining cDNA clones from an expression library. To meet this objective, a *T. vaginalis* cDNA library was constructed in λgt11 and probed with rabbit anti-serum raised against a purified preparation of CDF. Two immunoreactive clones were identified and were determined to be distinct, based upon molecular analysis. To establish whether either of the identified cDNA clones could represent possible CDF sequences, Northern blot hybridizations and sequence analysis were performed. Based upon the molecular size of CDF (200 kDa) and dependent upon its level of glycosylation, a clone containing CDF sequences would be expected to identify a transcript of up to 5.4 kb in Northern blot analysis. Furthermore, CDF sequences might be expected to show similarity to other known CPE-inducing cytotoxins, or to cysteine proteinases, in lieu of the molecule's biological and functional similarity to the *E. histolytica* histolysin proteinase.

Characterization of the weakly immunoreactive CDF-2 cDNA clone was hampered by a variety of problems. The size of the transcript and thereby the associated protein was never established for this clone due to high backgrounds in Northern blots, a problem attributed to the repetitive nature of the CDF-2 sequence. Analysis of the CDF-2 cDNA revealed a 3060 bp ORF that was incomplete since a translational start codon was absent from

the 5' end of the molecule. More significant was the absence of a translational stop codon, despite the presence of fourteen adenosine residues at the 3' end of the cDNA. Because lithium chloride precipitations were used during *T. vaginalis* RNA isolation, this sequence is not believed to have originated from DNA. Furthermore, the long ORF identified in the sequence strongly suggests the potential to encode a polypeptide. Rather, it is believed that the adenosine residues at the 3' end of the cDNA represents a lysine rich region of the protein. This sequence, upstream of the 3' polyadenylated tail of the mRNA, may have been used by the oligo-dT primer as an initiation site for cDNA synthesis. The size of the incomplete CDF-2 ORF and the predicted molecular weight of the polypeptide (approximately 111 kDa) indicates that this sequence has the potential to encode a *T. vaginalis* protein of high molecular weight.

No significant similarity existed between the sequence of the CDF-2 polypeptide and those of other cytotoxins or cysteine proteinases available in the database. The only homology exhibited by the CDF-2 polypeptide was to a repetitive serine-rich stretch in the human nucleolar phosphoprotein p130 (20% amino acid similarity). What is interesting is the repetitive nature of this *T. vaginalis* sequence. Like those of most higher eukaryotes, the genome of *T. vaginalis* contains highly repetitive sequences. C_0t analysis of *T. vaginalis* DNA has indicated that the genome consists of 13.3% highly repetitive, 53.3% moderately repetitive and 33.3% unique sequence (Wang and Wang 1985). One repetitive family, Tv-E650, has been identified to be a 650 bp A-T rich tandem repeat, specific to *T. vaginalis* and conserved among all strains studied (Pačes *et al.* 1992). The estimated copy number of this family is believed to be between 100-1000 per genome. Repetitive genome sequences do

appear to have code potential in *T. vaginalis*. Sequence analysis of a partial cDNA clone encoding the *T. vaginalis* surface immunogen P270 indicates that the immunodominant protein epitope is encoded within a 339 bp unit, tandemly repeated at least twelve times in the gene (Dailey and Alderete 1991). Analysis of another *T. vaginalis* cDNA clone encoding a possible cysteine proteinase indicates there to be at least five hexapeptide repeats at the carboxy terminus of this polypeptide (Garber *et al.* 1993).

If CDF-2 represents coding sequence, the potential role of the EEKPKL repeats in the polypeptide is unclear. Many parasitic protozoa have repetitive sequences in surface proteins that are believed to aid in immune evasion. Examples of these include the circumsporozoite protein of *Plasmodium knowlesi* (Zavala *et al.* 1983) which contains a tandemly repeated epitope, and the variant-specific surface protein of *G. lamblia* which carries numerous copies of a CXXC motif (Yang *et al.* 1994b). There are also protozoan proteins related to the cytoskeleton that contain repetitive sequences. The Tb-29 proteins of *Trypanosoma brucei* are associated with a membraneous network that appears to have a circumnuclear location. These proteins contain an octapeptide repeat of EARLRAEE which can be present a total of 60-79 times (Lee *et al.* 1994). Similarly, the microtubule-associated protein MARP-1 of *T. brucei* contains repeats of 38 amino acids which are believed to represent a novel microtubule-binding motif (Hemphill *et al.* 1992). Still other examples of protozoan polypeptides containing repetitive elements include a 101 kDa microtubule-associated protein of *G. lamblia* (Marshall and Holberton 1993) and several proteins of *Trypanosoma cruzi* (Hoft *et al.* 1995). Whatever role the repeats might play in the CDF-2 polypeptide, the presence of repetitive sequences in the cDNA clone hampered further molecular analysis.

Characterization of the immunoreactive CDF-1 cDNA clone suggests that it does not represent CDF sequences. Northern blot analysis identified a transcript approximately 1.1 kb in length that would correspond to a protein of only 40 kDa, barring glycosylation. Although a translational stop codon upstream of a polyadenylate tract indicated the potential of the CDF-1 cDNA to represent coding sequence, the clone was considered incomplete because it lacked a translational start codon. A comparison of the length of the cDNA insert to that of the transcript indicated that less than 150 bp of the 5' region of the CDF-1 gene was likely to be missing from the cDNA clone. Finally, a significant degree of homology was exhibited between the encoded CDF-1 polypeptide and the N-acetylneuraminate lyase (Neu5Ac lyase) of *E. coli* (35% identity) and *H. influenzae* (73% identity), suggesting that the *T. vaginalis* sequence may encode a lyase.

Although the preliminary molecular characterization of the CDF-2 clone proved inconclusive, it was apparent that the CDF-1 cDNA clone did not contain sequences representing CDF. Several factors are believed to have contributed to the lack of success in identifying an authentic CDF clone. One potential problem lies in the representation of CDF clones in the cDNA library. Although CDF is the predominant protein species in Western blots and radioimmunoprecipitations of cell-free filtrates, it is not easily detected in parasite lysates. Maximal expression of CDF appears to occur when organisms are grown in the presence of a eukaryotic cell monolayer (Garber *et al.* 1989; Garber and Lemchuk-Favel 1990). Since the source of the mRNA used in the library construction was axenically grown parasites, CDF expression and therefore, CDF mRNA levels may not have been optimal. The use of optimal growth conditions prior to RNA isolation has been found to be essential in the

cloning of the *T. vaginalis* AP65 adhesin molecule (Alderete *et al.* 1995a). This iron-regulated molecule was identified only in cDNA libraries prepared from mRNA isolated from parasites grown in high-iron medium.

A more significant factor that is likely to have affected the outcome of library screening is the apparent multiple specificities of the CDF anti-serum. It is generally accepted that successful immunological screening relies upon the quality of the antibody used. Polyclonal serum is advantageous because it contains antibodies reactive with numerous epitopes on the protein of interest. While this feature can greatly reduce the number of clones required for screening, it also presents the potential for recognition of otherwise unrelated polypeptides. The most critical property of serum used in library screening is its capability to detect the denatured protein. Serum reactive in Western blots can be expected to recognize a localized, linear epitope on an expressed fusion protein. Although the CDF anti-serum detected the protein in Western blots of filtrates, it had not been evaluated for its potential to react with intracellular *T. vaginalis* proteins prior to screening. As evident from the results of Western blot and radioimmunoprecipitation, the CDF anti-serum appears to recognize numerous *T. vaginalis* proteins. Although the serum was both pre-adsorbed with *E. coli* extract prior to screening, and employed at a high dilution (1:6000), the multiple specificities possessed by the anti-serum are believed to have resulted in the identification of the two unrelated cDNA clones.

At this time, the original objective of the study was re-assessed. With the under representation of CDF related sequences in the library a distinct possibility, and because of the multiple specificity of the anti-serum, it was decided that re-screening the library would

not improve the likelihood of identifying a genuine CDF cDNA clone. Therefore, the CDF-1 clone was chosen for further analysis. The reasons for this decision were: (i) the CDF-1 cDNA was almost a complete coding sequence, and (ii) the sequence encoded a polypeptide with significant similarity to a protein of known function. Consequently, the new objectives of the study became: (i) the molecular analysis of this *T. vaginalis* gene, which was renamed Tv*nanA*, and (ii) functional analysis of the Tv*nanA* encoded polypeptide to determine if it possessed the same activity attributed to its bacterial homolog.

CHAPTER 4: MOLECULAR CHARACTERIZATION OF THE *T. VAGINALIS nanA* GENE

INTRODUCTION

N-acetylneuraminate Lyase Enzymatic Reaction

N-acetylneuraminate lyase (Neu5Ac lyase; EC 4.1.3.3), an enzyme involved in sialic acid metabolism, catalyzes the cleavage of free sialic acid (N-acetylneuraminic acid) to the amino sugar N-acetyl-D-mannosamine and pyruvate. Although the cleavage reaction is normally favoured at equilibrium, the enzyme can also catalyze the condensation reaction if high levels of substrates are available (Warren 1986). Neu5Ac lyase belongs to the class I aldolase family based upon its ability to form a Schiff's base intermediate with substrate and its lack of a requirement for a metal ion co-factor. A Schiff's base occurs when the α-amino group of an amino acid reacts with an aldehyde. For Neu5Ac lyase, this reaction is proposed to occur between a lysine residue of the enzyme and the aldehyde group present at the C-2 position of N-acetylneuraminic acid (Izard *et al.* 1994; Nees *et al.* 1976). While in this intermediate form, cleavage occurs between the C-3 and C-4 bonds of sialic acid when a nucleophilic amino acid accepts a proton from the substrate's hydroxyl group at C-4 (Corfield and Schauer 1982; Nees *et al.* 1976). It is the C-4 of N-acetylneuraminic acid which appears to be critical during lyase cleavage. Modification of the hydroxyl group at this site by either acetylation or methylation has been shown to produce a molecule resistant to Neu5Ac lyase cleavage (Gross and Brossmer 1988; Shukla and Schauer 1986; Zbiral *et al.* 1992).

Distribution of Neu5Ac Lyase

Neu5Ac lyase has been identified in both eukaryotes and prokaryotes. Lyase activity has been detected in the kidney cortex of the rat, hog, guinea pig, rabbit and cow (Brunetti *et al.* 1962), as well as in rat intestine and human liver (Corfield and Schaeur 1982). Two undifferentiated cell lines, human promyelocytic leukemic cells (HL-60) and mouse embryonal teratocarcinoma cells (F9) have been shown to express Neu5Ac lyase activity after being induced to differentiate. HL-60 cells exposed to either phorbol ester or tetradecanoylphorbol 12-myristate 13-acetate had increased lyase activity (Warren 1986) and likewise, the Neu5Ac lyase activity in F9 cells, induced to differentiate with retinoic acid, increased approximately 13-fold (Warren 1985). The differentiated murine parietal endodermal cell line PYS-2 also possesses Neu5Ac lyase activity which appears to be sensitive to cell division. For PYS-2 cells, maximal lyase activity occurs during log phase growth and declines when cells become confluent (Warren 1985).

A variety of bacteria have been found to possess Neu5Ac lyase. These include *Vibrio cholerae* (Heimer and Meyer 1956), *Haemophilus paragallinarum* and *Haemophilus paravium* (Hinz and Muller 1977), *Clostridium perfringens* (Comb and Roseman 1960), *Corynebacterium diphtheriae* (Arden *et al.* 1972), *Proteus vulgaris* and *Proteus mirabilis* (Uchida *et al.* 1985) and *E. coli* (Uchida *et al.* 1984). Although several bacterial species containing Neu5Ac lyase also possess neuraminidase (ie. *C. perfringens* and *C. diphtheriae*), the presence of the sialidase does not appear to be a pre-requisite for the lyase enzyme. More important may be the fact that the majority of bacteria with Neu5Ac lyase activity commonly inhabit mucous membrane-associated sites where sialomucins exist.

Role/Function of Neu5Ac Lyase

Although its role in mammalian tissues and cells is not well established, it appears that, in bacteria, Neu5Ac lyase functions primarily in nutrient acquisition. The degradation of free sialic acid produces pyruvate, which can enter metabolic pathways to be used as a carbon and energy source, and N-acetyl-D-mannosamine, which can be utilized in the synthesis of peptidoglycan and other bacteria cell wall polymers (Vimr 1992, Vimr 1994). Bacterial sialic acid catabolism relies on at least two proteins; a sialic acid permease to transport free sialic acid into the cell, and Neu5Ac lyase (Martinez *et al.* 1995; Vimr and Troy 1985a, 1985b). The free sialic acid used as a nutritional source originates from sialoglycoconjugates that are present on the surface of eukaryotic cells and in the mucins that coat mucous membranes. Terminal sialic acid residues can be cleaved from sialoglycoconjugates by neuraminidases secreted by either the individual bacterium, or by other organisms occupying the same microbial niche.

In *E. coli*, Neu5Ac lyase may have additional functions. Although most prokaryotes do not appear to synthesize sialic acid, it has been identified in the capsules of *Neisseria meningitidis* groups B and C, and in *E. coli* K1 strains (Vimr and Troy 1985a, 1985b). In *N. meningitidis*, sialic acid synthesis occurs by the condensation of N-acetyl-D-mannosamine and phosphoenolpyruvate, a reaction catalyzed by the enzyme N-acetylneuraminic acid synthase. This enzyme does not appear to be present in *E. coli* K1 strains however, and sialic acid synthesis has instead been found to depend upon the condensation reaction catalyzed by Neu5Ac lyase (Rodríguez-Aparicio *et al.* 1995). Furthermore, sialic acid accumulation in *E. coli* has been shown to be toxic to the bacterium (Vimr and Troy 1985a, 1985b). The

bacteria must therefore possess a mechanism to regulate the intracellular concentration of sialic acid and this appears to be through induction of Neu5Ac lyase. Vimr and Troy (1985a, 1985b) demonstrated that Neu5Ac lyase was induced by intracellular sialic acid. Although the degree of induction was strain dependent, lyase activity was shown to be at least 1,000 times higher for induced bacteria compared to uninduced strains. Full induction appears to require an intracellular sialic acid concentration of at least 4 mM, and is repressed by the presence of glucose (Lilley *et al.* 1992; Vimr and Troy 1985b).

Characterization of Neu5Ac Lyases

Only two mammalian Neu5Ac lyases have been characterized. Using beef kidney cortex as an enzyme source, Sirbasku and Binkley (1970) purified a Neu5Ac lyase that demonstrated a pH optimum of 7.2-8.5, was not affected by the presence of metal ions such as Mg^{2+}, Zn^{2+} or Ca^{2+}, but was inhibited by Fe^{2+}, Hg^{2+} and Cu^{2+}. By Sephadex G-200 column chromatography, this lyase was established to be a protein of approximately 55 kDa. Neu5Ac lyase has also been purified from PYS-2 cells where it appears to be a soluble intracellular enzyme (Warren 1985). The PYS-2 cell lyase has an optimum pH of 8.0, was not inhibited by Mg^{2+}, Mn^{2+} or Ca^{2+}, and demonstrated a molecular mass of 63 kDa by Sephadex G-100 column chromatography.

The best characterized Neu5Ac lyases are those of *C. perfringens* and *E. coli*. The *C. perfringens* intracellular Neu5Ac lyase was first identified by Comb and Roseman (1960) and later purified by Nees *et al.* (1976). Similar to the *E. coli* enzyme, *C. perfringens* Neu5Ac lyase appears to be induced by free sialic acid (Nees and Schauer 1974; Vimr and

Troy 1985a). This bacterial lyase has a pH optimum of 7.2 and a pI of 4.7. It appears to be inhibited by metal ions such as Cu^{2+}, Fe^{2+} and Hg^{2+}, and is also inhibited by pyruvate in a competitive manner. The molecule exists in native form as a 99.2 kDa enzyme consisting of two identical 50 kDa subunits that are linked to produce a particle that is "dumb-bell"-shaped in electron micrographs of negatively stained preparations (Nees *et al.* 1976). The *C. perfringens* lyase is thought to possess two active centers, one per enzyme subunit, with a lysine residue believed to participate in Schiff's base formation. Photooxidation experiments and chemical modifications of the lyase both indicate that a histidine residue is the nucleophilic group involved in substrate cleavage (Nees *et al.* 1976).

The *E. coli* Neu5Ac lyase has been characterized at both the level of the gene and protein. The *E. coli* lyase gene, designated *nanA*, is a 891 bp ORF which encodes a 297 amino acid protein with a predicted molecular mass of 32.6 kDa (Kawakami *et al.* 1986a; Ohta *et al.* 1985). Typical of most prokaryotic genes, a Shine-Dalgarno sequence, a -10 Pribnow box, and a -35 region have all been identified in sequences upstream of the *nanA* gene, and are positioned in a context favoured for efficient transcription. The *nanA* gene appears to be organized in a sialocatabolic operon along with the *nanT* gene, that encodes a putative sialic acid permease (Martinez *et al.* 1995). As both genes appear to be regulated by sialic acid, it has been suggested that the operon is positively controlled by the cyclic AMP-catabolite repressor protein complex (Martinez *et al.* 1995). No putative regulatory regions for *nanA* have been identified to date. Recombinant plasmids capable of complementing *nanA*$^-$ mutant strains have been found to constitutively express Neu5Ac lyase (Aisaka and Uwajima 1986; Kawakami *et al.* 1986b). The constitutive expression from these

plasmids, which contain between 1.3-9 kb of cloned DNA, implies that either: (i) the regulatory regions are absent in the cloned fragments or that, (ii) the levels of wild type repressor are not adequate to repress the lyase gene expressed from a high copy number plasmid.

The *E. coli* Neu5Ac lyase protein is intracellular and has been purified by both traditional biochemical techniques and by genetically engineered expression from plasmid vectors (Aisaka *et al.* 1991; Lilley *et al.* 1992; Uchida *et al.* 1984). Similar to its *C. perfringens* counterpart, *E. coli* lyase appears optimally active at pH 6.5-7.7, has a pI of 4.5, and is inhibited by metal ions such as Ag^{+}, Hg^{2+}, Cu^{2+}, Fe^{2+} and by the presence of pyruvate (Aisaka *et al.* 1991; Uchida *et al.* 1984). The native lyase has been estimated to be between 98-105 kDa, and SDS-PAGE analysis suggests that the native form of the enzyme consists of three identical subunits of 32-35 kDa. The *E. coli* Neu5Ac lyase has also been shown to exhibit a 5-fold greater specific activity than that attributed to the *C. perfringens* lyase (Uchida *et al.* 1984). By X-ray crystallographic analysis, the precise structure of the *E. coli* lyase has recently been established (Izard *et al.* 1994). Rather than being a trimer, the enzyme exists as a tetrameric molecule with four co-planar subunits folded as α/β barrels. Each subunit appears to make contact with only two of the three remaining subunits. The α/β barrel motif, which is common to at least 20 enzymes, is composed of eight parallel β-strands surrounded by seven or eight α-helices (Farber and Petsko 1990). In the *E. coli* Neu5Ac lyase, the β-barrel core of the enzyme is circular and following the barrel, in the carboxy-terminus of the protein, there are three additional α-helices. The active site of the enzyme is considered to be a deep pocket lined primarily by amino acids Ala11, Ser47, Thr48, Tyr137,

Ile139, Thr167, Gly189 and Tyr190 (Izard *et al.* 1994). Lys165, which is situated on the floor of the pocket, is believed to be the catalytic residue that participates in Schiff's base formation. Unlike the *C. perfringens* lyase, which employs histidine as the nucleophilic acceptor during cleavage, *E. coli* Neu5Ac lyase does not contain exposed histidine residues in the active pocket (Izard *et al.* 1994; Nees *et al.* 1976). For this bacterial lyase, the nucleophilic acceptor for cleavage has been proposed to be Tyr137, however further structural analysis is required to confirm this.

T. vaginalis Sialic Acid Metabolism

Little is known concerning sialic acid metabolism in *T. vaginalis*. There have been reports that *T. vaginalis* and the related protozoan *Tritrichomonas foetus* secrete neuraminidases into culture medium, and that the release of this enzyme is augmented in the presence of eukaryotic cell monolayers (Costa e Silva Filho *et al.* 1989; Crampen *et al.* 1979; Dias Filho *et al.* 1995). The *T. vaginalis* neuraminidase present in culture supernatants is capable of cleaving sialic acid from the surface of erythrocytes and hydrolyzing the sialidase specific substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Costa e Silva Filho *et al.* 1989). Although it has been suggested that the neuraminidase is involved in *T. vaginalis* CPE, the enzyme has not yet been isolated and no specific function has been assigned to it. Sialic acid moieties have also been identified on the surface of *T. vaginalis* (Costa e Silvo Filho *et al.* 1986; Dias Filho *et al.* 1992). Binding has been observed between the parasite and wheat germ agglutinin (WGA), a lectin that recognizes both sialic acid and N-acetyl-glucosamine residues. To determine lectin-specificity, Dias Filho *et al.* (1992)

treated *T. vaginalis* with neuraminidase and then incubated organisms with fluorescent-labelled WGA. Treated parasites were unable to bind WGA, indicating that the lectin specifically recognized sialic acid residues on the surface of the organism.

Objectives

The possible presence of a *T. vaginalis* neuraminidase and the identification of sialic acid moieties on the parasite cell surface may suggest that: (i) *T. vaginalis* could utilize free sialic acid as a nutrient source and, (ii) the parasite may synthesize sialic acid. Prior to this study, no enzymes or genes involved in *T. vaginalis* sialobiology had been isolated or characterized. As the CDF-1/ Tv*nanA* cDNA clone, identified earlier, exhibited substantial sequence similarity to the *E. coli* Neu5Ac lyase, the objectives of the study became: (i) the characterization of this *T. vaginalis* gene, which potentially is involved in sialic acid catabolism, and (ii) functional analysis of the encoded polypeptide. Essential to meeting the first objective was the isolation of the genomic copy of the Tv*nanA* gene. Obtaining a genomic clone would not only supply the complete coding region of the gene, to be used for later expression and functional analysis, but would also provide information about the upstream and downstream flanking sequences that may be involved in the regulation of Tv*nanA* expression.

This chapter describes the characterization of the full-length Tv*nanA* gene, its 5' and 3' flanking sequences, and its transcriptional initiation site. As well, nucleotide sequence analysis of the Tv*nanA* gene and the protein homology of the encoded polypeptide with other bacterial lyases is discussed. Furthermore, analysis of transcriptional regulation by a potential

initiator element is detailed.

RESULTS

(i) Isolation of a genomic copy of the *T. vaginalis nanA* gene

The identification and isolation of a genomic copy of the *T. vaginalis nanA* gene was necessary for two reasons: (i) it would supply the sequence of the 5' end of the gene absent from the cDNA clone, and (ii) it would provide further information about *T. vaginalis* genome structure and organization. To obtain the genomic copy of Tv*nanA*, the results of Southern blot hybridizations were exploited. Using restriction endonucleases that did not cleave the cDNA copy of the gene, *T. vaginalis* high molecular weight DNA (>44 kb) was digested and agarose gel electrophoresis was performed. Gels were transferred to nylon membranes and blots were probed with randomly-primed ^{32}P-labelled Tv*nanA* cDNA (977 bp) (Figure 7). This probe identified single bands for each endonuclease digestion, indicating that the gene encoding the transcript was likely to be present in a single copy. Furthermore, Southern blots suggested that the complete *nanA* gene and its flanking sequences were contained within a 4.5 kb *EcoR* I fragment. In order to subclone this particular fragment, *EcoR* I digested *T. vaginalis* DNA of 4.3-6.0 kb was excised and eluted from agarose gels. DNA fragments were ligated to *EcoR* I linearized pGEM-7Zf(+) and used to transform competent bacteria. Utilizing the same cDNA probe employed in Southern blots, recombinant clones were screened by colony hybridization and a positive clone, designated pTvEco4.5, was identified. This clone was sequenced in its entirety using a series of nested deletion constructs. Subsequent sequence analysis identified not only the genomic copy of the

Figure 7. Southern blot hybridization to identify genomic DNA fragments containing the *T. vaginalis nanA* gene. High molecular weight *T. vaginalis* DNA (5 μg/well) was digested with various restriction endonucleases as indicated above each lane. DNA fragments were separated by electrophoresis on 0.8% agarose gels and transferred to nylon membrane. Blots were probed with the 977 bp *T. vaginalis nanA* cDNA insert that had been ^{32}P-labelled by random priming. Molecular weight markers were fragments of λ phage DNA digested with *Hind* III and are shown on the left.


EcoR I
Sal I
Sac I
Kpn I
kb
23
9.4
6.5
4.3
2.3
2.0

Tv*nanA* gene, but also a downstream ORF. This second genomic ORF (1218 bp) is predicted to encode a protein of 406 amino acids with a calculated molecular mass of approximately 47 kDa (Appendix III). Although the function of the polypeptide encoded by this ORF is unknown, the carboxy-terminus of the protein (amino acids 299-406) exhibits 35% identity and 59% similarity to the R19-22 repeats of the mouse ankyrin 3 protein (Peters *et al.* 1995). The repeat region of ankyrin proteins is believed to constitute a protein binding domain that can interact with molecules such as tubulin (Peters *et al.* 1995).

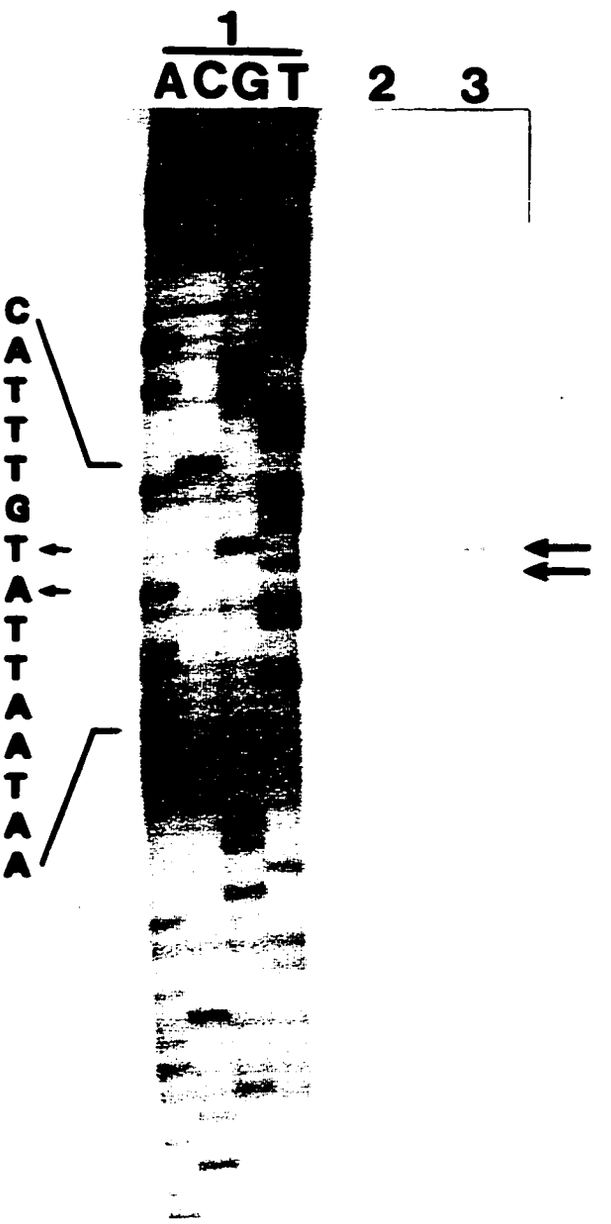
(ii) Tv*nanA* sequence analysis and genomic organization

Using sequences of both cDNA and genomic clones, a complete *T. vaginalis nanA* gene and its 5' and 3' flanking regions were characterized (Figure 8). Alignment of cDNA and genomic sequences indicated that this *T. vaginalis* gene lacked introns. The longest ORF is 954 bp and is predicted to encode a polypeptide of 318 amino acids with a calculated molecular mass of 35 kDa and an estimated pI of 6.0. To determine the transcriptional start site of the mRNA, primer extension was performed using total *T. vaginalis* RNA and an anti-sense primer (TvPE) designed to hybridize to the 5' proximal coding sequences (Figure 9). Two potential transcriptional initiation sites were identified which mapped to adjacent thymidine and adenosine residues, 17 and 18 nucleotides respectively, upstream of the translational start codon, therefore indicating a short 5' untranslated region (UTR). Although the region upstream of the Tv*nanA* gene is AT-rich, no consensus eukaryotic TATA or CAAT boxes were identified. The *T. vaginalis nanA* gene also exhibits a short 3' UTR of 28 nucleotides which contains a putative eukaryotic polyadenylation signal (AATAAA)

Figure 8. Nucleotide sequence of the *T. vaginalis nanA* gene and its flanking regions. This data is compiled from sequence analysis of the CDF-1/Tv*nanA* cDNA clone and the pTvEco4.5 genomic clone. The translational start and stop codons are boxed and shaded. Directional arrows indicate the sites of transcriptional initiation and the vertical arrowhead identifies the polyadenylate addition site. The potential Inr element and the putative polyadenylation signal are underlined in bold. The primer used in mapping the transcriptional initiation site is underlined. This nucleotide sequence is available in the EMBL, GenBank™ and DDJB data bases under the accession number U35878.

```
   1 CCAAAATTTCTCTAACATCTTCTCAATAGTTGAACGCTTTTCAAAAAATGTCATAAATTA
  61 TTAAAATTACATGCACATGCATATTAGATAATCCTATCGATGTTTGATTTGATGGGTATA
 121 GAAAATATTCAATGGGTATTCAGAAAAATACTCAAAGTAAACATAATTATTTTTGCCACT
 181 AATGTTCGTGTTCCTCGCGATTTCTATGGCGAAATCTGCAGCTGCAGAAGCCACTACCGG
 241 ACCAAAGGGAAAGAGTGCTAAAAGTTTAAAGGGTCTTTTTAGTGCTTTGTTAGTGTCATT
 301 CAATGAAGATGGTACAATCAACGAGAAAGGCTTACGTGAAATCGTTCGCTACAACATAGA
 361 CAAAATGAAAATTGATGGCTTATATGTTGGTGGCAGTACTGGTGAAAACTTCGAATTATC
 421 TACAGAAGAAAAGAAGCAAATCTTCCGCATTGCTAAGGATGAAGCCAAGGACCAGGTTGC
 481 ATTAATTGCCCAAGTCGGTAGCATTAACATCCACGAATCCATCGAATTAGGTAAATATGC
 541 CACAGAATTAGGCTACAATTGCTTATCCGCTGTTACACCATTCTACTACAAGTTCACCTT
 601 CCCAGAAATCAAGAACTACTACAACACAATTGTCAATGCCACAGGCATGAATATGATTGT
 661 TTATTCAATCCCAGCTTTAACAGGCGTCAGTATGACAGCTGATCAGTTCGGTGAACTCTT
 721 CGAGAATCCAAAGATCATCGGTGTCAAGTTCACAGCTGGTGATTTCTACTTATTAGAGCG
 781 TGTCAAGAGAGCTTACCCAGATCACCTTATTTGGGCCGGATTCGATGAAATGATGCTTCC
 841 AGCTTGCTCATTAGGTATTGATGGTGCCATCGGCAGCACATTCAATGTCAACGCTAAGCG
 901 TGCTAGACAAATCTTTGAATTGTCTAAAGCTGGCAAGTACGACGAAGCTCTTGAGGTTCA
 961 GCACGTTACAAACGATTTAATCGCTGGCATCCTCTCCAATGGCTTATACCTCACAATTAA
1021 GGAATTAATGAGACTCGATGGTGTTGATGCAGGATATTGCCGTGAGCCAATGACAAAGGC
1081 TTTGACCCCAGCTCAGGTTGCTTTCGCCAAGCAATTAAAGGAGAAATATCTTTTATAAAT
1141 TTATTAAATAAACTCTAATTTACGTTATCACTATAATATTTCAGTTTTTCAACGTTCT
```

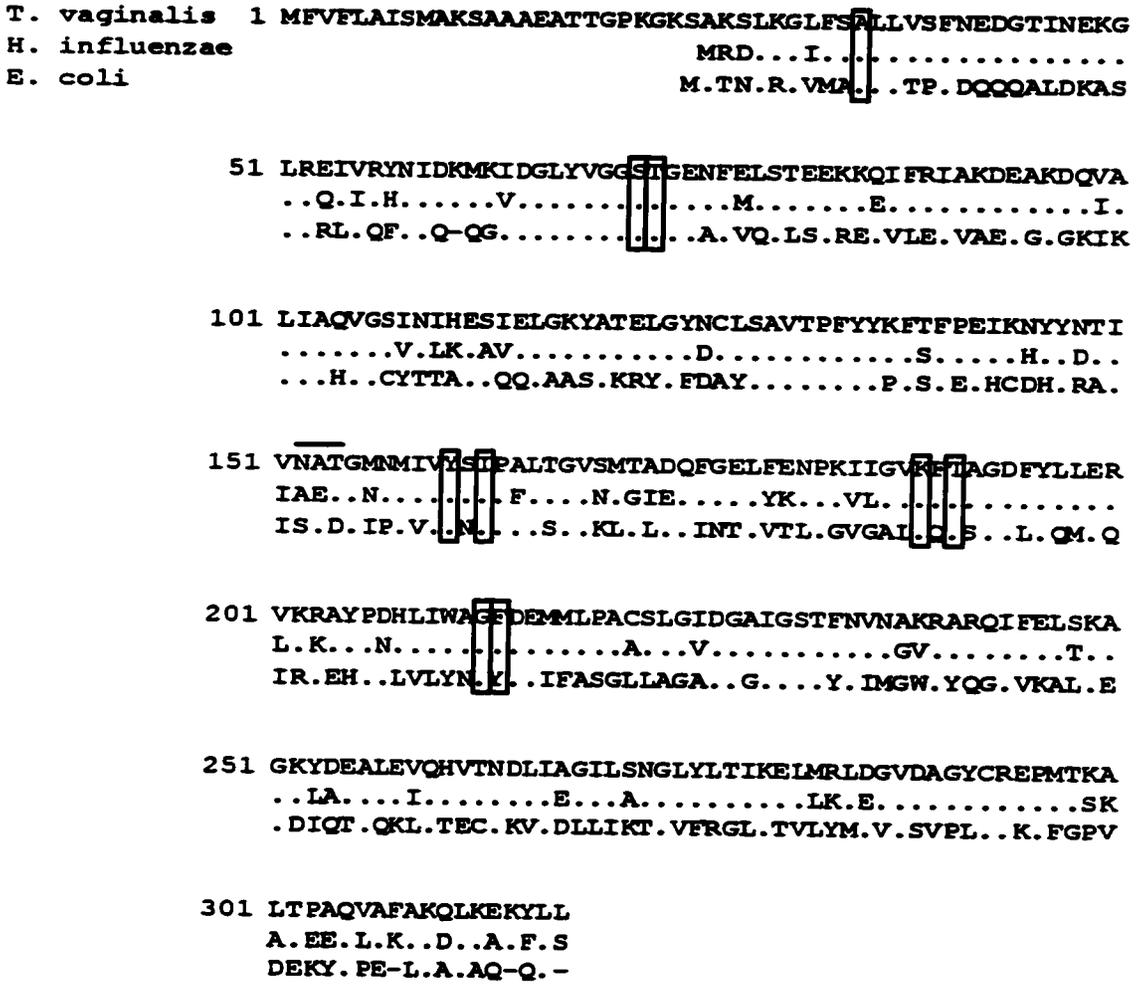
Figure 9. Primer extension analysis of the Tv*nanA* gene. 50 μg (lane 2) or 100 μg (lane 3) of total *T. vaginalis* RNA was hybridized with a radiolabelled anti-sense oligonucleotide primer (TvPE), reverse transcribed, and electrophoresis was performed on 8% polyacrylamide sequencing gel containing 8 M urea. The same primer was used in standard ^{35}S-dideoxy sequencing reactions using pTvEco4.5 as DNA template (lane 1) in order to determine the size of the extension products. The sequence and the sites of transcriptional initiation, indicated by arrows, are shown on the left.


1
ACGT
2
3
CATTTGTATTAATAA

(Proudfoot and Brownlee 1976) 13 nucleotides upstream of the polyadenylate addition site.

Codon usage in the Tv*nanA* ORF is similar to that described for other *T. vaginalis* genes (Katiyar and Edlind 1994). A strong bias was found toward G in the third position for Gln and Lys (73%), and towards A in the third position for Pro and Thr (81%). In the *T. vaginalis btub1* gene (Katiyar and Edlind 1994) a strong bias was also demonstrated for C in the third position for Phe, Tyr, Cys, His and Asn (100%). This bias was not as pronounced in the Tv*nanA* gene (74%). A BLAST non-redundant protein database search (Altschul *et al.* 1990; Gish and States 1993) using the Tv*nanA* encoded polypeptide sequence revealed 35% identity, 53% similarity to the Neu5Ac lyase of *E. coli* and 73% amino acid identity to what is believed to be the Neu5Ac lyase of *H. influenzae* (Figure 10). Optimal alignment introduced only 4 single amino acid gaps in the *E. coli* protein, three of which occur in the carboxy-terminus. The only major difference between the proteins is 24 additional amino acids, many of which are hydrophobic, at the amino-terminus of the *T. vaginalis* polypeptide. While the location and charge of this region suggested that it may function in protein localization, analyses by the methods of McGeoch (1985) and von Heijne (1986) indicated the absence of a eukaryotic N-terminal signal sequence. From X-ray crystallographic analysis, the *E. coli* Neu5Ac lyase has been predicted to exist in its native form as a tetrameric α/β barrel enzyme, with the potential catalytic residue of the enzyme (Lys165) located on the floor of a deep pocket lined primarily by amino acids Ala11, Ser47, Thr48, Tyr137, Ile139, Thr167, Gly189 and Tyr190 (Izard *et al.* 1994). Alignment of the bacterial lyases and the *T. vaginalis* polypeptide indicated that both the catalytic residue and those lining the active enzyme pocket are identical among the three molecules, with the exception of Tyr190 which

Figure 10. Alignment and comparison of the predicted *T. vaginalis nanA* encoded polypeptide and the deduced amino acid sequences of the Neu5Ac lyases from *H. influenzae* (Fleishmann *et al.* 1995) and *E. coli* (Kawakami *et al.* 1986a; Ohta *et al.* 1985). Amino acids that are identical in the *T. vaginalis* and bacterial lyases are indicated by dots. The potential catalytic residue and those amino acids believed to line the enzymatic pocket of the molecule are boxed. The potential N-linked glycosylation site of the *T. vaginalis* protein is indicated by a line above the sequence. The first 24 amino acids of the *T. vaginalis* protein constitute the unique N-terminal hydrophobic region.


T. vaginalis 1 MFVFLAISMAKSAAAEATTGPKGKSAKSLKGLFSALLVSFNEDGTINEKG
H. influenzae MRD...I.....
E. coli M.TN.R.VMA...TP.DQQQALDKAS
51 LREIVRYNIDKMKIDGLYVGGGIGENFELSTEEKKQIFRIAKDEAKDQVA
..Q.I.H.....V.....M.....E.....I.
..RL.QF..Q-QG.....A.VQ.LS.RE.VLE.VAE.G.GKIK
101 LIAQVGSINIHESIELGKYATELGYNCLSAVTPFYYKFTFPEIKNYYNTI
.....V.LK.AV.....D.....S.....H..D..
...H..CYTTA..QQ.AAS.KRY.FDAY.....P.S.E.HCDH.RA.
151 VNATGMNMIVVSTPALTGVSMTADQFGELFENPKIIGVKFTAGDFYLLER
IAE..N.....F....N.GIE.....YK...VL.....
IS.D.IP.V...N....S..KL.L..INT.VTL.GVGAL.Q.S..L.QM.Q
201 VKRAYPDHLIWAGFDEMMLPACSLGIDGAIGSTFNVNAKRARQIFELSKA
L.K...N.....A...V.....GV.....T..
IR.EH..LVLYN.Y..IFASGLLAGA..G....Y.IMGW.YQG.VKAL.E
251 GKYDEALEVQHVTNDLIAGILSNGLYLTIKELMRLDGVDAGYCREPMTKA
..LA....I.....E...A.....LK.E.....SK
.DIQT.QKL.TEC.KV.DLLIKT.VFRGL.TVLYM.V.SVPL..K.FGPV
301 LTPAQVAFAKQLKEKYLL
A.EE.L.K..D..A.F.S
DEKY.PE-L.A.AQ-Q.-

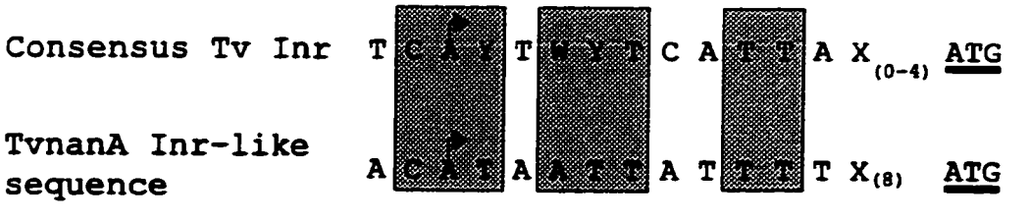
in both the *T. vaginalis* and *H. influenzae* proteins is replaced by phenylalanine. One potential N-glycosylation site is identified in the *T. vaginalis* polypeptide corresponding to amino acid residues 152-154.

(iii) Analysis of the Tv*nanA* Inr-like element

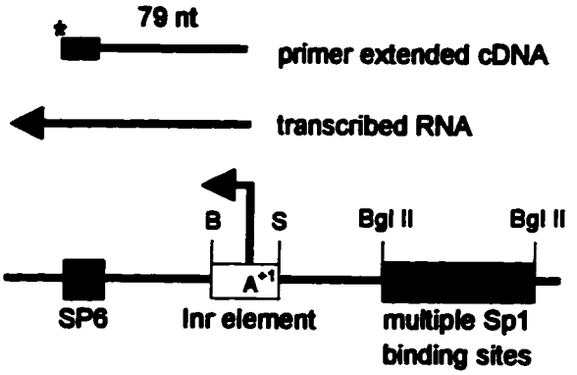
Nucleotide sequence analysis of the Tv*nanA* gene identified a sequence similar to the *T. vaginalis* initiator (Inr) element (Quon *et al.* 1994) in the 5' UTR of the gene, straddling the transcriptional initiation site (Figure 8 and Figure 11, panel A). The *T. vaginalis* Inr element, which has been identified in at least seven *T. vaginalis* genes (Appendix IV), consists of 13 nucleotides (TCA^{+1}YTWYTCATTA) that surround and contain the transcriptional initiation site. These elements are capable of directing accurate transcription at the initiation site in the absence of an upstream TATA box. In the Inr-like sequence of the Tv*nanA* gene (ACA^{+1}TAATTATTTT), 8 of the 13 residues identified in the consensus *T. vaginalis* Inr motif are conserved (Figure 11, panel A). This suggested that the sequence may be capable of accurately initiating transcription. To test the functionality of the Tv*nanA* Inr-like element, an oligonucleotide containing the sequence was cloned into the *BamH* I / *Sac* I sites of the plasmid p2032 (plasmid III, Smale *et al.* 1990). p2032 contains multiple copies of the 21 bp repeat region of the Simian virus 40 early promoter, which provides Sp1 transcription factor binding sites, and a SP6 promoter sequence (Figure 11, panel B). Once the Inr-like element was subcloned, the plasmid was tested for transcriptional initiation alongside plasmid p2121, which contains the functional mammalian terminal deoxynucleotidyltransferase (TdT) Inr element (Smale and Baltimore 1989), and plasmid p2549, which contains a non-functional Inr

Figure 11. Analysis of the potential Tv*nanA* Inr element. Panel A. Comparison of the nucleotide sequence of the consensus *T. vaginalis* (Tv) Inr element (Quon *et al.* 1994) and the sequence surrounding the Tv*nanA* transcriptional start. Directional arrows indicate the site of transcription initiation, nucleotides similar in both sequences are boxed and shaded, and the translational start codon is underlined in bold. Y, cytosine or thymidine; W, adenosine or thymidine; X, any of the four nucleotides. Panel B. Schematic of the strategy used in assessing Inr capability. Inr elements are cloned into the *BamH* I (B) / *Sac I* (S) sites of plasmid p2032 (Smale and Baltimore 1989) which contains the SP6 promoter sequence (solid box) and multiple binding sites for the Sp1 transcription factor (hatched box). RNA is transcribed *in vitro* from the plasmid template, hybridized to a 5'-^{32}P labelled SP6 primer (solid box with an asterick) and then reverse transcribed. Efficient transcription from the Inr element is determined by the presence of a 79 nt labelled cDNA fragment. Panel C. Autoradiograph of a 8% polyacrylamide / 8 M urea sequencing gel containing primer extension analysis products used to assess Inr sequence capability. TdT, the terminal deoxynucleotidyltransferase Inr element; R2549, a non-functional Inr sequence; TvnanA, the *T. vaginalis nanA* gene Inr-like sequence.

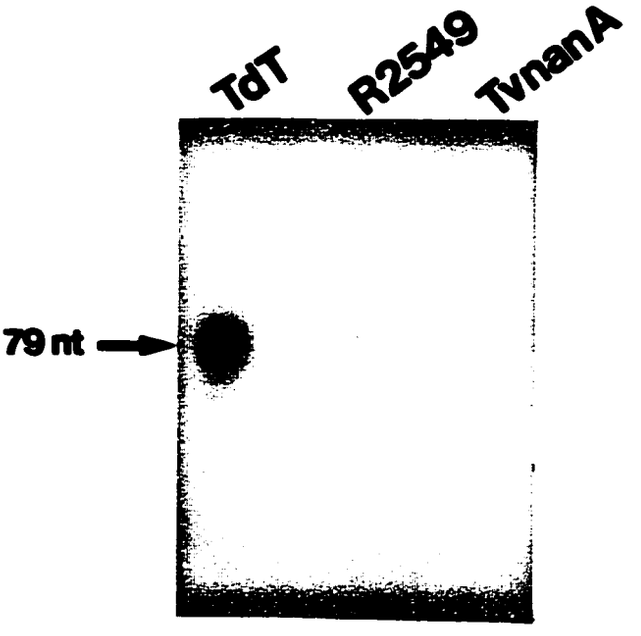
A


Consensus Tv Inr
T C A Y T W Y T C A T T A X (0-4) ATG
TvnanA Inr-like sequence
A C A T A A T T A T T T T X (8) ATG

B


79 nt
primer extended cDNA
transcribed RNA
B
S
Bgl II
Bgl II
A+1
SP6
Inr element
multiple Sp1 binding sites

C


TdT
R2549
TvnanA
79 nt

sequence (Javahery *et al.* 1994; Quon *et al.* 1994). Plasmids were transcribed *in vitro* using HeLa cell nuclear extracts and the resulting RNA transcripts were hybridized to a 5'-^{32}P-labelled SP6 primer and reverse transcribed. If a functional Inr element was present, transcription would commence at the initiate site and SP6 primer extension would yield a cDNA product of 79 nt in length. When samples were analyzed by electrophoresis on 8% polyacrylamide, 8 M urea sequencing gels and autoradiography, only the TdT Inr element was able to initiate accurate transcription and generated the expected 79 nt cDNA product (Figure 11, panel C).

DISCUSSION

Using cloned cDNA sequences and a cloned segment of overlapping genomic DNA, the complete sequence of the coding and flanking regions of the Tv*nanA* gene were determined. This gene appears to be present as a single copy in the *T. vaginalis* genome. Furthermore, the colinearity observed between the cDNA and genomic sequences of Tv*nanA* indicates that the gene lacks introns. This finding is not surprising since all of the genes described to date for the three amitochondrial protozoa; *E. histolytica*, *G. lamblia* and *T. vaginalis*, appear to be devoid of introns (Bruchhaus *et al.* 1993; Holberton and Marshall 1995; Hrdy and Müller 1995a, 1995b; Johnson *et al.* 1990; Katiyar and Edlind 1994; Lahti *et al.* 1992; Länge *et al.* 1994). Although introns are typical in the genes of higher eukaryotes, their absence in these protozoa may be indicative of the ancient evolutionary divergence proposed for these organisms.

In *E. coli*, the *nanA* gene is located upstream of the *nanT* permease gene in a

sialocatabolic operon (Martinez *et al.* 1995). Although sequencing of the genomic pTvEco4.5 clone revealed a second ORF downstream of the Tv*nanA* gene, the polypeptide encoded by this sequence did not exhibit similarity to the putative *E. coli nanT* permease or to other transporter proteins. This suggests that *T. vaginalis* genomic organization is typically eukaryotic, with genes that are involved in the same metabolic pathway situated throughout the genome rather than in regulatory operons.

The Tv*nanA* gene is predicted to be transcribed as a single polyadenylated mRNA that contains short, AT-rich 3' (28 nt) and 5' (17-18 nt) UTRs. Short non-coding regions appear to be a feature common to all *T. vaginalis* genes characterized to date. Prior to characterization of the *nanA* gene, the longest *T. vaginalis* 3' UTRs that had been described belonged to the ferredoxin (18 nt) and the succinyl-coenzyme A synthetase β-subunit (20 nt) genes (Johnson *et al.* 1990; Lahti *et al.* 1992). Despite the short length of the 3' UTRs, all *T. vaginalis* mRNAs that have been analyzed contain a sequence that resembles the eukaryotic polyadenylation signal, AATAAA (Johnson *et al.* 1990, 1994; Lahti *et al.* 1992; Proudfoot and Brownlee 1976). The Tv*nanA* gene is not an exception and contains the same signal 13 nt upstream of the polyadenylate addition site. The position of the polyadenylation signal with respect to the polyadenylate addition site is also in agreement with the 10-30 nt spacing established for the majority of eukaryotic mRNAs (Birnstiel *et al.* 1985).

The 5' UTRs in eukaryotic genes normally range up to 100 nt in length, however the 5' UTRs in *T. vaginalis*, *G. lamblia* and *E. histolytica* mRNAs are exceptionally short (Katiyar *et al.* 1995). In at least six *T. vaginalis* genes, the transcriptional initiation site maps within 17 nt upstream of the translational start codon (Alderete *et al.* 1995a; Johnson *et al.*

1990, 1994; Katiyar and Edlind 1994; Lahti *et al.* 1992, 1994). The transcriptional initiation site of the Tv*nanA* gene is consistent with this observation as the potential transcriptional start sites are located 17 and 18 nt upstream of the translational initiation codon. The penultimate nucleotide in capped eukaryotic mRNAs is generally a purine residue and since *T. vaginalis* mRNAs are capped (Quon *et al.* 1994), it is likely that Tv*nanA* transcription is initiated at the adenosine residue rather than at the adjacent thymidine residue. The short 5' non-coding regions found in mRNAs of amitochondrial protozoa have lead to speculation that the ribosomes of these organisms do not bind and scan for translational initiation codons in the same manner described for ribosomes of higher eukaryotes (Katiyar *et al.* 1995; Kirk-Mason *et al.* 1989). Katiyar *et al.* (1995) identified the sequence (T)TCA upstream or overlapping the translational start codon in ten *T. vaginalis* genes and hypothesized that this sequence is important in ribosome binding, since a complementary sequence has also been identified in the terminal hairpin loop of the small 16S-like subunit of *T. vaginalis* rRNA. Although further work is required to determine if this sequence is a potential ribosome binding site, it is not present in the 5' non-coding region of the Tv*nanA* gene.

Of particular interest was the finding that the sequence in the Tv*nanA* 5' UTR was similar to the consensus *T. vaginalis* Inr sequence. Inr motifs are capable of directing transcriptional initiation in the absence of a TATA box, the common eukaryotic regulatory element that initiates RNA polymerase II transcription. Genes without a TATA box have been described and there appear to be two classes of promoters that function in the absence of TATA elements: (i) those that contain GC-rich sequences that provide binding sites for the Sp1 transcription factor, and (ii) those without a GC-rich region but which contain sequence

elements (Inr), surrounding the initiation site, which promotes basal levels of transcription (Azizkhan *et al.* 1993; Smale and Baltimore 1989). Inr elements have been shown to act in a unidirectional manner and, in concert with either TATA boxes or Sp1-binding GC boxes, to enhance transcription (Azizkhan *et al.* 1993; O'Shea-Greenfield and Smale 1992; Smale *et al.* 1990). The mechanism of Inr-mediated transcription has not been fully elucidated. However, some of the known events resemble what has been described for transcription promoted by TATA boxes. The initial template recognition appears to involve the TATA-binding protein (TBP) and transcription factor (TF) IID. DNase I footprint analysis indicates that TFIID, which normally interacts with the TATA box, can bind with a lower affinity to Inr elements (Kaufmann and Smale 1994; Smale *et al.* 1990). It was also demonstrated that TBP can bind 30 nucleotides upstream of an Inr element, despite the absence of TATA-like sequences (Zenzie-Gregory *et al.* 1993). It is thought that this non-specific binding of TBP to the DNA, in addition to the binding of TFIID and subsequently TFIIB, stabilizes the pre-initiation complex situated at the Inr element through protein-protein interactions (Kaufmann and Smale 1994; Zenzie-Gregory *et al.* 1992, 1993). Following template recognition, the steps involved in both the final assembly of the initiation complex and in the commencement of transcription appear to be identical to those described for TATA box promoters (Jiang *et al.* 1993; Zenzie-Gregory *et al.* 1992).

Following the analysis of a series of mutated Inr elements, Javahery *et al.* (1994) proposed the loose consensus sequence Py Py A^{+1} N T/A Py Py for mammalian Inr elements. The cytosine at the -1 position (C^{-1}), adenosine at +1 (A^{+1}) and thymidine at +3 (T^{+3}) were identified as the most conserved nucleotides in eukaryotic Inr elements. The T^{+3} and A^{+1}

positions appear to be the most critical in imparting Inr activity (Javahery *et al.* 1994). The replacement of thymidine at the +3 position with guanine, cytosine or adenosine results in 97%, 93% and 78% reduction in Inr activity respectively. Similarly, the replacement of adenosine at the +1 position with thymidine or guanine results in a 81% and 90% reduction in Inr strength. Without these two crucial nucleotides (T^{+3}, A^{+1}), Inr activity is conferred only if the sequence surrounding the transcriptional initiation site contains a high proportion of pyrimidines.

The presence of Inr elements in *T. vaginalis* may not be surprising when one considers that typical eukaryotic TATA and CAAT boxes have not been identified upstream of *T. vaginalis* genes (Alderete *et al.* 1995a; Hrdy and Müller 1995a, 1995b; Johnson *et al.* 1990; Katiyar and Edlind 1994; Lahti *et al.* 1992). The consensus *T. vaginalis* Inr sequence contains the three nucleotides (C^{-1}, A^{+1}, T^{+3}) found to be critical for mammalian Inr activity (Javahery *et al.* 1994; Quon *et al.* 1994). Furthermore, the presence of an Inr element in *T. vaginalis* genes encoding proteins of disparate function and location suggests that the Inr element is essential for basal transcription in this organism. In the Tv*nanA* Inr-like element, 8 of the 13 residues present in the consensus motif were conserved, however this element was unable to direct transcription in the *in vitro* assay system.

There are several possible explanations for the inability of the Tv*nanA* Inr-like element to direct transcription. In the Tv*nanA* sequence an adenosine replaces the thymidine residue at the +3 position, which is considered to be the most important residue in conferring Inr strength. Other Inr elements with an adenosine residue at this site demonstrate a maximum of 22% of the transcriptional activity attributed to the consensus sequence (Javahery *et al.*

1994). Since only the 13 nt sequence element was tested in the Inr assay system, it is also possible that Inr elements divergent from the *T. vaginalis* consensus sequence, like Tv*nanA*, may require the AT-rich region upstream of the gene for efficient transcription. While the HeLa nuclear extract system has been used to identifiy other *T. vaginalis* Inr elements, there are limitations in testing the short parasitic sequences in a heterologous system which contains different transcription factors and accessory proteins. Finally, most of the *T. vaginalis* genes associated with active Inr elements, which include a β-tubulin gene, a P-glycoprotein gene and several genes encoding hydrogenosomal enzymes, are considered to be constitutively expressed. Since the Tv*nanA* gene may be regulated like its bacterial homolog, the possibility arises that transcriptionally regulated *T. vaginalis* genes may require other regulatory elements either distinct from or in addition to Inr elements. A more precise understanding of *T. vaginalis* gene regulation and expression will only be possible after a transfection system has been developed for the organism.

The polypeptide encoded by the Tv*nanA* gene has considerable sequence similarity to the Neu5Ac lyases of *E. coli* and *H. influenzae*. The *E. coli* protein, which is involved in sialic acid catabolism, exists as a tetrameric α/β barrel enzyme. The active region of the molecule is predicted to be a deep pocket with the potential catalytic residue (Lys165) located on the pocket floor (Izard *et al.* 1994). Requiring only minimal gaps, alignment of the bacterial lyases and the *T. vaginalis* polypeptide indicated that, with the exception of Tyr190, the catalytic residue and the residues lining the enzyme pocket were identical in all three molecules. These data suggest that the *T. vaginalis* protein could possess Neu5Ac lyase activity.

CHAPTER 5: FUNCTIONAL ANALYSIS OF THE *T. VAGINALIS* Neu5Ac LYASE

INTRODUCTION

The protein sequence similarity between *E. coli* Neu5Ac lyase and the *T. vaginalis* polypeptide, which included conservation of residues present at the active site of the bacterial enzyme, led to the hypothesis that the *T. vaginalis* protein possessed Neu5Ac lyase activity. Therefore, the second objective of this study was to express the *T. vaginalis* polypeptide and determine if the protein possessed enzymatic activity.

The *E. coli* GST fusion protein system, initially developed by Smith and Johnson (1988), was chosen for protein expression. This system involves the synthesis of a fusion protein that contains a foreign polypeptide linked to the carboxy-terminus of the 26 kDa GST protein from *Schistosoma japonicum*. Because of its fusion to the GST carrier, the expressed protein can be purified under non-denaturing conditions using glutathione affinity chromatography. The original vectors designed for GST fusion expression (Smith and Johnson 1988) contained: (i) the ampicillin-resistance gene, which provides a selectable marker, (ii) the IPTG-inducible *tac* promoter directly preceding the GST coding sequence minus its termination codon, (iii) a polylinker sequence, downstream of the GST, that includes a 5' protease-sensitive cleavage site, a multiple cloning region and a series of stop codons in the 3' end, and (iv) the *lacI*^{q} repressor gene, added to avoid basal transcription of potentially toxic fusion proteins. The expression vector employed in this study, pGEX-KG, (Guan and Dixon 1991) is a derivative of one of the original GST vectors (pGEX-2T, Smith and Johnson 1988). This plasmid was constructed to carry a series of five tandemly repeated glycine

residues, termed a glycine kinker, directly following the thrombin protease cleavage site in the polylinker. The addition of the glycine kinker to the fusion protein appears to facilitate more efficient thrombin cleavage possibly by altering protein structure and thereby creating a more accessible site for thrombin recognition .

Since its development, GST fusion proteins have been successfully used in a variety of applications. The high relative expression and the ease of purification by affinity chromatography has provided a simple means of obtaining pure protein preparations that can be employed as immunogens for antibody production. Mice, rabbits and sheep immunized with GST fusion proteins have all demonstrated good immunological responses to the foreign polypeptide portion of the fusion protein (Fikrig *et al.* 1990; Johnson *et al.* 1989; Toye *et al.* 1990). Additionally, purified GST fusion proteins have been used to study protein-protein binding interactions (Kaelin Jr. *et al.* 1991) as well as the biological activity attributed to the expressed foreign polypeptide. Enzymatic activity has been demonstrated with a variety of protease cleaved and uncleaved fusion polypeptides including rat protein tyrosine phosphatase (Frangioni and Neel 1993; Guan and Dixon 1991), bovine β-1,4-galactosyltransferase (Boeggeman *et al.* 1993) and human erythropoietin (Bill *et al.* 1995).

This chapter details the construction and expression of two *T. vaginalis* GST fusion protein clones, one containing the full-length Tv*nanA* coding sequence and the other a deletion mutant that lacks the N-terminal hydrophobic region of the polypeptide. The ability of each fusion protein to degrade sialic acid, therefore indicating Neu5Ac lyase activity, and to produce a cell detaching effect on eukaryotic cell monolayers is examined. Finally, in view of Western blot and enzyme assay results, the molecular mass and location of the *T. vaginalis*

Neu5Ac lyase is discussed.

RESULTS

(i) Construction and expression of *T. vaginalis* GST fusion proteins

To determine if the Tv*nanA* gene encoded a polypeptide with Neu5Ac lyase activity, the gene was expressed as a GST fusion protein. The complete coding region of the Tv*nanA* gene, contained within an *EcoR* I / *Hind* III fragment in the plasmid pGEM-TvLyase was isolated, purified and subcloned between the *EcoR* I and *Hind* III sites downstream of the GST and glycine kinker sequences in the pGEX-KG expression plasmid (Guan and Dixon 1991) (Figure 12). In accordance with the molecular mass of the GST protein (26 kDa) and the predicted mass of the *T. vaginalis* polypeptide (35 kDa), the size of the fusion protein expressed by this construct, designated pTvNeu5AcLy, was estimated to be 61 kDa. Bacteria transformed with pTvNeu5AcLy were grown and induced with 0.1 mM IPTG. Following a 2½ hr induction period, bacteria were pelleted, sonicated and the bacterial lysates were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. As demonstrated in Figure 13 (lanes 1 versus 2), a fusion protein of the correct size was apparent in bacterial sonicates only after IPTG induction. When sonicates from induced bacterial cultures were clarified by centrifugation, the majority of the GST fusion protein was found in the pellet as opposed to the supernatant (Figure 13, lanes 3 versus 4). This finding indicated that the fusion protein was insoluble under standard lysis conditions.

Many GST fusion proteins have been reported to be insoluble to varying degrees (Frangioni and Neel 1993), and several methods aimed at improving fusion protein solubility

Figure 12. Schematic of the plasmids used in the construction of the GST fusion protein clones. pGEM-TvLyase (pGEMTvLy) contains the complete coding region of the *T. vaginalis nanA* gene (open box) in an *EcoR* I / *Hind* III cassette. The GST expression plasmid pGEX-KG (Guan and Dixon 1991) consists of the *tac* promoter (arrow) directly upstream of the GST coding sequence (filled box). Replacing the GST stop codon is a polylinker sequence containing a thrombin cleavage site, the glycine kinker, a multiple cloning region and a series of three stop codons. The pGEM-TvLyase *nanA* coding cassette was subcloned between the *EcoR* I and *Hind* III sites present in the pGEX-KG polylinker to produce the pTvNeu5AcLy fusion construct. The deletion clone pTvΔaa24Neu5AcLy is identical to pTvNeu5AcLy except that the first 72 nt of the *nanA* coding sequence have been removed.

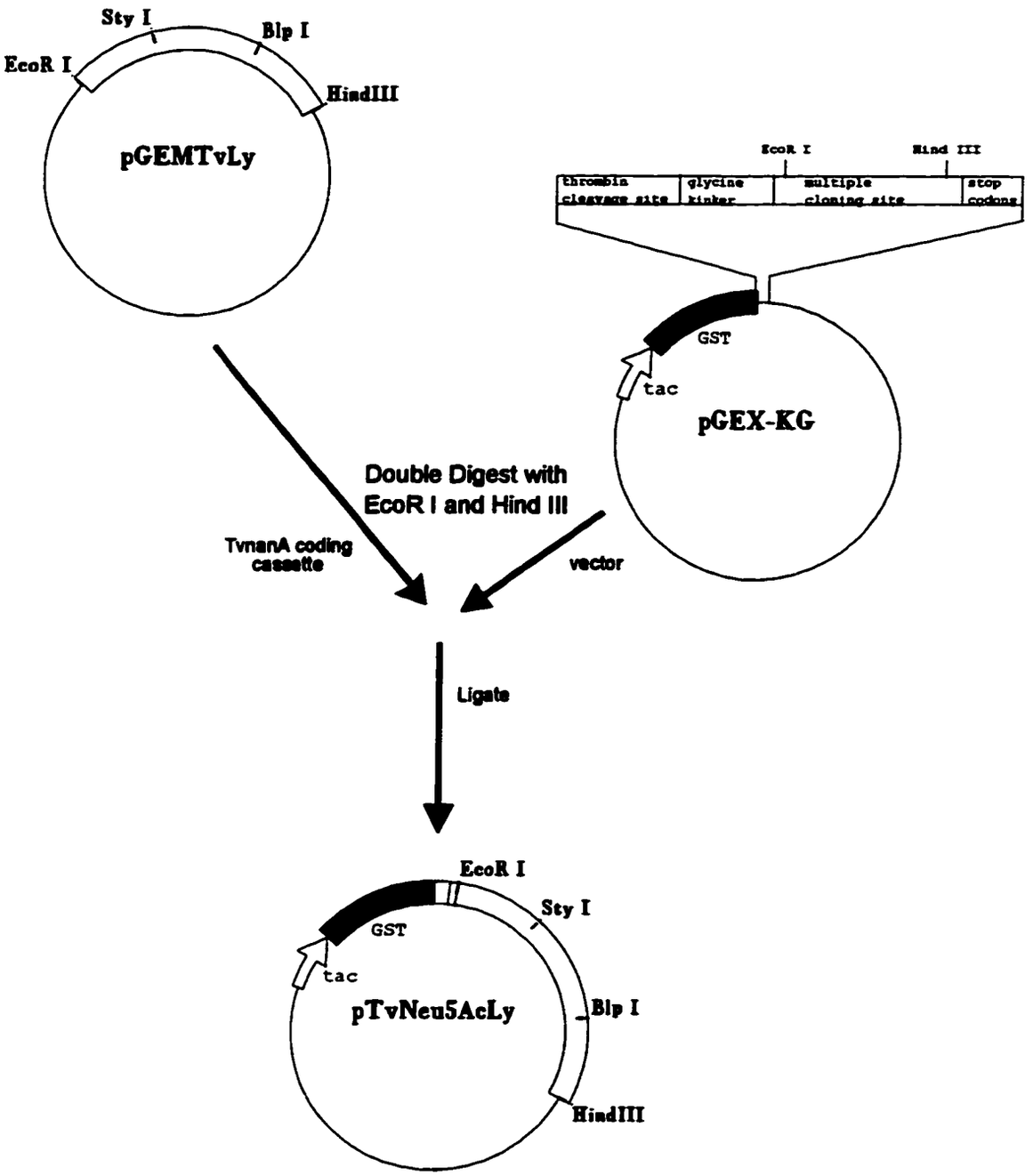
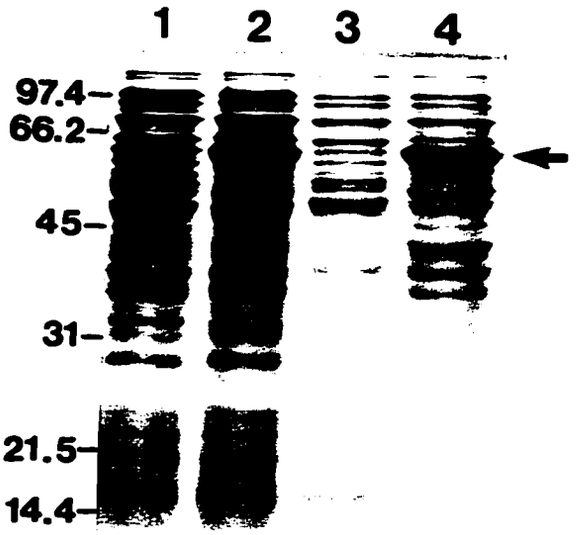

Sty I
Blp I
EcoR I
HindIII
pGEMTvLy
EcoR I
Hind III
thrombin cleavage site
glycine linker
multiple cloning site
stop codons
GST
tac
pGEX-KG
Double Digest with EcoR I and Hind III
TvnanA coding cassette
vector
Ligate
EcoR I
Sty I
GST
tac
pTvNeu5AcLy
Blp I
HindIII

Figure 13. Expression of the full-length pTvNeu5AcLy fusion protein. Bacteria transformed with the pTvNeu5AcLy fusion protein construct were grown and induced with IPTG. Pelleted bacterial cells were washed, sonicated and clarified by centrifugation. Samples were analyzed by SDS-PAGE on 11% polyacrylamide gels and gels were stained with Coomassie Brilliant Blue. Lane 1, sonicates of uninduced cells; lane 2, sonicates of induced cells; lane 3, clarified sonicate of induced cells; lane 4, resuspended pellet of induced cell sonicate. The arrow indicates the position of the GST fusion protein (~61 kDa). The positions of molecular weight markers (BioRad) are shown to the left of the figure.

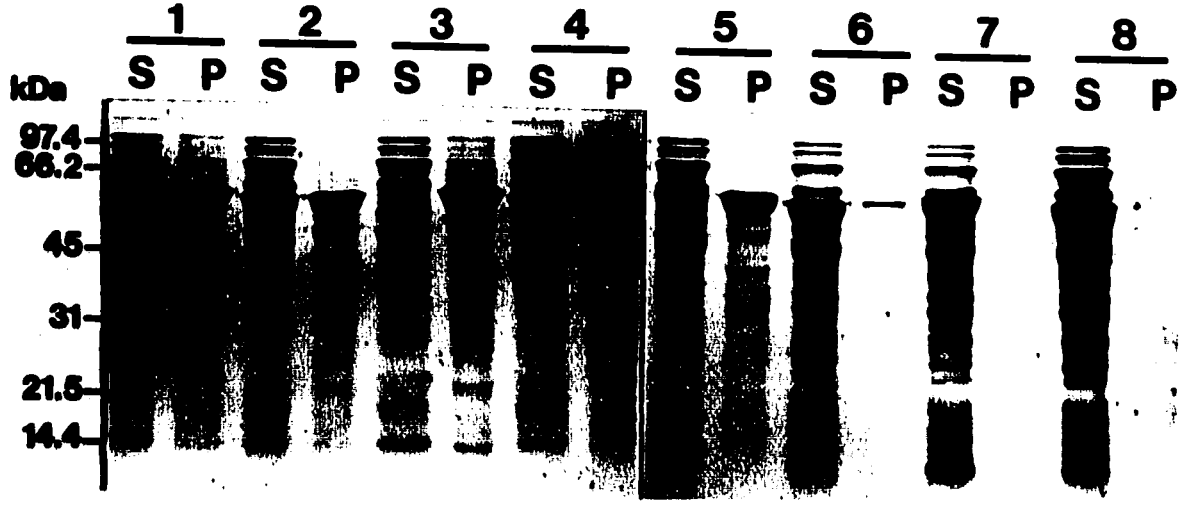

1
2
3
4
97.4
66.2
45
31
21.5
14.4

have been described. One approach to increasing solubility is by modification of the bacterial growth conditions namely: (i) altering the induction time, (ii) decreasing the concentration of IPTG used for induction and, (iii) decreasing the growth temperature during induction. Although all three growth parameters were modified and tested in several combinations, no significant increases in fusion protein solubility were attained. Two other approaches were subsequently taken to enhance fusion protein solubility. Since strongly hydrophobic or highly charged regions of proteins have been shown to have a deleterious effect on GST fusion protein solubility (Smith and Johnson 1988; Smith and Corcoran 1994), it was considered possible that the N-terminal hydrophobic portion of the *T. vaginalis* polypeptide affected the solubility of the fusion protein. A plasmid encoding a second GST fusion protein, lacking the hydrophobic region, was therefore constructed. Using pGEM-TvLyase as a template, the 5' region of the Tv*nanA* gene was amplified by PCR with a specific internal primer, corresponding to nucleotides 367-386 of the gene, and a primer designed to produce an in-frame *EcoR* I restriction site 72 nt downstream of the translational start site. The amplicon was digested with *EcoR* I and *Sty* I and the DNA fragment cloned between the corresponding sites in pGEM-TvLyase (Figure 12). This new *EcoR* I / *Hind* III cassette was then subcloned into the appropriate restriction sites in pGEX-KG. The size of the fusion protein expressed by this deletion construct, designated pTvΔaa24Neu5AcLy, was estimated to be 58.6 kDa.

The addition of detergent after bacterial cell lysis has also been shown to aid in fusion protein solubilization. Smith and Johnson (1988) demonstrated that addition of 1% of the non-ionic detergent Triton X-100 was efficient in solubilizing several GST fusion proteins expressing *Plasmodium falciparum* polypeptides. Likewise, Frangioni and Neel (1993) were

able to successfully solubilize GST fusions of protein tyrosine phosphatase 1B with the ionic detergent Sarkosyl while maintaining enzymatic activity. *T. vaginalis* GST fusion protein constructs were therefore evaluated for their solubility in the presence and absence of the forementioned detergents (Figure 14). Bacteria transformed with either pTvNeu5AcLy or pTvΔaa24Neu5AcLy were grown, induced with IPTG, and then sonicated in the presence of each detergent. Sonicates were clarified and the supernatants and pellets were analyzed by Coomassie Brilliant Blue staining following SDS-PAGE. A comparison of samples 1 and 2 (Figure 14) indicates the relative solubility of the two fusion proteins. While the vast majority of the full-length fusion protein was insoluble and found in the pellet, under standard lysis conditions (sample 1), the pTvΔaa24Neu5AcLy fusion protein (sample 2) appeared to be more soluble and was present in both supernatants and pellet fractions. Therefore the N-terminal hydrophobic region of the *T. vaginalis* polypeptide appeared to reduce the solubility of the *T. vaginalis* GST fusion protein. The non-ionic detergent Triton X-100 did not significantly enhance the solubility of either fusion protein construct (Figure 14, samples 3 and 4). The addition of the ionic detergent Sarkosyl markedly improved the solubility of both fusion proteins, however the optimal concentration for solubilization was dependent upon the construct. For the full-length pTvNeu5AcLy fusion protein, complete solubilization was achieved only with concentrations of 0.5% Sarkosyl or higher (Figure 14, sample 5 compared to 7). For the pTvΔaa24Neu5AcLy fusion protein, 0.1% Sarkosyl was sufficient for protein solubilization (Figure 14, sample 6 compared to 8).

Figure 14. *T. vaginalis* GST fusion protein solubility. IPTG-induced bacteria containing the *T. vaginalis* fusion protein constructs were sonicated in the presence of detergent and clarified by centrifugation. Protein samples were analyzed by SDS-PAGE on 11% polyacrylamide gels and gels were stained with Coomassie Brilliant Blue. Odd-numbered samples represent pTvNeu5AcLy fusion proteins while even-numbered samples are pTvΔaa24Neu5AcLy fusion proteins. Samples 1 and 2, no detergent; samples 3 and 4, 1% Triton X-100; samples 5 and 6, 0.1% Sarkosyl; samples 7 and 8, 0.5% Sarkosyl. S, supernatant; P, pellet. The positions of molecular weight markers (BioRad) are shown to the left of the figure.


1
2
3
4
5
6
7
8
S P S P S P S P S P S P S P S P
kDa
97.4
66.2
45
31
21.5
14.4

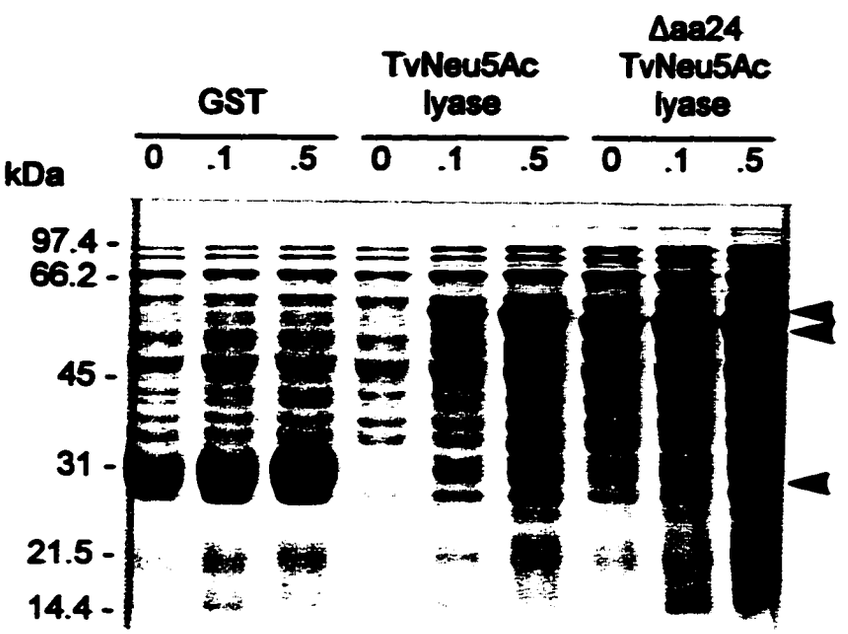
(ii) Neu5Ac lyase activity of the *T. vaginalis* GST fusion proteins

Once it was established that the *T. vaginalis* fusion proteins were expressed and could be solubilized by the addition of low concentrations of Sarkosyl, clones were tested to determine if the fusion proteins possessed Neu5Ac lyase enzymatic activity. IPTG-induced bacteria expressing either the GST protein or the GST fusion proteins were sonicated in the presence of Sarkosyl. Supernatants were then assayed for Neu5Ac lyase activity by incubation with free sialic acid and subsequently measuring the production of the enzymatic cleavage product N-acetyl-D-mannosamine using a modified Morgan-Elson colour reaction (Brunetti *et al.* 1962; Reissig *et al.* 1955). Protein samples were also analyzed by Coomassie Brilliant Blue staining after SDS-PAGE. While both GST and GST fusion proteins were soluble (Figure 15, panel A), only bacteria transformed with the fusion protein constructs demonstrated Neu5Ac lyase activity as evidenced by the production of the sugar N-acetyl-D-mannosamine (Figure 15, panel B). Bacteria transformed with the parental pGEX-KG plasmid, and which may have contained endogenous bacterial Neu5Ac lyase, were consistently found to possess minimal lyase activity. Interestingly, the pTvΔaa24Neu5AcLy fusion protein demonstrated the greatest Neu5Ac lyase activity. On average, this activity was found to be 5- to 6-fold higher than that of the wild-type fusion protein (pTvNeu5AcLy) regardless of the concentration of Sarkosyl used for protein solubilization.

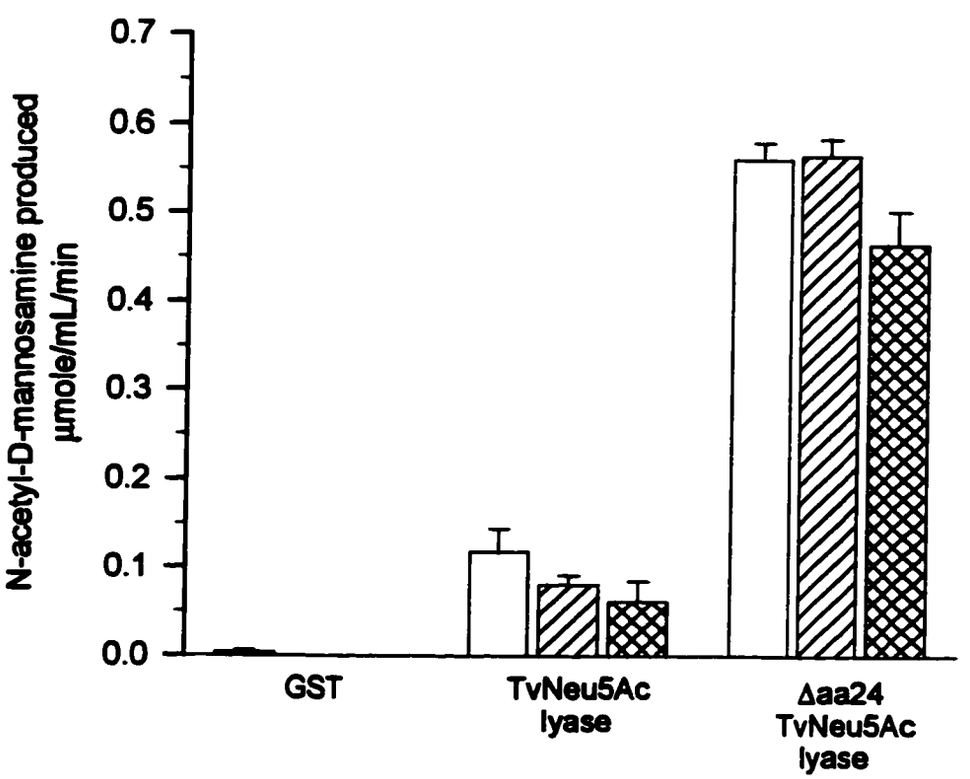
To further assess enzymatic activity, large scale fusion protein purifications were performed using the pTvNeu5AcLy and pTvΔaa24Neu5AcLy constructs. Bacteria grown in one litre cultures were induced with IPTG and then sonicated in the presence of Sarkosyl (0.5% Sarkosyl for pTvNeu5AcLy and 0.1% Sarkosyl for pTvΔaa24Neu5AcLy). Previous

Figure 15. Expression and Neu5Ac lyase activity of GST and *T. vaginalis* GST fusion proteins. Panel A. Supernatants containing either the GST protein (GST), the pTvNeu5AcLy fusion protein (TvNeu5Ac lyase), or the pTvΔaa24Neu5AcLy fusion protein (Δaa24 TvNeu5Ac lyase) were analyzed by SDS-PAGE on 11% polyacrylamide gels and gels were stained with Coomassie Brilliant Blue. The percentage of Sarkosyl used for protein solubilization is indicated above each lane. Arrows indicate the positions of the GST protein (26 kDa) and the fusion protein products (61 kDa and 58.6 kDa). The positions of molecular weight markers (BioRad) are shown to the left of the figure. Panel B. The same protein samples were assayed for Neu5Ac lyase activity by incubation with free sialic acid and subsequently measuring the formation of the enzymatic cleavage product N-acetyl-D-mannosamine using a colormetric reaction. Open bars indicate that no Sarkosyl was added to the lysis buffer; hatched bars, 0.1% Sarkosyl; cross-hatched bars, 0.5% Sarkosyl. Results are the average of four individual experiments with error bars indicating standard deviations.

A


GST
TvNeu5Ac lyase
Δaa24 TvNeu5Ac lyase
0 .1 .5 0 .1 .5 0 .1 .5
kDa
97.4
66.2
45
31
21.5
14.4

B


N-acetyl-D-mannosamine produced µmole/mL/min
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
GST
TvNeu5Ac lyase
Δaa24 TvNeu5Ac lyase

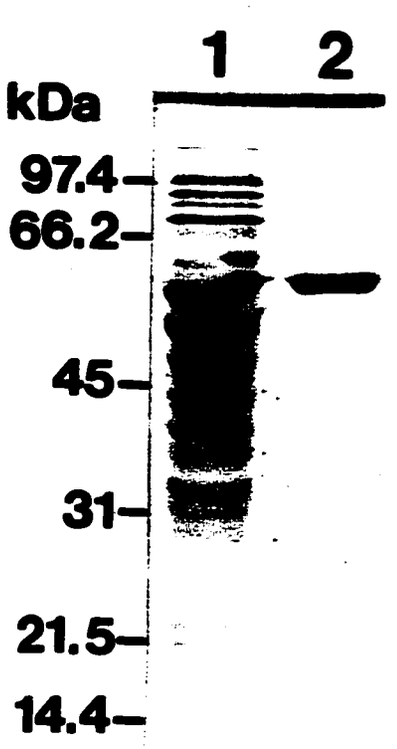
work by Frangioni and Neel (1993) indicated that while Sarkosyl enhanced fusion protein solubility, the presence of the detergent adversely affected binding of the fusion protein to glutathione beads. This group demonstrated that Sarkosyl could be sequestered by the addition of Triton X-100, and that inclusion of Triton X-100 in bacterial sonicates allowed for more efficient binding of fusion protein to beads. Following titration experiments, the optimal concentrations of Triton X-100 required for fusion protein binding were established to be 1% for pTvNeu5AcLy and 0.5% for pTvΔaa24Neu5AcLy. After detergent treatment, fusion proteins were bound to glutathione Sepharose 4B beads and eluted with reduced glutathione. Fusion protein samples were then dialyzed against PBS and concentrated. Despite several attempts using various conditions, thrombin cleavage of the purified GST fusion proteins proved inefficient. As enzymatic activity had already been established in crude bacterial sonicate supernatants, it did not appear that the addition of the GST protein to the amino terminus of the *T. vaginalis* polypeptide affected Neu5Ac lyase activity. After affinity purification, samples analyzed by SDS-PAGE and Coomassie Brilliant Blue staining identified only the GST fusion protein (Figure 16, panel A). Comparisons of the specific Neu5Ac lyase activities of bacterial cell sonicates to that of the purified fusion proteins indicated a 2-fold increase after purification of the pTvNeu5AcLy fusion protein, while the pTvΔaa24Neu5AcLy fusion protein showed a 9-fold increase in specific activity after purification (Figure 16, panel B).

(iii) CDF activity of *E. coli* Neu5Ac lyase and *T. vaginalis* GST fusion proteins

The original Tv*nanA* cDNA clone was identified with an anti-serum prepared against

Figure 16. Affinity purification and specific Neu5Ac lyase activities of *T. vaginalis* GST fusion proteins. Panel A. Supernatant containing the pTvΔaa24Neu5AcLy fusion protein (lane 1), and the fusion protein after glutathione Sepharose 4B affinity purification and concentration (lane 2) were analyzed by SDS-PAGE on 11% polyacrylamide gels and gels were stained with Coomassie Brilliant Blue. The positions of molecular weight markers (BioRad) are shown to the left of the figure. Panel B. A table presenting the specific Neu5Ac lyase activities of both the pTvNeu5AcLy and pTvΔaa24Neu5AcLy fusion proteins from bacterial sonicate supernatants and purified fusion protein preparations.

A



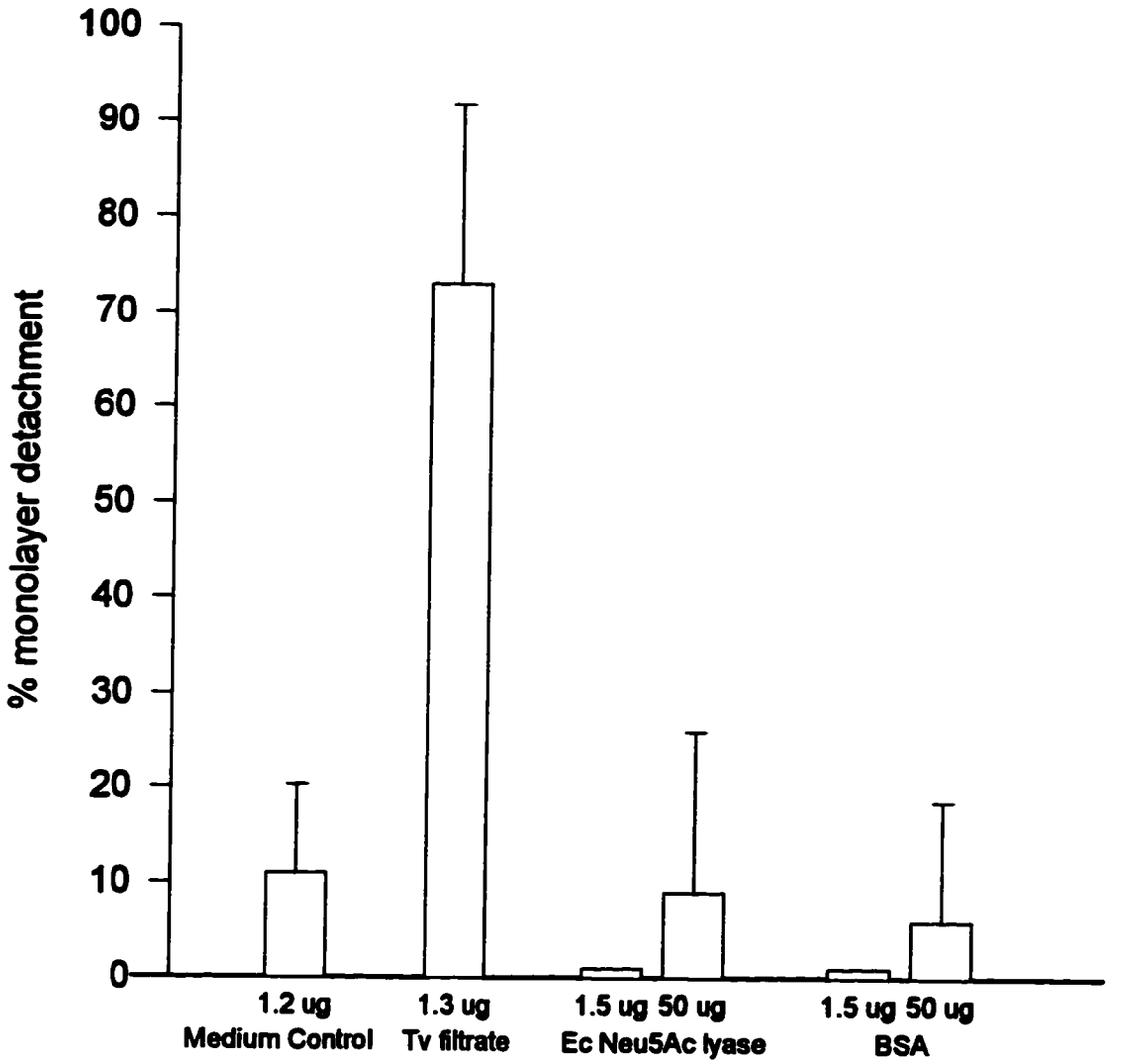
B

	Neu5Ac Lyase Specific Activity (U/mg)	
	pTvNeu5AcLy (average of 2 purifications)	pTv Δaa24Neu5AcLy (average of 3 purifications)
Bacterial Sonicate Supernatant	0.01 ± 0.005 (sd)	0.252 ± 0.03 (sd)
Purified GST Fusion Protein	0.023 ± 0.003 (sd)	2.25 ± 1.02 (sd)

a purified preparation of CDF. Therefore, the possibility existed that this *T. vaginalis* protein, while not the 218 kDa polypeptide identified as the major component of the CDF preparation, could possess bi-functional Neu5Ac lyase and cell detaching activities. The occurrence of bi-functional proteins is not without precedent. Both the glyceraldehyde-3-phosphate dehydrogenase of group A streptococci (Pancholi and Fischetti 1992, 1993) and the alcohol dehydrogenase 2 protein of *E. histolytica* (Arhets *et al.* 1995) appear to have dual functions and activities. To test for cell detaching activity, samples were either added to confluent monolayers of McCoy cells, or incubated with freshly seeded cells. Following 24 hour incubation, cells were washed and adherent monolayers were fixed with formaldehyde, stained with crystal violet and solubilized. By measuring the absorbance of solubilized samples, the percentage of monolayer detachment could be calculated from the formula: [1-(A_{595} sample/A_{595} control)] x 100%, where A_{595} control represents 100% confluence of monolayers incubated with normal growth medium.

Prior to the construction and expression of the GST fusion proteins, commercially available *E. coli* Neu5Ac lyase was examined to determine if the bacterial enzyme had a cytotoxic effect on cell monolayers. As evident in Figure 17, 1.5 μg (representing 0.035 Units of enzyme activity) or 50 μg (representing 1.15 Units of enzyme activity) of *E. coli* Neu5Ac lyase did not produce significant cell monolayer detachment. McCoy cell monolayers incubated with either the bacterial lyase, similar concentrations of an irrelevant protein (BSA) or with medium control filtrate exhibited less than 20% monolayer detachment. Notable cell detachment (>70%) was only apparent when *T. vaginalis* filtrates were examined.

Figure 17. CDF activity of *T. vaginalis* filtrate and commercially available *E. coli* Neu5Ac lyase. Confluent McCoy cell monolayers were incubated with the indicated samples in serum-free growth medium. After 24 hours medium was removed and adherent cells were fixed, stained and solubilized. The absorbance of solubilized samples was recorded and the percentage of monolayer detachment was calculated. Numbers below each bar represent the total protein concentration for each sample. Medium control, medium control filtrate; Tv filtrate, *T. vaginalis* cell-free filtrate; Ec Neu5Ac lyase, commercially available *E. coli* lyase (Sigma); BSA, bovine serum albumin. The results are the average of four independent analyses performed in duplicate with error bars indicating standard deviations. 1.5 μg of *E. coli* Neu5Ac lyase represents 0.035 Units of enzyme activity and 50 μg of *E. coli* Neu5Ac lyase represents 1.15 Units of enzyme activity.

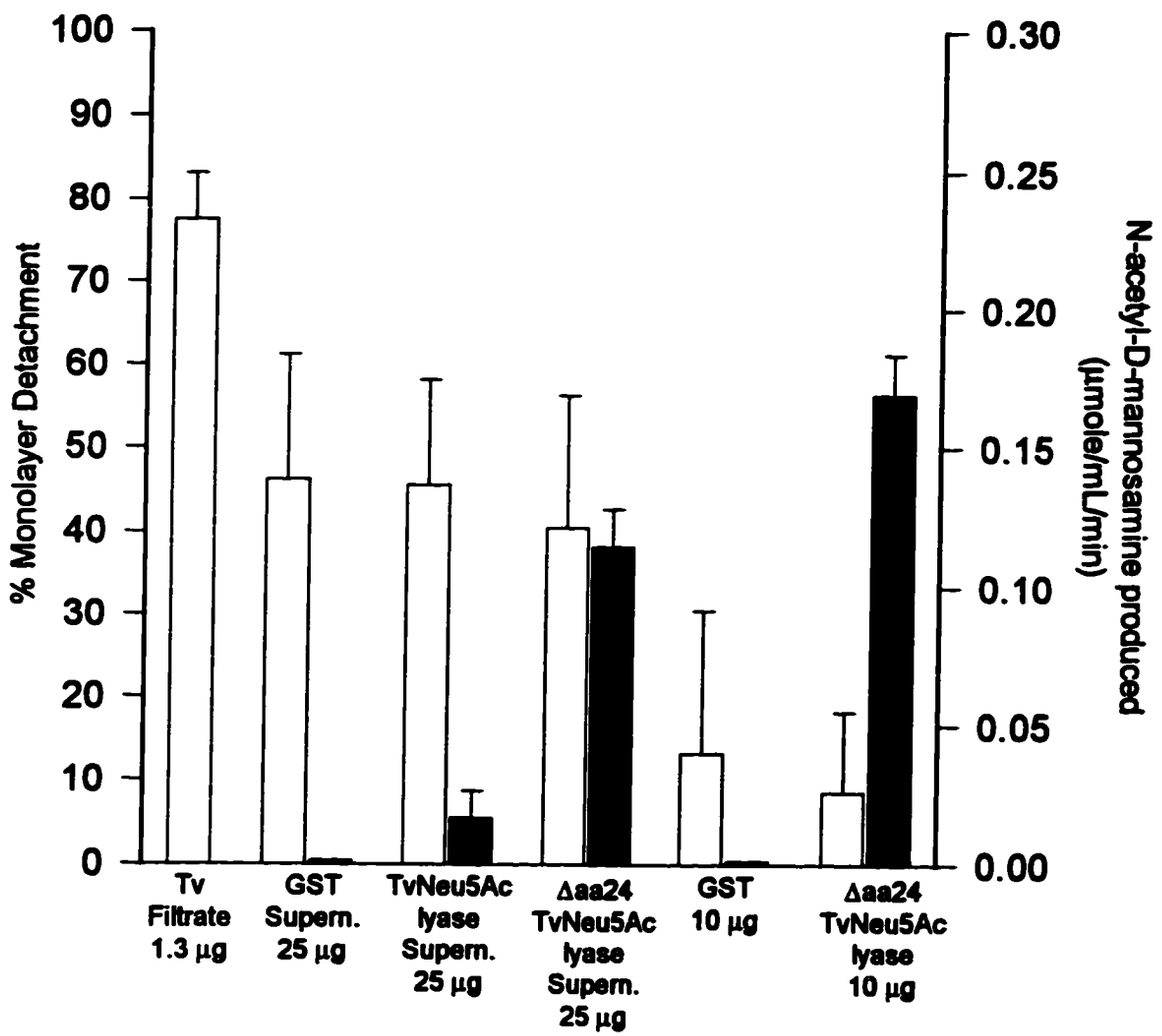

% monolayer detachment
100
90
80
70
60
50
40
30
20
10
0
1.2 ug
Medium Control
1.3 ug
Tv filtrate
1.5 ug
50 ug
Ec Neu5Ac lyase
1.5 ug
50 ug
BSA

Similar results were obtained when *T. vaginalis* GST fusion proteins were assayed for cell detaching activity (Figure 18, open bars). The addition of supernatant from sonicates of IPTG-induced bacteria (25 μg total protein) to freshly seeded McCoy cells resulted in less than 50% monolayer detachment. This effect appeared to be independent of GST fusion protein expression. The majority of the monolayer detachment produced by these samples may be attributed to the presence of other bacterial proteins included in the crude sonicate preparations. Affinity purified GST protein (10 μg) and pTvΔaa24Neu5AcLy fusion protein (10 μg) both exhibited less that 20% monolayer detachment. With approximately 7-fold less total protein (1.3 μg), *T. vaginalis* filtrate was able to produce over 70% monolayer detachment. Although the possibility existed that fusion proteins were inactive in cell detachment assays, samples simultaneously assayed for Neu5Ac lyase were found to be enzymatically active (Figure 18, filled bars).

(iv) Preliminary experiments to localize and size the *T. vaginalis* Neu5Ac lyase

To begin localization of the *T. vaginalis* Neu5Ac lyase, enzymatic assays were performed on *T. vaginalis* cells and culture filtrates. *T. vaginalis* filtrate that possessed CDF activity, was concentrated and assayed for Neu5Ac lyase activity. Despite employing up to 10 μg of filtrate protein, no lyase activity was detected in filtrate preparations. Since bacterial Neu5Ac lyase activity appears to be intracellular, enzyme assays were subsequently performed on sonicates of *T. vaginalis* grown in axenic culture. Approximately 3-4 x 10^8 parasites were sonicated and the clarified supernatant was utilized in protein determinations and in lyase enzyme assays. Neu5Ac lyase activity was detected only when substantial amounts of total

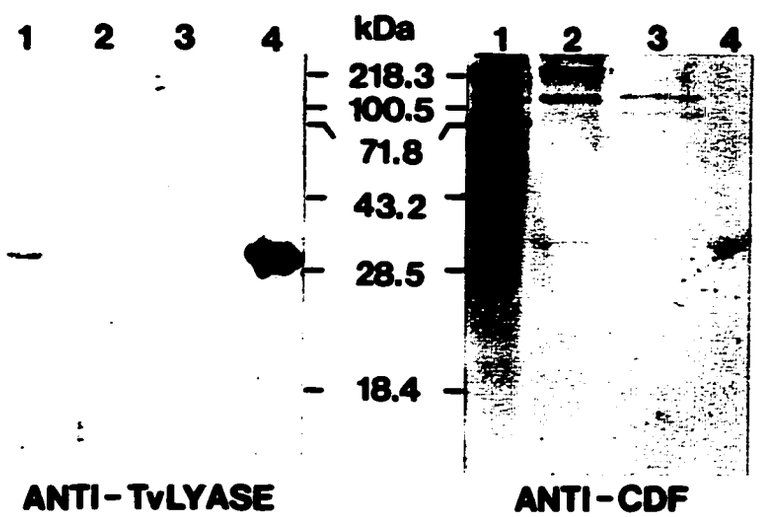
Figure 18. CDF and Neu5Ac lyase activities of fusion proteins. Samples were incubated with freshly seeded McCoy cells in serum-free growth medium. Following a 24 hour incubation, medium was removed and adherent cells were fixed, stained and solubilized. Absorbance of solubilized samples was recorded and the percentage of monolayer detachment was calculated for each sample (open bars). The same samples were simultaneously assayed for Neu5Ac lyase activity by measuring the production of the enzymatic cleavage product N-acetyl-D-mannosamine following incubation with free sialic acid (filled bars). Tv filtrate, *T. vaginalis* cell-free filtrate; GST Supern., 25 μg of protein from the supernatant of a bacterial sonicate containing the GST protein; TvNeu5Ac lyase Supern., 25 μg of protein from the supernatant of a bacterial sonicate containing the pTvNeu5AcLy fusion protein; Δaa24 TvNeu5Ac lyase Supern., 25 μg of protein from the supernatant of a bacterial sonicate containing the pTvΔaa24Neu5AcLy fusion protein; GST, 10 μg of affinity purified GST protein; Δaa24 TvNeu5Ac lyase, 10 μg of affinity purified pTvΔaa24Neu5AcLy fusion protein. The results are the average of four independent experiments with error bars indicating standard deviations.


% Monolayer Detachment
N-acetyl-D-mannosamine produced (µmole/mL/min)
100
90
80
70
60
50
40
30
20
10
0
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Tv Filtrate 1.3 µg
GST Supern. 25 µg
TvNeu5Ac lyase Supern. 25 µg
Δaa24 TvNeu5Ac lyase Supern. 25 µg
GST 10 µg
Δaa24 TvNeu5Ac lyase 10 µg

parasite protein were assayed, with 500 μg of *T. vaginalis* protein generating just 1.08 nmole/mL/min ± 0.0008 (sd) of N-acetyl-D-mannosamine. Furthermore, the Neu5Ac lyase specific activity of undiluted *T. vaginalis* sonicate supernatant was determined to be only 0.0013 Units/mg ± 0.00013 (sd).

The availability of *T. vaginalis* Neu5Ac lyase fusion proteins also provided the opportunity to produce anti-serum with specificity for the parasitic lyase. Using affinity purified pTvΔaa24Neu5AcLy protein as an immunogen, anti-serum was raised in rabbits and subsequently employed in Western blot analysis. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes and probed with a 1:50 dilution of either CDF anti-serum or *T. vaginalis* Neu5Ac lyase anti-serum that had been adsorbed with the GST protein prior to use (Figure 19). As previously demonstrated, the CDF anti-serum identified several proteins in *T. vaginalis* lysates and filtrates. In addition to two predominant proteins of approximately 30 and 70 kDa, various high molecular weight protein species were recognized in *T. vaginalis* lysates. In cell-free filtrates, the 218 kDa "CDF" protein was identified amongst several high molecular weight proteins as was a 35 kDa protein species. The CDF anti-serum was also examined for its ability to react with *E. coli* Neu5Ac lyase (Figure 19, ANTI-CDF lane 4) and was found to react weakly with the bacterial enzyme. The same protein samples were used to establish the specificity of the Neu5Ac lyase anti-serum (Figure 19, ANTI-TvLYASE). In parasite lysates probed with the anti-serum, a predominant protein species of approximately 35 kDa was identified along with a series of higher molecular weight species between 45-70 kDa. Only two protein species were found to be reactive in cell-free filtrates, one of 38-40 kDa and another of approximately 100 kDa. The 218 kDa "CDF"

Figure 19. Characterization of the *T. vaginalis* Neu5Ac lyase by Western blot analysis. *T. vaginalis* proteins were separated by SDS-PAGE on 11% polyacrylamide gels and proteins were transferred to nitrocellulose. Membranes were incubated with a 1:50 dilution of either CDF anti-serum (ANTI-CDF) or *T. vaginalis* Neu5Ac lyase anti-serum (ANTI-TvLYASE) which had been adsorbed with GST protein prior to being used. Immunologically reactive proteins were detected with an alkaline phosphatase-conjugated secondary antibody and NBT/BCIP. Lane 1, *T. vaginalis* cell lysate (30 μg total protein); lane 2, *T. vaginalis* cell-free filtrate (5 μg total protein); lane 3, medium control filtrate (5 μg total protein); lane 4, *E. coli* Neu5Ac lyase (1 μg total protein). The positions of high range prestained protein molecular weight standards (Gibco/BRL) are shown between the two blots.


1
2
3
4
kDa
218.3
100.5
71.8
43.2
28.5
18.4
1
2
3
4
ANTI-TvLYASE
ANTI-CDF

protein present in *T. vaginalis* filtrates was not recognized by the Neu5Ac lyase anti-serum, however this serum did react strongly with *E. coli* lyase.

DISCUSSION

To verify that the polypeptide encoded by the *T. vaginalis nanA* gene possessed Neu5Ac lyase activity, the *nanA* gene was used to express GST fusion proteins. The pTvNeu5AcLy construct, containing the full-length Tv*nanA* coding region, generated a fusion protein that was insoluble under standard lysis conditions and required the addition of 0.5% Sarkosyl to promote efficient solubilization. The ionic detergent Sarkosyl has been considered to be an inhibitor of enzymatic activity, however Frangioni and Neel (1993) demonstrated that tyrosine phosphatase-GST fusion proteins solubilized with 1.5% Sarkosyl were enzymatically active. As the effects of Sarkosyl on other classes of enzymes (ie. lyases) was unknown, a second approach to enhance fusion protein solubility was also examined. The insolubility of many GST fusion proteins has been associated with the presence of either highly charged or strongly hydrophobic regions and the elimination of these regions has greatly improved fusion protein solubility (Smith and Johnson 1988). The *T. vaginalis nanA* encoded polypeptide includes a 24 amino acid N-terminal hydrophobic stretch that appears to be unique to the parasite lyase. Therefore, a second GST fusion construct was produced that expressed a mutant fusion protein lacking the N-terminal hydrophobic region. As expected, the solubility of this altered polypeptide was greater than that of the full-length fusion protein and required the presence of only 0.1% Sarkosyl for maximal solubilization.

Bacterial cell sonicates containing either of these fusion polypeptides reproducibly

demonstrated Neu5Ac lyase activity. In the absence of detergents, sonicates of cells expressing the *T. vaginalis* full-length fusion gene product exhibited Neu5Ac lyase activity that was 30-fold higher than the activity in control cells carrying the parental plasmid pGEX-KG. These results established that the *T. vaginalis nanA* gene encoded a functional Neu5Ac lyase. Interestingly, the deletion of the N-terminal hydrophobic region of the *T. vaginalis* lyase appeared to augment lyase activity. The fusion protein with the N-terminal deletion exhibited a 5-fold increase in lyase activity over that of the wild-type fusion protein. While this increased activity may be simply a reflection of increased solubility and/or reduction in protein aggregation, it does bring into question the role of the N-terminal hydrophobic region in the *T. vaginalis* protein.

Although it has not been established if the N-terminal hydrophobic sequence is in fact present in the mature *T. vaginalis* protein, its absence in bacterial lyases, and its detrimental effect on the activity of the GST fusion protein would suggest that the sequence is not included in the active *in vivo* protein. There are two potential explanations for how or why the N-terminal region of the *T. vaginalis* Neu5Ac lyase would be removed. First, the hydrophobic region could be a parasitic targeting sequence that functions in protein localization. While analysis by both the methods of McGeoch (1985) and von Heijne (1986) indicated that the N-terminal region is not a typical eukaryotic secretory signal , this does not exclude the possibility that *T. vaginalis* employs different sequences to target proteins to cellular compartments. *T. vaginalis* targeting sequences have been defined only for the hydrogenosomal proteins. Similar to mitochondrial proteins and their leader sequences, *T. vaginalis* hydrogenosomal proteins possess short pre-sequences of 6-9 amino acids, which

are cleaved from the mature protein (Hrdy and Müller 1995b; Johnson *et al.* 1990; Lahti *et al.* 1992; Länge *et al.* 1994). Typically, the hydrogenosomal pre-sequences include Met-Leu or Met-Leu-Ser at the N-terminus of the sequence and an arginine residue at -2 relative to the cleavage site. The pre-sequence also forms an amphiphatic α-helix that is believed to facilitate translocation into the hydrogenosome after the protein is synthesized on free polyribosomes (Lahti and Johnson 1991). Interestingly, the sequences of several *T. vaginalis* cysteine proteinases appear to have neither a typical eukaryotic secretory signal nor a hydrogenosome pre-sequence despite the fact that the enzymes appear to be targeted to lysosomes (Mallinson *et al.* 1994). The *T. vaginalis* AP65 adhesin protein, which is located on the surface of the organism, is also synthesized without a typical eukaryotic secretory signal. Instead, this protein contains a hydrogenosome targeting pre-sequence that appears to be cleaved from the mature surface-associated protein. Alderete *et al.* (1995a) speculate that the pre-sequences of adhesin proteins may allow translocation into vesicles that are subsequently exported to the cell surface. While the N-terminal portion of the *T. vaginalis* Neu5Ac lyase, from amino acids 4-17, is predicted to contain an α-helix (Frishman and Argos 1995), this region does not include residues typical of the hydrogenosome pre-sequence.

An alternative explanation for the role of the N-terminal hydrophobic region of the *T. vaginalis* Neu5Ac lyase would be if the enzyme exists as a zymogen. The possibility that N-terminal cleavage would be required to produce a mature, active enzyme is consistent with the observation that activity of the Neu5Ac lyase is increased when the N-terminal portion of the polypeptide is deleted. For zymogens with substrate binding pockets such as chymotrypsinogen/chymotrypsin, the active binding pocket is not properly formed in the

precursor protein and requires proteolytic cleavage for the active residues to realign (Price and Stevens 1989). If the *T. vaginalis* Neu5Ac lyase consists of α/β barrel domains, similar to its bacterial homolog (Izard *et al.* 1994), the presence of the N-terminal region with its α-helical structure could interfere with either enzyme folding or with substrate binding in the active pocket. Since as many as 23 distinct protease activities have been identified in *T. vaginalis* isolates (Neale and Alderete 1990), it is possible that one of these proteases activates the parasite Neu5Ac lyase.

Since the Tv*nanA* cDNA clone was initially identified with anti-serum prepared against a purified preparation of *T. vaginalis* filtrate with CDF activity, and because the localization of the lyase was undefined in the parasite, the possibility existed that the *T. vaginalis* Neu5Ac lyase could possess CDF activity. Metabolic enzymes have been identified on the surface of a variety of microorganisms. In bacteria, there are numerous examples of this phenomenon. The α-hemolytic *Streptococcus gordonii* possesses a surface bound glucosyltransferase that demonstrates binding to human umbilical vein endothelial cells in addition to having enzymatic activity (Vacca-Smith *et al.* 1994). Likewise in group A streptococci, a major surface protein has been identified as an enzymatically active glyceraldehyde-3-phosphate dehydrogenase. This surface enzyme appears multi-functional as it exhibits both dehydrogenase and ADP-ribosylating activity as well as possessing the ability to bind to eukaryotic proteins such as fibronectin, actin and myosin (Pancholi and Fischetti 1992, 1993). There are also reports of a *Porphyromonas gingivalis* surface-associated glutamate dehydrogenase (Joe *et al.* 1994) and a *Streptococcus pneumoniae* surface active neuraminidase (Cámara *et al.* 1994).

Similarly, metabolic enzymes have been found on the surface of several parasites. The major larval surface protein of *Schistosoma mansoni* has been characterized by sequence analysis as a glyceraldehyde-3-phosphate dehydrogenase with approximately 73% amino acid similarity to its human homolog (Goudot-Crozel *et al.* 1989). Whether this protein exhibits enzymatic activity or any of the binding properties associated with the group A streptococcal surface glyceraldehyde-3-phosphate dehydrogenase remains unknown. Recently, Arhets *et al.* (1995) identified an alcohol/acetaldehyde dehydrogenase on the surface of *E. histolytica*. This enzyme is capable of binding to extracellular matrix proteins and possesses iron-independent alcohol dehydrogenase activity (Yang *et al.* 1994a). The AP65 adhesin protein of *T. vaginalis* may also be a bi-functional protein as it exhibits both ligand-binding ability and significant protein sequence similarity to malic enzyme (Alderete *et al.* 1995a). When purified *T. vaginalis* GST fusion proteins and commercially purchased *E. coli* Neu5Ac lyase were tested in cell cytotoxicity assays less than 20% monolayer detachment was demonstrated, establishing that neither the bacterial nor the parasitic lyases had CDF activity. It therefore appears that the predominant and quite possibly the sole function of the *T. vaginalis* protein is the degradation of free sialic acid.

The lack of significant Neu5Ac lyase activity in *T. vaginalis* cell-free filtrates and the activity associated with parasite sonicates suggest that the *T. vaginalis* lyase is intracellular, and coincides with the location of the enzyme in bacteria (Uchida *et al.* 1985). The lyase activity observed in *T. vaginalis* sonicates was extremely low, and required large amounts of parasite protein to be detected. This low level of *T. vaginalis* lyase activity may be explained by analogy with the bacterial Neu5Ac lyase. Uchida *et al.* (1985) have demonstrated that

Neu5Ac lyase activity is detected in bacterial supernatants only when organisms are grown in medium containing N-acetylneuraminic acid as the sole carbon source. Likewise, Vimr and Troy (1985a) showed that induction of the *E. coli nanA* gene required the presence of sialic acid. This induction, of up to 1000-fold over basal levels in *E. coli*, was repressed by glucose (Martinez *et al.* 1995; Vimr and Troy 1985a). Similarly, the *T. vaginalis nanA* gene may require the presence of sialic acid for induction. Furthermore, it may be envisioned that the *T. vaginalis nanA* gene is repressed when parasites are cultivated in growth medium that contains glucose as a primary source of carbon. This situation would account for the low levels of endogenous enzyme activity in the parasite.

Finally, anti-serum raised against Neu5Ac lyase GST fusion protein detected a protein of approximately 35 kDa in *T. vaginalis* lysates. Since the size of the *T. vaginalis* Neu5Ac lyase is predicted to be 35 kDa, barring glycosylation, and because the anti-serum also reacts with the *E. coli* Neu5Ac lyase, the data suggest that the 35 kDa *T. vaginalis* protein is the lyase. The relatively weak signal in Western blots and the large amounts of total protein (30 μg) used in the analysis is also consistent with the possibility that the parasite Neu5Ac lyase is repressed under normal growth conditions. Western blot analysis, using the lyase anti-serum, also identified a protein in *T. vaginalis* filtrates that is slightly larger than the 35 kDa protein recognized in parasite lysates. Whether this secreted protein species represents a glycosylated or an uncleaved form of the *T. vaginalis* Neu5Ac lyase remains unclear at this time.

Comparing the results of Western blots probed with anti-serum prepared against either the Neu5Ac lyase-GST fusion protein or CDF does not help to explain why the initial λgt11

Tv*nanA* cDNA clone was identified. The CDF anti-serum shows very weak reactivity with the *E. coli* Neu5Ac lyase at a relatively low dilution of anti-serum (1:50) compared to that employed during library screening (1:6000). A 30 kDa protein is detected in *T. vaginalis* lysates with the anti-CDF serum, however it appears to be slightly smaller than both the predicted Neu5Ac lyase and the intracellular protein species recognized by the lyase anti-serum. Furthermore, a protein of approximately 35 kDa is weakly recognized in *T. vaginalis* cell-free filtrates. Unfortunately, the significance of these observations remains unclear, in lieu of the multiple specificities possessed by the CDF anti-serum.

CHAPTER 6: CONCLUSIONS

The original objective of this study was to characterize the *T. vaginalis* CDF gene by analyzing cDNA sequences isolated from a λgt11 library. Using anti-serum prepared against a purified preparation of *T. vaginalis* CDF, two distinct cDNA clones were identified. By Northern blot analysis, the *T. vaginalis* cDNA sequences in the strongly reactive CDF-1 (Tv*nanA*) clone did not appear to be associated with a transcript capable of encoding the >200 kDa CDF protein. Additionally, the longest ORF identified in the CDF-1/Tv*nanA* sequence showed significant protein sequence similarity only to the Neu5Ac lyase of *E. coli*. CDF-2, the immunologically weaker of the two clones, contained a 3060 bp ORF that was considered to be incomplete due to the absence of translational start and stop codons. Given the length of the cDNA, the lack of a start codon was not surprising, however the absence of a termination codon was unexpected. This cDNA appears to have been synthesized by oligo-dT priming on a tract of adenosine residues encoding a lysine-rich stretch of protein rather than from the polyadenlyate tail of the mRNA. Although the size of the incomplete ORF suggests that it could encode a large protein (>100 kDa), both nucleotide and protein sequence analysis failed to indicate any similarity to known cytotoxins or to cysteine proteases capable of producing a cell-detaching effect. The inability to detect a transcript associated with this cDNA further compounded the difficulty in establishing a relationship between the CDF-2 clone and the CDF protein.

The fact that two distinct clones were identified during primary screening of the library suggested that the CDF anti-serum was not as specific as previously thought. Further

characterization of the CDF anti-serum indicated that the serum reacts with several *T. vaginalis* proteins other than the high molecular weight polypeptide believed to be CDF. While the multiple specificities of the CDF anti-serum likely contributed to the identification of two distinct cDNA clones, other explanations exist for selecting clones that may not have been "CDF" specific. The unique presentation of phage fusion protein epitopes could possibly account for the identification of unrelated clones. Likewise, protein reactivities may have differed depending upon the immunological screening technique used. While the anti-serum was able to detect CDF in Western blots and immunoprecipitations, both procedures used detergents to facilitate protein solubilization and denaturation. Detergents were not present during immunological screening of the λgt11 library and this may have accounted for differences in anti-serum reactivity to native phage fusion proteins. Several different approaches could be used to obtain a CDF clone. A panel of monoclonal antibodies (MAbs) could be generated against the purified CDF protein and then used to screen the expression library. This approach generally requires larger numbers of recombinant phage to be screened however, the availability of MAbs reactive to various regions of the protein would allow simple validation of presumptive clones. A second approach to identify a CDF clone would be by N-terminal sequencing of purified CDF. The protein sequence could then be used to design a series of degenerative oligonucleotide primers for library screening. Finally, because of the similarity in biological activity between CDF and the *E. histolytica* histolysin, it can be envisioned that the two proteins also share sequence similarity in functional domains. If this assumption is correct, the recently identified sequence of histolysin (Reed *et al.* 1993) could be used to generate conserved oligonucleotide primers that could be employed in PCR

amplification or *T. vaginalis* library screening. While application of any of the forementioned approaches could have yielded a CDF clone, it was deemed more prudent at the time to continue the characterization of the CDF-1/Tv*nanA* clone.

Utilizing cloned cDNA and genomic DNA sequences, the *T. vaginalis nanA* gene was shown to be a single copy gene that lacks introns. Transcribed as a 1.1 kb mRNA, the Tv*nanA* gene contains both short 5' (17-18 nt) and 3' (28 nt) UTRs with a eukaryotic polyadenylation signal in the 3' non-coding region and an Inr-like element straddling the transcriptional initiation site. Although 8 of the 13 residues identified in the *T. vaginalis* consensus Inr motif were conserved in the Tv*nanA* Inr-like element, the sequence was not capable of initiating accurate transcription in *in vitro* assays. The estimated 35 kDa polypeptide encoded by the longest ORF (954 bp) of the Tv*nanA* gene shows striking similarity to the bacterial Neu5Ac lyases of *E. coli* and *H. influenzae*. Optimal alignment of these protein sequences required minimal gaps and showed conservation of the residues predicted to line the active pocket of the enzyme among all three molecules.

After molecular characterization of the Tv*nanA* gene, the final objective of the project was to demonstrate whether the *T. vaginalis* encoded polypeptide possessed Neu5Ac lyase activity. Expressing the wild-type protein and a version of the polypeptide with an N-terminal deletion as GST fusions, it was shown that the *T. vaginalis* protein products were enzymatically active. These molecules did not appear to be bi-functional however, as neither fusion protein had demonstrable CDF activity. Preliminary experiments to localize and size the *T. vaginalis* Neu5Ac lyase suggested that the enzyme is an intracellular protein of approximately 35 kDa.

Although these data clearly establish that *T. vaginalis* contains a Neu5Ac lyase, questions remain concerning the nature of the gene, its gene product and the role of the enzyme in the organism. The low levels of lyase activity detected in parasites grown in glucose-containing medium suggests that the Tv*nanA* gene, like its bacterial homolog, is regulated or induced by free sialic acid. Modification of *T. vaginalis* growth medium and the use of sialic acid as an alternative carbon source may provide the conditions necessary to confirm that *T. vaginalis* Neu5Ac lyase is inducible. If Tv*nanA* gene expression is regulated, a unique opportunity to examine cis-acting regulatory elements in *T. vaginalis* would be available, once a transfection system can be developed. A second interesting aspect of the *T. vaginalis* Neu5Ac lyase is the function of the N-terminal region of the protein. Localization of the parasite lyase could indicate whether or not this hydrophobic sequence is used as a protein targeting signal. Immunofluorescence using Neu5Ac lyase fusion protein anti-serum was attempted, however the protein could not be detected. The failure to localize the *T. vaginalis* Neu5Ac lyase may be due in part to low levels of protein expression caused by sub-optimal induction conditions. To establish if the N-terminal region is present in the mature form of the *T. vaginalis* lyase, the size of *in vitro* transcription / translation gene products can be compared to the size of radiolabelled parasite proteins immunoprecipitated with the lyase anti-serum. Immunoprecipitation may also permit N-terminal sequencing of the parasite lyase protein to determine if and where N-terminal proteolytic cleavage could be occurring.

Although little is known about sialic acid metabolism in *T. vaginalis*, the newly identified Neu5Ac lyase may be considered to have two potential functions: (i) nutrient

acquisition and/or, (ii) sialic acid synthesis and regulation. The site of *T. vaginalis* infection, the human vagina, is a dynamic environment in constant fluctuation due to the menstrual cycle. In order to survive the constant changes in its environment, *T. vaginalis* may be capable of utilizing a wide variety of alternative energy sources depending upon their availability during the hormonal cycle. Similar to the situation apparent in the gastrointestinal tract, vaginal epithelium is coated with a mucus gel layer primarily composed of glycoproteins termed mucins (Corfield *et al.* 1992; Gipson *et al.* 1995). Sialomucins, a specific sub-type of mucin that contain sialic acid as the terminal saccharide residue, have been shown to be the most abundant glycoprotein in human colonic mucus and are considered to be a potential energy source for mucin-degrading enteric bacteria (Corfield *et al.* 1988, 1992). Although vaginal mucins are not as well characterized, the presence of sialomucins in vaginal mucus can be envisioned as a potential energy source for *T. vaginalis.* In this situation, the neuraminidase secreted by the organism (Costa e Silva Filho *et al.* 1989) would cleave sialic acid residues from the mucin glycoproteins and the resulting free sialic acid would be transported into the parasite, most likely by a permease similar to that identified in *E. coli* (Martinez *et al.* 1995). The intracellular free sialic acid would then be degraded by Neu5Ac lyase to produce usable carbon and energy sources.

The identification of sialic acid moieties on the surface of *T. vaginalis* (Dias Filho *et al.* 1992) also suggests a second possible function for the parasite Neu5Ac lyase. Sialic acid residues present on *T. vaginalis* glycoconjugates could be synthesized by the condensation reaction catalyzed by Neu5Ac lyase. This possibility is not without precedent. Sialic acid residues present in the capsules of *E. coli* K1 strains are synthesized by a similar mechanism

involving Neu5Ac lyase (Rodríguez-Aparicio *et al.* 1995). Alternatively, other enzymes could also account for the presence of sialic acid residues on the parasite surface. N-acetylneuraminic acid synthase is used to produce the sialic acid residues present in the capsules of *N. meningitidis* groups B and C, while the trans-sialidases possessed by trypanosomes can cleave host cell sialic acid residues and transfer the saccharides to the parasite surface. The possibility that *T. vaginalis* utilizes Neu5Ac lyase to synthesize endogenous sialic acid therefore requires further study. Considering the importance of sialic acids in microbial pathogenesis, the identification of the *T. vaginalis* Neu5Ac lyase may provide impetus for the identification and characterize of other enzymes involved in the sialobiology of this parasite.

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APPENDIX I

OLIGONUCLEOTIDE SEQUENCES

Sequencing Primers:

T7	5' TAA TAC GAC TCA CTA TAG GG 3'
SP6	5' GAT TTA GGT GAC ACT ATA G 3'
KM1-1	5' TCA ATG AAG ATG GTA CAA TC 3'
KM1-2	5' GCT ACA ATT GCT TAT CCG CT 3'
KM1-3B	5' TTA GAG CGT GTC AAG AGA GCT 3'
KM1-4	5' ATC ATC GGT GTC AAG TTC AC 3'
KM1-5	5' GCG CTG GCA TCC TCT CCA AT 3'
CDF-1R1	5' TTT TCC TTT TTA ACG TAA AT 3'
CDF-1R2	5' TGG AGA GGA TGC CAG CGA TT 3'
CDF-1R3	5' AAC TTG ACA CCG ATG ATC TT 3'
CDF-1R4	5' GAT AAG CAA TTG TAG CCT AA 3'
CDF-1R5	5' CTA TGT TGT AGC GAA CGA TT 3'
GenCDF1	5' TGC CAG ATT GTA TCT ATA AT 3'
GenCDF2	5' CAT CCA TGA TTG CTC TGT AT 3'
GenCDF3	5' GCA CCA TAT CAA GTA AAA CT 3'

Primer Extension Analysis

TVPE 5' TTT AGC ACT CTT TCC CTT TGG TCC GGT AGT 3'

PCR Primers for GST Fusion Protein Constructs

TvLyase-1 5' AAG ATC GGA ATT CTC ATG TTC GTG TTC CTC 3'

TvLyase-2 5' ACT GTC AAG CTT ATA AAA GAT ATT T 3'

TvLyaseΔsig 5' AGA TCG GAA TTC TGA GTG CTA AAA GTT TAA 3'

Inr Construct Oligonucleotides

TvLyInrA 5' GAT CCA AAA TAA TTA TGT TTA CCA GCT 3'

TvLyInrB 5' GGT AAA CAT AAT TAT TTT G 3'

R2549A 5' GAT CCG TAG AAA AGG TGG GGG CCA GCT 3'

R2549B 5' GGC CCC CAC CTT TTC TAC G 3'

*EcoR*I Adaptors (Amersham)

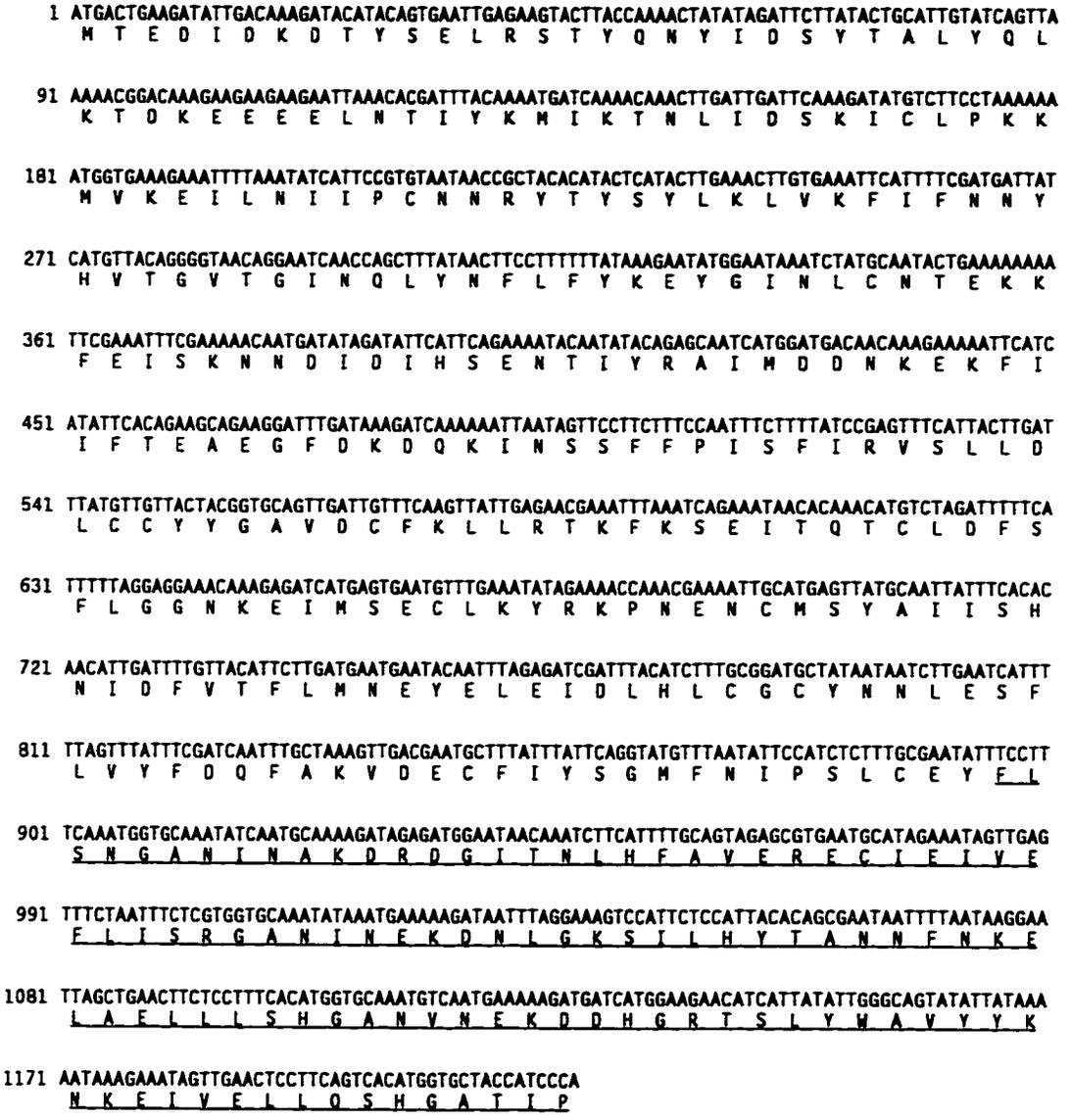
5' AAT TCG AGG ATC CGG GTA CCA TGG 3'

APPENDIX II

ρ-Dimethylaminobenzaldehyde (DMAB) stock solution

DMAB stock solution was prepared by dissolving 10 g of DMAB in 100 mL of glacial acetic acid that contains 12.5% (v/v) 10 N HCl. This stock was stored at 4°C for up to one month. For Neu5Ac lyase enzyme assays, the DMAB stock solution was diluted 1:10 in glacial acetic acid prior to use.

Appendix III. Nucleotide sequence and the deduced amino acid sequence of the *T. vaginalis* genomic ORF. The underlined region of the *T. vaginalis* polypeptide from amino acids 299-406 exhibits 35% identity and 59% similarity to the repeat domain (R19-22) of the mouse ankyrin 3 protein (Peters *et al.* 1995). This nucleotide sequence is available in the EMBL, GenBank™, and DDJB data bases under the accession number U44916.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F L G G N K E I M S E C L K Y R K P N E N C M S Y A I I S H
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N I D F V T F L M N E Y E L E I D L H L C G C Y N N L E S F
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F L I S R G A N I N E K D N L G K S I L H Y T A N N F N K E
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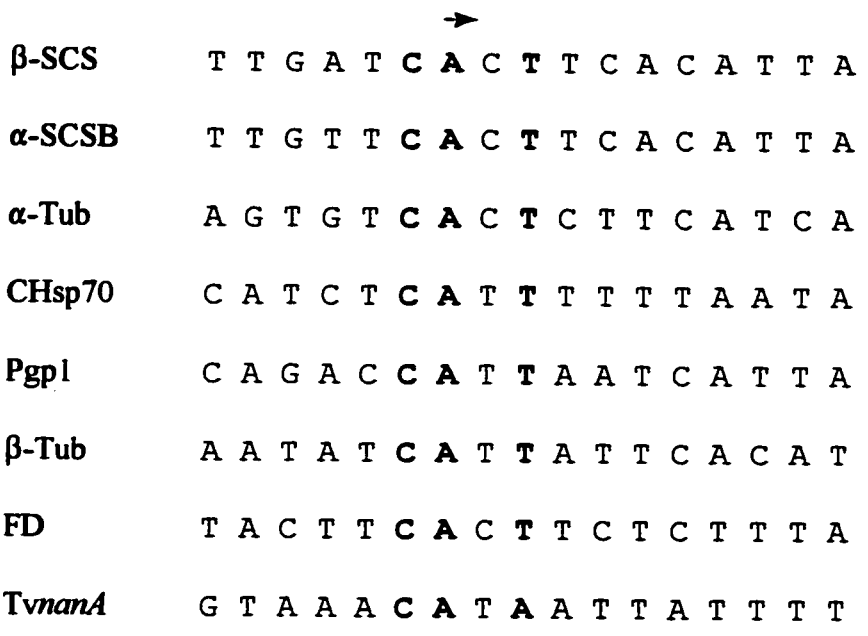
L A E L L L S H G A N V N E K D D H G R T S L Y W A V Y Y K
1171 AATAAAGAAATAGTTGAACTCCTTCAGTCACATGGTGCTACCATCCCA
N K E I V E L L Q S H G A T I P

Appendix IV. Alignment and comparison of the seven *T. vaginalis* Inr elements identified by Quon *et al.* (1994) and the Tv*nanA* Inr-like element. The residues most critical in determining Inr strength are bold-faced and the directional arrow indicates the transcriptional start site. β-SCS, β-Succinyl-coenzyme A synthetase gene; α-SCSB, α-subunit of the succinyl CoA synthetase gene; α-Tub, α-tubulin gene; CHsp 70, heat-shock protein 70; Pgp1, p-glycoprotein gene; β-Tub, β-tubulin gene; FD, ferredoxin gene.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TvnanA G T A A A C A T A A T T A T T T T