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<https://doi.org/10.15760/etd.5908>

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AN ABSTRACT OF THE THESIS OF J. Lindsley Bennett for the Master of Science
in Biology presented July 2, 1990.

Title: The Electrokaryotype and Molecular Characterization of
Trypanosoma cervi Isolates Using Recombinant DNA Techniques.

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE:

David T. Clark, Chair

Lester J. Newman

Robert L. Millette

David H. Peyton

The distribution of trypanosomes infecting wild ruminants of North America has only recently been investigated. Many isolates have been mensurally studied and were determined to be conspecific with *Trypanosoma cervi*. Widely divergent forms exist however, between host species and seasonally within a host. To determine the validity of all inclusions in the taxon and the extent of intraspecific variability, trypanosome isolates of moose, reindeer, antelope, muledeer, Roosevelt Elk

and two discrete transplant populations of Rocky Mountain elk were characterized and differentiated using recombinant DNA techniques.

The isolates were extensively cultured and DNA was extracted from the cells with several methods. Chromosome profiles were prepared using Pulse Field Gradient electrophoresis and compared to those of *T. brucei*, *T. lewisi*, *T. cruzi* and *Leishmania* species. Whole-cell DNA was digested with endonuclease restriction enzymes and examined for highly repeated sequences. Southern blots of the karyotypes and whole-cell DNA digests were examined for gene localization and restriction fragment length polymorphisms using heterologous DNA and oligonucleotide probes. The probes include; *T. brucei* derived α - and β -tubulin, ribosomal fragments, the universal minicircle consensus sequence, an INGI/RIME retroposon sequence and the 76 bp barren region repeat sequence. The results substantiate the classification of *Trypanosoma cervi* and further characterize the group. Chromosome profiles of all *T. cervi* isolates are more similar to *Leishmania* and *T. cruzi* than to those of *T. brucei* or *T. lewisi*. While all isolates studied conform to the general *T. cervi* characteristics, intraspecific variability has been demonstrated at the molecular level, especially in the case of muledeer and reindeer isolates. These differences are most apparent in the minicircle size, restriction site polymorphisms and molecular karyotypes.

THE ELECTROKARYOTYPE AND MOLECULAR CHARACTERIZATION OF *TRYPANOSOMA CERI*
ISOLATES USING RECOMBINANT DNA TECHNIQUES

by

J. LINDSLEY BENNETT

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1990

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ACKNOWLEDGEMENTS

I thank Jack Myers and my supervisors, David Clark, Lester Newman and Robert Millette for their advice, motivation and support throughout the period of this work. I am indebted to Newton Kingston for imparting purpose, stocks and instruction, and to Ted Kistner and Pat Paradis for grappling with the elk. I am grateful to Don Mattson and Ken Stuart for their generosity in donating facilities, supplies and expertise, and to John Scholler for his time and guidance. I would like to thank Curtis Patton and Steve Reed for providing comparative species and Paul Su for the photography. Finally, I thank my family and fellow employees at Seattle Biomedical Research Institute for their friendship, love, humor and encouragement.

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CHAPTER I

INTRODUCTION

Diseases often occur with little effect in their natural hosts but can be devastating for man and his cattle. Consequently, parasitic agents of game animals are often ignored until their zootic potential has been realized. For example, trypanosomatids often elicit little reaction in their natural hosts yet are well studied due to their contribution to widespread human mortality and loss of cattle (Nelson, 1988). Because of their pervasiveness, the causative agents of human diseases such as African Sleeping Sickness, leishmaniasis and Chagas' disease are included among the 6 infectious complexes most deserving of research and control (WHO, 1982).

The tracing of trypanosome epidemics and epizootics as well as the effective application of trypanocides depends upon accurate identification. For this reason, reliable classification is tantamount to productive research. When classified with the taxonomic criteria of mensural detail, host specificity, metabolism and pathological syndromes, many trypanosome species prove indistinguishable in concurrent infections (Clayton, 1988). Recently however, the World Health Organization has established substantial guidelines for classification, including the combined use of electrophoretic karyotyping and recombinant DNA techniques (WHO, 1987).

All trypanosomatids are of interest. Recently, a new group was isolated from the blood of all species of the North American Cervidae. Many of the isolates have been mensurally studied and so determined to be *Megatrypanum stercorarians* conspecific with *Trypanosoma cervi* (new species, Kingston and Morton, 1975; Kingston et al., 1985). Other approaches towards further characterization of *T. cervi* include sterol and fatty acid extract studies by Drs. G. Holz and D. Beach, Department of Microbiology, State University of New York, Health Science Center, Syracuse, NY; trans Atlantic collections by Dr. N. Kingston, Department of Veterinary Sciences, University of Wyoming, Laramie; transmission studies by Drs. R. Bose, K.T. Friedhoff and S. Olbrich, School of Veterinary Medicine, University of Hannover, Hannover, Germany and isoelectric focusing by Dr. D. H. Molyneux, Department of Biological Sciences, University of Salford, Salford, UK.

This study represents a taxonomic investigation of *T. cervi* isolates using molecular techniques developed in the characterization and differentiation of African and South American trypanosomatids (Morel et al., 1980; Van der Ploeg et al., 1984; Gibson et al., 1985; Scholler et al., 1986; Paindavoine et al., 1986; De Jonckheere, 1987; Aymerich and Goldenberg, 1989). Included in the samples were isolates of muledeer, reindeer, moose and pronghorn antelope provided by Dr. N. Kingston, Department of Veterinary Sciences, University of Wyoming, Laramie, as well as trypanosomes isolated from blood clots of Washington Roosevelt elk (new host record) and two discrete transplant populations of Wyoming Rocky Mountain elk, located in New Mexico (NM.R.Mt. elk) and eastern Oregon (O.R.Mt. elk). The Roosevelt and Rocky Mountain elk

populations were sampled by private veterinarians, Dr. T. Kistner and Dr. P. Paradis.

In preparation for the study, trypanosomes were isolated from the donated blood clots under the guidance of Dr. D. Clark and acclimated to culture conditions in the laboratory of Dr. R. Millette at Portland State University, Portland, OR. Deoxyribonucleic acid (DNA) was initially isolated from the cultured cells and examined under the guidance of Dr. J. Myers, also of Portland State University. All available *T. cervi* isolates were then extensively cultured through the generosity of Dr. D. Mattson at the Oregon State University Veterinarian Diagnostic Lab, Corvallis, OR. DNA was extracted from the cell pellets and analyzed according to the instruction of Mr. J. Scholler in the laboratory of Dr. K. Stuart at the Seattle Biomedical Research Institute, Seattle, WA. Species used for comparison include *T. brucei*, *T. cruzi*, *T. lewisi* and *Leishmania*. *T. lewisi* was provided by Dr. C. Patton, School of Medicine, Yale University, New Haven, CT. The *Leishmania*, *T. cruzi* and *T. brucei* samples were obtained through the laboratories of Dr. S. Reed and Dr. K. Stuart of the Seattle Biomedical Research Institute.

STATEMENT OF PURPOSE

The purpose of this comparative study is to establish the generic and specific characteristics of *T. cervi* at the molecular level and explore the intraspecific variability of available North American *T. cervi* isolates. *T. cervi* was compared as a group with representatives of other species, sections and genera of the family Trypanosomatidae. Included in the intraspecific study were isolates of

discrete populations of the same host species and an antelope isolate which had not been mensurally studied or classified. To accomplish this I carried out the following studies: 1) Chromosome profiles were prepared and compared to those of representative species, 2) Southern blots of the karyotypes were probed to establish the location of several highly conserved genes in the chromosome profiles, 3) total DNA was digested and examined for patterns of highly repeated sequences and finally; 4) restriction fragment length polymorphisms of the conserved genes were inspected to determine the extent of divergence among the seven *T. cervi* isolates.

CHAPTER II

CLASSIFICATION AND CULTURE OF *TRYPANOSOMA CERV*

TRYPANOSOME CLASSIFICATION AND EVOLUTION

Trypanosomes are unicellular, flagellate protozoans belonging to the Phylum Sarcomastigophora, Class Zoomastigophorea, Order Kinetoplastida and Family Trypanosomatidae. Members of the Kinetoplastida are characterized by a kinetoplast containing a large aggregate of mitochondrial DNA. Trypanosomatids have a mono-flagellum, free or attached to the body as an undulating membrane. All genera of the family are parasitic and differentiated based on details of life cycle and developmental stages in both vertebrate and invertebrate hosts (see Figures 1, 4, 5 and 6). Lee et al., (1985) include nine genera in the Trypanosomatidae; *Leptomonas*, *Herpetomonas*, *Phytomonas*, *Blastocrithidia*, *Rhynchoidomonas*, *Endotrypanum*, *Crithidia*, *Leishmania* and *Trypanosoma*.

Members of the family Trypanosomatidae are highly adaptive and have been found in every class of the vertebrates, as well as plants, arthropods, nematodes and other invertebrates. All but three of the nine genera are monogenetic (have only one host). The digenetic lifestyle however, is a more recent development and two hosts are required for full development. This can include sexual recombination which has only recently been demonstrated in the insect host of African trypanosomes (Jenni et al., 1986) and is of great importance in

Figure 1. Developmental stages of trypanosomatids. From Lee et al., 1985. Stage definition is based on relative positions of K: kinetoplast, N: nucleus, and F: flagellum.

phenotypic characterization and genomic manipulation (Sternberg et al., 1989).

Digenetic trypanosomes are thought to have evolved from monogenetic plant trypanosomes vectored by feeding insects. Some trypanosomes no longer require an insect for transmission or development and are vectored directly through contact between hosts. These trends are depicted in a phylogenetic tree of five representative species from four genera of the family Trypanosomatidae (Figure 2). Important evolutionary steps include:

- 1) the acquisition of an insect host (monogenetic life cycle),
- 2) introduction of the promastigote stage of cell development,
- 3) development of a digenetic life style, and
- 4) acquisition of an accelerated rate of evolutionary substitutions in *Trypanosoma*. Dates of two important nodes inferred from the fossil/geological record are shown (Lake et al., 1988).

Figure 2. Phylogenetic tree of the family Trypanosomatidae.
From Lake et al., 1988.

The genus *Trypanosoma* is composed of digenetic trypanosomes with both trypomastigote and epimastigote stages. The importance of stage specific morphological transformation lies in host infection, gene exchange and the concurrent activation or inactivation of mitochondrial genes and differential metabolic pathways. Thus, the trypanosome can switch between fermentative and oxidative metabolism to facilitate acclimation to its host habitat (see Figure 3).

Figure 3. Stage specific metabolism in *T. brucei*. From Bogitsh and Cheng, 1990.

The trypanosomes are divided into two sections (Stercoraria and Salivaria) based on mode of development, transmission, details of respiration, chemotherapeutics, morphology and culture. The two sections are further typified by their pathogenicity in man or domestic animals (see Table I).

Members of the section Salivaria are exclusively extracellular and develop in the anterior station (salivary glands or foregut) of their invertebrate host. This section is composed mostly of trypanosomes with an infective metacyclic stage, which is transmitted between the mammalian hosts by the hypodermic feeding habits of the insect vector, usually a tsetse fly (*Glossina*) or horseflies (Tabanidae).

TABLE I
CHARACTERISTICS OF SPECIES OF *TRYPANOSOMA*

Species	Definitive hosts	Lab. hosts	Vectors	Distribution	Pathology
<u>Section STERCORARIA</u>					
<i>T. theileri</i>	cattle, antelope	none	tabanid flies	cosmopolitan	nonpathogenic (?)
<i>T. cervi</i>	cervids, antelope	none	tabanids (?)	cosmopolitan	nonpathogenic (?)
<i>T. melophagium</i>	sheep	none	<i>Melophagus ovinus</i> (ked)	cosmopolitan	nonpathogenic
<i>T. lewisi</i>	rats	rats	fleas	cosmopolitan	nonpathogenic
<i>T. musculi</i>	mice	mice, *rats	fleas	cosmopolitan	nonpathogenic
<i>T. nabiassi</i>	rabbits	rabbits	<i>Spilopsyllus cuniculi</i>	cosmopolitan	nonpathogenic
<i>T. rangeli</i>	man, monkey, dog, opossum	rats, mice	triatomid bug	Central & S. America	nonpathogenic
<i>T. cruzi</i>	man, armadillo, dog, opossum, cats, raccoon	rodents and other mammals	triatomid bug	Americas	pathogenic: Chagas' disease
<i>T. theodori</i>	goats	?	<i>Lipoptena capreoli</i>	Palestine	nonpathogenic
<u>Section SALIVARIA</u>					
<i>T. vivax</i>	cattle, sheep, equine, goat, dog, antelope	*rodents	tsetse-flies	Trop. Africa, Mauritius, Antilles	pathogenic: Souma
<i>T. uniforme</i>	antelope, sheep, cattle, goat	none	tsetse-flies	Central & E. Africa, Angola	pathogenic
<i>T. congolense</i>	warthog, cattle, sheep, zebra, equine	rodents	tsetse-flies	Trop. Africa	pathogenic
<i>T. dimorphon</i>	cattle, sheep, equine, pig	rodents	tsetse-flies	Trop. Africa	pathogenic
<i>T. simiae</i>	warthog, pig, camel; possibly cattle, equine	monkey, rabbit	tsetse-flies	Trop. Africa	pathogenic
<i>T. suis</i>	pigs	none	tsetse-flies	Zaire, Tanzania	pathogenic
<i>T. brucei</i>	domestic mammals, antelope	rodents	tsetse-flies	Trop. Africa	pathogenic: Nagana
<i>T. rhodesiense</i>	man, antelope	rodents	tsetse-flies	E. Africa	pathogenic: sleeping sickness
<i>T. gambiense</i>	man	rodents	tsetse-flies	Trop. Africa	pathogenic: sleeping sickness
<i>T. evansi</i>	dog, cattle, equine	rodents	tabanid flies, vampire bats	S. America	pathogenic: Mal de Caderas
<i>T. equiperdum</i>	equine	rabbit, dog	(venereal transmission)	S. Europe, Asia, N. Africa	pathogenic: dourine

Modified from Smyth, 1976.

Most of the 'African' trypanosomes are included in this group, which is exemplified by the causative agent of African Sleeping Sickness, *Trypanosoma brucei* (Figure 4).

Figure 4. Life cycle of *Trypanosoma brucei*. From Bogitsh and Cheng, 1990.

Although Stercorarians are a more diverse group, they typically develop in the posterior station (hindgut and rectum) of their invertebrate hosts. Stercorarians are usually vectored by the fecal contamination of a wound or mucous membrane and unlike the Salivarians,

may also have intracellular amastigote or epimastigote stages in the mammalian host. Both *Trypanosoma cruzi* and *Trypanosoma lewisi* have been

Figure 5. Life cycle of *Trypanosoma cruzi*. From Bogitsh and Cheng, 1990.

placed in this group but represent different subgenera, *Schizotrypanum* and *Herpetosoma*, respectively (Lee et al., 1985). Both have intracellular stages, though *T. lewisi* invades flea cells and *T. cruzi* invades mammalian cells. Their life cycles are diagrammed in Figure 5 and Figure 6.

Figure 6. Life cycle of *Trypanosoma lewisi*. Developmental phases in blood of rat and in rat fleas. Typical trypomastigote form in blood of rat; 1. Division stages in rat blood; 2-4. Epimastigote form released after schizogony; 5. Newly ingested trypomastigote in stomach of flea; 6. Dividing form in intestine of flea; 7. Invasion of stomach epithelial cell; 8, 9. Intracellular division of amastigote stages within intestinal epithelial cells; 10, 11. Epimastigote and division stages that pass down the intestine into rectum; 12. Epimastigote attached to rectal wall; 13. Dividing epimastigote attached to rectal wall; 14. Clump of epimastigotes released from rectal wall, hanging together by their flagella; 15, 16. Transitional stages in metamorphosis into trypomastigotes; 17. From Lee et al., 1985.

Megatrypanum is a subgenus of the section Stercoraria which includes some of the most phylogenetically primitive mammalian trypanosomes. Included in the subgenus are *T. melophagium* from sheep, *T. theileri* from cattle, *T. thiodori* from goats, *T. cyclops* from macaques, *T. rangeli* from humans and *T. cervi* (Figure 7). The Megatrypanum are large trypanosomes ($>30\mu\text{m}$) with a long, pointed posterior end. The kinetoplast is typically located far from the posterior end and relatively close to the nucleus (see Figure 8).

Figure 7. Trypanosomatid classification. Compiled from Clayton, 1988; Lee et al., 1985; and Van der Ploeg et al., 1984.

Besides their common morphological features, the Megatrypanum seem to share the quality of producing little or no pathogenicity in their mammalian hosts (Lee et al., 1985).

Figure 8. Representative Megatrypanum, *Trypanosoma cervi*. From white-tailed deer in the southeastern USA (Kingston et al., 1977).

Trypanosoma cervi

Unidentified trypanosomes were first recovered from the blood of white-tailed deer by Kistner et al. (1969) in southeastern United

States. Subsequent isolates included those from muledeer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and elk (*Cervus canadensis*) from Wyoming, Michigan, and New Mexico; reindeer (*Rangifer tarandus*), Wyoming and Alaskan moose (*Alces alces shiras* and *A. a. gigas*) and more recently, pronghorn antelope (*Antilocapra americana*) and bison (*Bison bison*) (Kingston et al., 1981). Since 1981, *T. cervi*-like trypanosomes have been isolated from at least two families of the order Artiodactyla (Antilocapridae and Cervidae) including all North American cervid species (Kingston et al., 1982a; Schmidt and Roberts, 1985).

Most of these isolates were mensurally characterized from the trypomastigotes available in blood smears. All of the bloodstream forms were determined to be conspecific with *Trypanosoma cervi* (new species, Kingston and Morton, 1975; Kingston et al., 1985) which has been subgenerically classified as a *Megatrypanum* in the section *Stercoraria* (see Figure 7). Some doubt persisted however, in the taxonomic designation of *T. cervi* because widely divergent sizes and forms existed between bloodstream isolates of host species and even from season to season within a single host (see Tables II and III). The single specific designation did appear valid when based on the Kinetoplast Index (KI) which was comparable between isolates, except in the case of moose (see Table III). Often however, a sufficient number of trypomastigotes could not be recovered for critical statistical analysis (Kingston et al., 1985) because the natural parasitemias of *T. cervi* are typically so low. As in the case of antelope, these isolates were available in the morphologically diverse cultured forms only and could not be used for the critical measurements.

Trypanosoma theileri is the type species of Megatrypanum. Although morphological comparison of *T. theileri* with *T. cervi* isolates support the validity of separate specific designations (see Table IV; KI values 1.9 and 2.7 respectively), prodigious similarities between *T. cervi* and *T. theileri* biology suggests a common origin. It is possible that the two trypanosomes are merely host variants of the same species. For example, like *Trypanosoma cervi*, the host range of *T. theileri* appears to be restricted to the order Artiodactyla but in the case of *T. theileri*, infection may be more common among the bovids. In fact, *T. theileri* has been found to be cosmopolitan in cattle from the tropics to the arctic and is 10 to 90% prevalent within individual herds of North America (Griebel et al., 1989). Similarly, *T. cervi* can be 100% prevalent in individual herds (Kingston et al., 1985b; 1985c and see results, Table V) and distributed in the North American continent from east to west (Kingston et al., 1981 and see results, Table V), from the arctic to South America and even into Europe (Kingston et al., 1985b; 1985c; Bose et al., 1987). Although the complete life cycle of neither *T. cervi* nor *T. theileri* is known, tabanid flies carrying epimastigotes of *T. theileri* and other Megatrypanum have been used in experiments to mechanically transmit infections (Bose et al., 1987; 1987b; Kingston et al., 1986). Furthermore, infections of *T. cervi* or *T. theileri* have been found in bovine and cervid fetuses and newborn calves, thereby implying transplacental infection as well as infection through an insect vector (Kingston et al., 1981; 1982b; 1986).

Parasitemias of *T. theileri* and *T. cervi* are rarely detected in the peripheral blood of healthy animals but can be found associated with concurrent disease. Under such conditions, fetal abortion and (p.21)

TABLE II
COMPARISON OF MENSURAL VALUES* OF *TRYPANOSOMA CERVI* ISOLATES

	PK	KN	PN	NA	BL	FF	L	W	FF:BL	NI	KI
<i>Trypanosoma cervi</i>											
Elk (<i>Cervus canadensis</i>)†	12.6† (5-20)	7 (4-9)	19.2 (11-32)	25.2 (20-30)	54.4 (32-56)	6.5 (3-12)	52 (40-61)	4.4 (3-8)	1:8.1	0.77 (0.5-1.3)	2.86 (1.7-5)
Mule deer (<i>Odocoileus hemionus</i>)‡	7.5 (3-15)	5.8 (2-13)	13.1 (8-19)	17 (10-26)	30.3 (21-42)	7 (1-16)	37.3 (26-51)	5.2 (2-9)	1:6.03	0.79	2.43
White-tailed deer (<i>Odocoileus virginianus</i>)§	13.5 (5-27)	8.3 (2-14)	22 (14-34)	26.3 (12-41)	48.2 (31-74)	9.5 (3-21)	57.7 (40-83)	5.6 (3-15)	1:5.7	0.88	2.86
Grand mean	10.4 (3-27)	6.99 (2-14)	17.2 (8-34)	21.5 (10-41)	38.7 (21-74)	7.9 (1-21)	46.6 (26-83)	5.3 (2-15)	1:6.13	0.82	2.64

Kingston et al., 1977.

*Expressed in μm .

Figures out of parentheses represent means; figures in parentheses represent ranges.

PK = posterior end to kinetoplast distance, KN = kinetoplast-to-nucleus distance, PN = posterior end-to-nucleus distance, NA = nucleus-to-anterior end distance, BL = body length, FF = length of free flagellum, L = overall length, FF:BL = free flagellum to body length ratio, W = width, NI = PN/NA (nuclear index), KI = PN/KN (kinetoplast index).

TABLE III
COMPARISON OF MEANS OF MENSURAL VALUES* BETWEEN WYOMING MOOSE AND ALASKA
MOOSE TRYPANOSOMES AND TRYPANOSOMES FROM OTHER DEER

	<i>x</i>										
	PK±	KN	PN	NA	BL	FF	L	W	FF:BL	NI	KI
Wyoming moose (N = 4)	7.5 ± 0.96	7.0 ± 0	14.5 ± 0.96	15.2 ± 1.03	30.3 ± 1.7	8.3 ± 0.75	38.5 ± 1.85	2.8 ± 0.25	1:3.8 ± 0.36	0.9 ± 0.06	1:2.1 ± 0.14
Alaska moose (N = 38)	16.7 ± 0.85	7.5 ± 0.28	24.0 ± 0.93	30.4 ± 0.99	54.5 ± 1.54	9.8 ± 0.59	64.0 ± 1.56	7.0 ± 0.50	1:6.5 ± 0.50	0.8 ± 0.37	1:3.3 ± 0.14
Composite: Wyoming moose + Alaska moose (N = 42)	15.8 ± 0.86	7.5 ± 0.26	23.1 ± 0.95	29.0 ± 1.13	52.2 ± 1.78	9.6 ± 0.54	61.6 ± 1.84	6.6 ± 0.49	1:6.2 ± 0.47	0.8 ± 0.35	1:3.2 ± 0.14
Other deer: Composite (N = 132)	10.2 ± 0.42	6.9 ± 0.19	17.1 ± 0.50	21.5 ± 0.55	38.8 ± 0.95	7.7 ± 0.29	46.4 ± 1.05	5.1 ± 0.18	1:6.1 ± 0.35	1:0.8 ± 0.02	1:2.6 ± 0.08
Composite grand mean (N = 174)	11.5 ± 0.43	7.0 ± 0.16	18.5 ± 0.48	23.3 ± 0.55	42.0 ± 0.94	8.2 ± 0.26	50.1 ± 1.03	5.5 ± 0.19	1:6.1 ± 0.29	0.8 ± 0.2	1:2.7 ± 0.07

Kingston et al., 1985.

*Expressed in μm .

Other deer include values from elk, mule deer, white-tailed deer, and reindeer.

PK = posterior end to kinetoplast distance, KN = kinetoplast-to-nucleus distance, PN = posterior end-to-nucleus distance, NA = nucleus-to-anterior end distance, BL = body length, FF = length of free flagellum, L = overall length, FF:BL = free flagellum to body length ratio, W = width, NI = PN/NA (nuclear index), KI = PN/KN (kinetoplast index).

TABLE IV
COMPARISON OF MENSURAL VALUES* OF BLOODSTREAM TRYPOMASTIGOTES RECOVERED
FROM BISON AND *TRYPANOSOMA THEILERI* FROM BOVINES AND *T. CERVI* FROM
CERVIDAE IN NORTH AMERICA.

	PK*	KN	PN	NA	BL	FF	L	W	FF:BL	NI	KI
Bison N = 27	5.7	6.4	12.4	15.9	28.0	12.4	40.3	3.1	1:2.43	0.8	1.9
SD	2.3	0.97	2.5	3.9	6.0	3.1	6.5	1.5	0.92	0.2	0.4
Range	2-11	4-9	8-18	9-29	17-47	7-17	24-58	2-9	1:1.35-4.71	0.542-1.143	1.286-3
Bovine N = 304	7.4	8.9	16.2	20.2	36.4	14.2	50.5	3.3	1:2.8	0.8	1.9
SD	3.3	2.6	5.1	6.3	10.5	4.5	12.7	2.0	2.3	0.2	0.4
Range	0-17	2-20	5-33	7-36	13-59	1-37	16-90	1-13	1:0.89-39.0	0.4-1.7	1-4
All deer† N = 174	11.5	7.1	18.5	23.3	42.0	8.2	50.1	5.5	1:6.1	0.8	2.7
SD	5.6	2.1	6.3	7.3	12.4	3.4	13.6	2.5	3.8	0.2	0.9
Range	3-27	2-14	8-36	10-43	21-74	1-21	26-83	1-15	1:0-27	0.4-1.6	1.2-7

Kingston et al., 1986.

*Expressed in μm .

Other deer include values from elk, mule deer, white-tailed deer, and reindeer.

PK = posterior end to kinetoplast distance, KN = kinetoplast-to-nucleus distance, PN = posterior end-to-nucleus distance, NA = nucleus-to-anterior end distance, BL = body length, FF = length of free flagellum, L = overall length, FF:BL = free flagellum to body length ratio, W = width, NI = PN/NA (nuclear index), KI = PN/KN (kinetoplast index).

TABLE V

INCIDENCE OF TRYPANOSOMES IN SAMPLED RUMINANTS

HOST SPECIES	NUMBER SAMPLED	PERCENT INFECTED	SEX		AGE			DATE SAMPLED	SOURCE
			M	F	CALVES	YEARLING	ADULTS		
Roosevelt Elk	29	55.2	-	-	-	-	-	880107	Jewell, OR
Roosevelt Elk	54	51.9	-	-	-	-	-	880120	Jewell, OR
Rocky Mt. Elk	5	80	3	2	2	1	2	880203	Elk Creek, OR
Rocky Mt. Elk	5	20	2	3	4	1	-	880203	La Grande, OR
Rocky Mt. Elk	87	67.8	-	87	-	-	87	880313	New Mexico
Muledeer	13	15.4	4	9	5	-	8	880203	La Grande, OR
Sheep	25	0	-	-	-	25	-	880509	W. Valley, OR
Sheep	16	0	-	-	16	-	-	880509	Hermiston, OR
Sheep	12	0	-	-	12	-	-	880509	California
Sheep	15	20	-	-	-	15	-	880509	N. Zealand
Sheep	15	0	15	-	-	-	15	880601	Corvallis, OR
Beef	28	3.6	-	-	-	-	-	880509	Washington
Llama	21	0	-	21	-	-	21	880620	Corvallis, OR

Blood clot culture technique defined in Methods.

DATE = year/month/day

compromised herd fecundity may be the consequence of infection with either *T. theileri* or *T. cervi* (Kingston et al., 1982b; Griebel et al., 1989). For this reason, these parasites could be of importance in wild life management and to the cattle industry. Inclusion of *T. theileri* for comparative study was intended; however, attempts to isolate *T. theileri* failed and alternative sources were not available.

GENERAL CULTURE METHODS

The ingredients of solutions and abbreviations used in this and following chapters are listed in the appendix.

Culture of *Trypanosoma cervi* in vitro

Trypanosoma cervi is typically cryptic in the peripheral blood of it's host and must be cultured to collect enough cells for molecular characterization. Continuous cultivation of the *T. cervi* isolates was generally conducted according to the methods of McHolland-Raymond et al., (1978) at 37°C in a media overlay of a feeder cell monolayer. In some cases, Keratinocyte Growth Medium (Clonetics Corporation) was used to reduce clumping (agglutination). The trypanosomes were cultivated in Corning tissue culture flasks or roller bottles and were observed daily to check concentrations and maintain pH values between 6 and 7 with 6% (w/v) NaHCO₃. Most fungicides reportedly prove lethal to trypanosome cultures but antibiotics were routinely used and are listed below (McHolland-Raymond et al., 1978). The trypanosomes were harvested from culture by centrifugation (1500x g, 10 min), washed in TDB and pelleted in a microfuge (1300 RPM, 1 min). The dry pellets were snap frozen in liquid nitrogen and stored at -80°C or in liquid nitrogen. A harvested

culture could be continued by adding just enough fresh media to cover the feeder cell layer and incubating until the trypanosomes were sufficiently dense ($>10^6/\text{ml}$) to further dilute. A culture could be repeatedly managed as long as the feeder cell layer remained healthy and intact.

Medium. The principal overlay was Medium 199, complete with Hank's Balanced Salt Solution and glutamine (Gibco). Preparation of a one liter package of Medium 199 (Gibco) included supplementation with 0.35gm sodium bicarbonate, 0.5% (w/v) Bacto-peptone (Gibco), 0.2 $\mu\text{g}/\text{ml}$ vitamin B12 (Gibco), 10% (v/v) heat inactivated serum, and the antibiotics 100 U/ml Penicillin G (Gibco), 100 mg/ml Streptomycin sulfate (Gibco) and 250 $\mu\text{g}/\text{ml}$ Gentocin (Schering Veterinary). All the components except serum were dissolved in approximately 500 ml ddH₂O, the pH was adjusted to 7.0 and the preparation was brought to 900 ml final volume. All media were filter sterilized (0.22 μm) and stored at 4°C until the time of use when serum was added to 10%, v/v.

Serum requirements. The cultures were most productive when supplied with fetal calf serum (FCS). However, other serum sources included newborn calf, steer, adult ovine, Roosevelt elk, horse, human, llama and mule deer. Partially defined serum supplements such as Serum Plus (Hazleton) and Seru-Max (Sigma) were also used. Unless otherwise specified, the serum was heat inactivated at 56°C for 30 min before addition to the media.

Feeder Cell Isolation and Cultivation

The feeder cell monolayers were usually derived from fetal calf bone marrow. Occasionally, lamb coryplexus or immortal rat myoblast

cells were used for the monolayers. Initially, stocks of the cells were donated by Dr. N. Kingston of the University of Wyoming Department of Veterinary Medicine or Dr. Donald Mattson of the Oregon State University Veterinarian Diagnostic Lab, Corvallis, OR. These gifts were destroyed by freezer malfunction so feeder cells were prepared from slaughter-house animals.

To collect cells from an animal, a fetal calf (3 month gestation to term) femur was cleaned of muscle tissue and surface sterilized by 15 second submersion in boiling, sterile water. Using aseptic technique, the bone was opened with a saw and the marrow was scraped out. Clumps of marrow cells were dispersed by gentle trituration with the large end of a pipette in Hank's MEM (Gibco) supplemented with 20% active FCS. About 3 ml of the suspension was distributed per 25cm² culture flask and maintained at 37°C for about 1 week of undisturbed incubation.

Following the 1 week incubation, the cells were gently fed by the addition of 3 ml of MEM media supplemented with 20% active FCS. Old media was not removed from the flask until the attached cells had spread to near confluence. Confluent cells were trypsinized (0.1 mg/ml Type IX crystalline porcine pancreas trypsin, Sigma #T-0134 in Versene Saline) and split 1:2 for amplification or frozen for stocks. Well established cells were maintained at a slower growth rate by changing the media to Earle's MEM (Gibco) supplemented with 5% active FCS and antibiotics. Viable cells could be recovered from a fetus stored at 4°C or on ice as long as one week after collection from the mother.

Trypanosome Isolation from Blood Clots

Using aseptic technique, peripheral blood was drawn from the jugular vein or heart into 10 ml or 15 ml VacuPack tubes. The samples were kept in a waterbath at 37°C for at least two hours after collection to allow full clot development. After clotting, the samples could be stored at 4°C or processed immediately as follows. The clot was detached from the tube wall by rapid rotation between the palms. The serum was removed from the clot and replaced with Veal Infusion Medium or Brain Heart Infusion (Gibco). The clot cultures were maintained at room temperature for as long as two weeks, and periodically screened for the presence of trypanosomes by microscopic examination of wet mount preparations of the media overlay. About 1 ml of positive clot overlay was transferred to a recently trypsinized feeder cell culture for further cultivation. Three to four day old feeder cell monolayers seem to encourage trypanosome cultures.

Stabilate Preparation and Use

A stabilate is a cryopreserved stock of cells used to regenerate cultures. Stabilate preparations include those for feeder cell and trypanosome cultures and were prepared according to methods outlined in the Nalge Cryopreservation Manual (Dagget et al, 1987). Each feeder cell stabilate contained half of the cells from a 1:2 split of a confluent culture. The final stabilate concentrations for feeder cells was 10% (v/v) DMSO and 20% (v/v) active FCS in Hank's MEM.

Trypanosome stabilates suitable for culture inoculation were prepared from culture densities of 10^6 to 10^8 per ml. The culture

volume was mixed with DMSO to a final 7% (v/v) concentration and 10% (v/v) inactive FCS serum.

Slow freezing was recommended in both cases. The newly prepared (unfrozen) stabilate was allowed equilibration at 4°C for 45 minutes, then wrapped in about an inch of cotton and placed at -80°C overnight. Long-term storage of stabilates was at -80°C or in liquid nitrogen. Cryopreservation vials (1.5 ml, Nalgene) with outer threads and/or O-rings were used to deter contamination. A quick thaw of a stabilate by immersing the vial in lukewarm water with constant agitation improved the recovery of stabilate cells. When completely thawed, the suspension of cells was immediately transferred to fresh growth medium. Other than 1:10 volume dilution in the fresh growth medium volume, no measures were taken to remove the stabilate concentration of DMSO when a stabilate was used.

RESULTS

Trypanosome Isolation from Blood Clots

See Table V for a summary of clot culture results. Although morphological identification was not possible, these results probably represent the incidence of three species of the subgenus *Megatrypanum*; *T. cervi* in cervids, *T. melophagium* in sheep and *T. theileri* in cattle. The isolation of *T. cervi* from Roosevelt Elk constitutes a new host record as well as an extension of the geographical range. The previous western limit had been the state of Wyoming. This extension formally establishes the range of *T. cervi* from the east to west coast of the United States and North from Florida and Texas to New York, Michigan and now, Oregon.

Culture of *Trypanosoma cervi* in vitro

Growth curves were not specifically determined but, generally, the doubling times of cultures ranged from 5 to 7 hours and final concentrations of trypanosomes averaged 1×10^7 /ml. Serum tests were performed to determine the optimum culture conditions for each isolate and are summarized in relative descriptive assessments in Table VI.

TABLE VI
COMPARISON OF NUMBERS, AGGLUTINATION AND MORPHOLOGY* OF CULTURED
TRYPANOSOMA CERVI ISOLATES IN DIFFERENT SERA

SERA	O.R.Mt. ELK	NM.R.Mt. ELK	ROOS. ELK	REIN DEER	MULE DEER	MOOSE	PRONGHORN ANTELOPE
A- ROOS. ELK	+	ND	+	+	+	+	ND
I- ROOS. ELK	++	++	++	++	+	++	++
A- STEER	+	ND	+	+	+	+	ND
I- STEER	++	++	++	++	++	++	++
A- OVINE	++	ND	++	++	++	++	ND
I- OVINE	++	++	++	++	++	++	++
A- FCS	++	++	++	++	++	++	++
I- FCS	++	++	++	++	++	++	++
A- HORSE	+	+	+	+	+	+	+
A- LLAMA	+	L	+	+	L	L	+
A- HUMAN	L	L	L	L	L	L	L

I = inactivated sera, A = active sera.

ND = not done

L = lysis

ORDER OF TEST: numbers (growth); - = none to very few, + = numerous, ++ = dense
agglutination; - = none (diffuse or attached to cell layer)
+ = clusters, ++ = tight balls
morphology; - = promastigote, + = several forms present,
++ = trypomastigote only

Although fetal calf serum gave the most productive results, the purpose of the serum testing was to explore the possibility of a cheaper alternative. The results indicate that either horse or ovine sera would be suitable but ovine serum proved to be the most accessible and permissive alternative. While culture of these trypanosomes in active sera proved to be inhibitive to growth at first, the results over time were much the same as culture with inactivated sera.

Inactivated ovine serum was used exclusively in most cases but had to be supplemented with Keratinocyte Growth Medium (KGM) to enhance trypanosome growth and reduce the agglutination that occurred in all sera over extended culture. The ratios of KGM to serum were established for each isolate and in the case of the antelope isolate was as high as 1:1 (combined volume of 20% final medium volume). When added to the sera test cultures, all isolates appeared to benefit from the application of Keratinocyte Growth Medium. This is not surprising in light of the recent identification of epidermal growth factor receptor homologues in trypanosomes. It is now known that these receptors are required for growth regulation and differentiation (Hide et al., 1989).

The response of *T. cervi* isolates to heterologous host derived factors is interesting when considering their implications for host suitability and colonization. Some sera caused complete lysis of the trypanosomes while others induced morphological transformation from the promastigote to trypomastigote form. Trypomastigotes were rarely agglutinated but their growth was typically inhibited and they were usually found attached to the feeder cell layer. Based on the response of Roosevelt elk isolates cultured in (the native) Roosevelt elk serum,

this condition appears to be 'natural'. Culture of all elk isolates in active steer serum also resulted in inhibited growth and complete transformation to the trypomastigote form. All trypanosomes in these cultures remained attached to the feeder cell layer. Inactivation of both steer and elk serum appeared to slightly moderate these effects. That ovine serum is permissive for cultivation without complete transformation while steer serum is not suggests that *T. cervi* could be more closely related to the cattle parasite *T. theileri* than to the sheep *T. melophagium*.

Generally, the muledeer isolate was the most productive, morphologically distinct and flexible in terms of culture requirements while the moose and antelope isolates were the most fastidious. If cultured without KGM in serum other than FCS, these isolates formed massive aggregates and died off. In llama serum (a recently introduced host species) Oregon Rocky Mountain elk, antelope and reindeer isolates persisted in trypomastigote form. The Roosevelt elk isolate never transformed. Of particular note is that only the antelope isolate could survive active human serum. Pronghorn antelope is the only native North American host among the *T. cervi* isolate hosts, and is restricted to the continent. All other isolate hosts migrated across the Bering land bridge to the Americas from the European continent (Thomas and Toweill, 1982). If the invading cervid hosts introduced *T. cervi* to the North American continent, then it is quite likely that the infection of pronghorn antelope is a relatively recent event. The antelope isolate has not yet been studied mensurally and Kingston et al. (1981) speculate that the antelope trypanosome could be any one of *T. theileri*, *T. melophagium*, *T. cervi* or different from all of these.

CHAPTER III

CHROMOSOME PROFILE

INTRODUCTION

This chapter contains the methods used for the resolution of trypanosome chromosomal elements, as well as a discussion of the results. During mitosis, chromosomes of the family Trypanosomatidae condense only slightly and are difficult to see with conventional microscopic methods (Vickerman et al. 1970). The recent development of pulsed field gel electrophoresis (PFGE) however, has permitted the visualization of trypanosome chromosomes, as well as details of size, numbers and location of genes (Schwartz and Cantor, 1984). Although there is some doubt that PFGE results actually represent a chromosome profile, Aymerich and Goldenberg (1989) sanction their validity;

It is now generally accepted that the DNA bands separated by pulse field gel electrophoresis correspond to the intact chromosomes since they hybridize to specific genes as well as to telomeric probes.

While this technique has permitted the karyotyping of trypanosomes and other protozoans (Van der Ploeg et al. 1984a; 1984b), it is also useful in genetic studies.

METHODS

The ingredients of solutions and abbreviations used in this and following chapters are listed in the appendix.

Pulse Field Gradient Electrophoresis

Standard electrophoretic methods are limited in the ability to resolve DNA fragments larger than about 50 kb but PFG electrophoresis generates electric fields in alternating orientations that can separate megabase pair sized DNA molecules up to at least 7 megabase pairs (Birren et al., 1988). Several parameters interact to determine the size of the resolved chromosomes but the primary factors are voltage and duration of switching times during electrophoresis. Although the process is still not fully understood, the separation of DNA molecules in a PFG is presumably facilitated by an elastic type of migration termed reptation (Smith et al., 1989).

Electrophoresis was performed with various switching times at 300 volts for about 20 hours or as otherwise specified in the results. The resulting molecular migrations were measured using computer aided analysis which was standardized with HindIII restriction enzyme digests of lambda phage C/ts857Sam7 (BRL), lambda phage (cl857S7) annealed DNA ladders (BRL), *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* chromosome profiles (FMC) or *Leishmania* (L. b. b. M2903 and L. b. g. CUMC1-A-1) chromosome profiles of determined values (Scholler et al., 1986).

Pulse Field Gradient Blocks. Intact trypanosome chromosomes were isolated from pulse field blocks which were prepared by embedding the live cells in agarose (Scholler et al., 1986). The cells were collected from culture by centrifugation at 3,000 x g for 10 min at room temperature. The pellets were washed by resuspending in PBSG or Trypanosoma Dilution Buffer (TDB) and finally concentrated in 1.5 ml snap-cap tubes by 1 min microfuge centrifugation, 12,000 x rpm. The cells were resuspended in PBSG prewarmed to 42°C at a concentration of 1×10^9 cells per ml and then mixed 1:1 with 1% (w/v) SeaPlaque low gelling temperature agarose (FMC BioProducts) in 0.06 M PBS + 50 mM EDTA and kept at 42°C. The cell/agarose suspension was quickly transferred to a gel forming apparatus (7.5 x 5 cm with 2 mm gap), cooled at 4°C for 15 min and sliced into 3 x 5 mm blocks. The blocks were transferred to an equal volume of the primary lysis buffer (5 ml lysis mix for 5 ml of blocks). The preparation was incubated from 12 to 24 hours at 50°C. The blocks were then washed 2 or 3 times with TE buffer and placed in the secondary lysis buffer. The blocks were again soaked from 12 to 24 hours at 50°C in this preparation, then washed 2 or 3 times with TE buffer and stored at 4°C in 0.5 M EDTA, pH 9.5. Blocks stored in this manner should produce intact chromosomes for at least 2 years. If frozen, the chromosomes break and quickly degrade.

Pulse Field Gels. Agarose gels of 0.4% or 0.8% (SeaKem ME, FMC) were prepared with 0.5 x TBE buffer. Sample blocks were loaded into the preformed well and sealed in place with 0.7% agarose. After electrophoresis, the gels were stained in 1 x TBE + 0.5 µg/ml ethidium bromide (EtBr) for 30 min, destained 30 min in dH₂O and photographed on a ultraviolet transilluminator using a 667 Polaroid camera and film.

DNA from the gels was subsequently blotted onto nylon membranes (Nytran) as described in the Southern blotting technique, below.

Southern Blotting

The transfer of DNA from the agarose gels to Nytran membranes by capillary action was performed according to the methods described by Maniatis, et al. (1982). This was done by soaking the agarose gels for 30 min in each of the following solutions: 0.25 M HCl and then in denaturing buffer. After soaking the gels, the DNA transfer 'sandwich' was assembled (as diagrammed in Maniatis et al., 1982, pp. 385) in the following manner: A filter paper wick supported by a glass plate was propped over a dish containing denaturing buffer. After prewetting the filter paper with denaturing buffer, all bubbles were rolled out of the filter paper with a pipette. The gel was placed on the filter paper and bubbles between the filter paper and gel were removed with the pipette as described. A Nytran membrane was prewetted in dH_2O and then soaked in denaturing buffer. The prepared membrane was then placed on the gel and all bubbles removed as described. A filter paper prewetted in denaturing buffer was placed on the membrane and bubbles removed as described. Dry paper towels were thickly layered on the filter paper, upon which another glass plate and weights were placed. This transfer 'sandwich' was maintained overnight, or until the denaturing buffer had been adsorbed by the paper towels. After DNA from the gels had been so transferred to the membranes by capillary action, the "membrane blots" were washed for 15 min in 2 x SSPE. After drying the Nytran membranes, the DNA was permanently bound to them by baking for one hour at 80°C under vacuum.

RESULTS

Representatives of the *T. cervi* isolates, subgeneric sections (Salivarian = *T. brucei*, Stercorarian = *T. lewisi*) and *Leishmania* were resolved with Pulse Field Gel Electrophoresis at various switching times to determine the relative karyotypic profiles. Included in Figure 10 is a profile of the Stercorarian *T. cruzi*, kindly provided by Dr. Juan Engel of the Laboratory of Parasitic Diseases, National Institutes of Health.

The following gels (A and B, Figure 9) explore the comparative intergenic range and general profile of *T. cervi* chromosomal DNA. The size ranges of *T. brucei* and *L. b. guyanensis* profiles are known (Scholler et al. 1986) and were used to help approximate *T. lewisi* and *T. cervi* values from another gel prepared with commercial standards (not shown). The overall range of resolved elements in panel A extends from 25 to about 850 kb at the compression zone.

In lane 5, panel A of Figure 9, the *T. brucei* minichromosome cluster spans 50 to 150 kb and the intermediate band is about 350 kb. The minichromosome cluster of *T. lewisi* is even smaller, ranging from about 25 to 100 kb with 8 resolved elements situated between 150 kb and approximately 450 kb. The smallest *L. b. guyanensis* chromosome is approximately the same size as the smallest of the *T. cervi* elements, which is found in the muledeer karyotype at approximately 275 kb. The next largest muledeer band is about 400 kb while the reindeer profile starts at approximately 340 kb.

Figure 9. Electrophoretic separation of *Leishmania* and *Trypanosoma* chromosomes. (A) PFG 881219: 42 sec. pulse frequency, 22.5 hours, 300V, 0.8% gel; *L. braziliensis guyanensis* (1), *L. braziliensis* Ba 147 (2), *T. lewisi* (3), *T. cervi* isolate reindeer (4), *T. brucei* (5) and *T. cervi* isolate muledeer (6).

(B) PFG 890319: 60 minute pulse frequency, 80 hours, 100V, 0.4% gel; *T. brucei* (1), *T. lewisi* (2), *Schizosaccharomyces pombe* (3), *Saccharomyces cerevisiae* (4), *Tetraymena thermophila* (5), *L. braziliensis guyanensis* (6), *T. cervi* isolates muledeer (7) and O.R.Mt. elk (8).

Resolution of the upper karyotype elements are depicted in Panel B. The standard bands of *Schizosaccharomyces pombe* are 5.7, 4.7 and 3.5 Mb while the upper limit of the *Saccharomyces cerevisiae* profile is about 1.6 Mb. When compared by computer with these markers, the megabase pair sized chromosomes of *T. brucei* appear to be approximately 1.4, 2.0, 3.0 and 4.0 Mb. *T. lewisi* is again similar to *T. brucei* in element distribution but appears to have only 3 mega bands of approximately 2.0, 3.5 and 4.7 Mb. Both *T. cervi* and *L. b. guyanensis* appear to have maximum element sizes of approximately 2.2 Mb. The *Leishmania* values are in general agreement with the findings of Samaras and Spithill (1987), 22-28 bands distributed between 200 and 2200 kb.

In a comparison of several *T. cruzi* strains, Aymerich and Goldenberg (1989) found that the elements of *T. cruzi* karyotypes (15-18 elements) are variable in number but generally distributed within a range of 500 to more than 1500 kb. The photograph in Figure 10 is of a *T. cruzi* strain that exhibits physiological changes upon challenge and withdrawal of the drug Nifurtimox (Bayer trade name = Lampit). The smallest chromosome (about 440 kb) in lanes 2, 3, 5 and 6 has been induced by the drug. The next largest chromosome is about 550 kb and the largest resolved element in the profile is about 1.1 Mb (personal communication, J. Engel, NIH, 1990).

Figure 10. Electrophoretic separation of *T. cruzi* chromosomes. *Saccharomyces cerevisiae* (1 and 9), induced cells cultured with Lampit (2 and 8), induced cells recently cleared of Lampit (3 and 7), induced cells cleared of Lampit for an extended time (4). 2.5 minute pulse frequency, 96 hours, 1% gel. Courtesy of J. Engel, NIH.

The set of gels in Figure 11 demonstrate the full range and variability of chromosomes within the general *T. cervi* profile. The karyotype profile of the seven *T. cervi* isolates is composed of at least 19 to 25 elements which range in size from about 275 kb to 2.2 Mb. The antelope and moose isolates have the least number of elements (at least 19), Roosevelt elk has at least 21, Oregon Rocky Mountain elk has approximately 23, New Mexican Rocky Mountain elk and muledeer have at least 24 each and reindeer has the most, at least 25.

Figure 11. Comparison of chromosome profile among *T. cervi* isolates. (A) PFG 881102: 42 second pulse frequency, 24 hrs, 300V, 1% gel; *L. braziliensis guyanensis* (1), *T. cervi* isolates antelope (2), reindeer (3), muledeer (4), (5), Lambda marker, moose (6), NM.R.Mt. elk (7), O.R.Mt.elk (8), Roosevelt elk (9), *T. brucei* (10).

(B) PFG 880731: 55 second pulse frequency, 20 hrs, 300V, 0.8% gel; *L. b. braziliensis* (1) *T. cervi* isolates antelope (2 and 3) and moose (4 and 5).

(C) PFG 880727: 70 second pulse frequency, 20 hrs, 300V, 0.6% gel; *L. b. braziliensis* (1), *T. cervi* isolates O.R.Mt.elk (2), Roosevelt elk (3) and NM.R.Mt. elk (4).

The smallest chromosome in the *T. cervi* profile (275 kb) is unique to the muledeer isolate (panel A, lanes 4 and 5). Otherwise, the

smallest chromosome of each *T. cervi* isolate ranges between 340 to 380 kb. The rest of the chromosomes of each isolate are distributed over the size range in a roughly even manner except for a major break spanning about 200kb and located between 850 and 1050 kb. Generally, 12 to 15 median size chromosomes are distributed between 275 to 850 kb while 7 to 8 chromosomes are distributed above 1050 kb.

CHAPTER DISCUSSION

Pulse Field Gel Electrophoresis is a problematic technique in that the inhomogeneity of the pulsed field results in DNA migration mobilities which may impart location dependent attributes. Sample concentration, temperature and switching times can also alter chromosome mobilities. Although measures were taken to curtail these effects, there is potential for a margin of error in size determinations by comparative migration across the gel. Therefore, all values presented here should be considered approximations.

T. brucei and *T. lewisi* profiles are characterized by a few very large chromosomes (1.4 to > 4.7 Mb), several to many small (mini) chromosomes (25 to 150 kb) and a very few, if any mid sized chromosomes. This pattern is thought to characterize the salivarian trypanosomes (Dickin and Gibson, 1989). Although *T. lewisi* is classified as a stercorarian, it appears to have a salivarian profile (Figure 9, panels A and B). Other characteristics of *T. lewisi* suggest that the species may be more closely related to the salivarians. In contrast, the chromosome profiles of *T. cruzi*, *Leishmania* and *T. cervi* representatives (Figure 9) are composed of mid sized to large chromosomes (250 kb to

2.2 Mb). Other stercorearians which also share this pattern are *T. cyclops* and *T. rangeli* (Van der Ploeg et al. 1984).

While similar to both *T. cruzi* and *Leishmania* sp. in element numbers and size distribution, the karyotype pattern of *T. cervi* actually lies somewhere between the two. For example, *T. cruzi* isolates generally have 15 to 18 chromosomal elements distributed from 500 to greater than 1.5 Mb (Aymerich and Goldenberg, 1989), *T. cervi* isolates have 19 to 25 elements which range between 275 kb to 2.2 Mb and *Leishmania* sp. have 22 to 33 elements which span 200 kb to 2.2 Mb (Scholler et al., 1986; Samaras and Spithill, 1987).

All seven isolates conform to the general *T. cervi* karyotype however, not one is exactly alike any other within the group. Minor chromosome size variations within the general range of distribution characterize each host and geographical isolate. Although the intraspecific polymorphisms may not be stable over prolonged periods of culture, the limited variability within the karyotype motif suggest a close genetic relatedness between the isolates as well as plasticity of the genome in the course of evolution.

A caveat of this study was the failure of attempts to clone the isolates (not described). Consequently, all cultures must be considered as multiclonal populations and the molecular karyotypes probably represent a mosaic strain structure. Therefore, while it is obvious from the relative fluorescence of the ethidium bromide stained PFGs that certain *T. cervi* chromosomes are not present in equimolar amounts (see staining intensity of large chromosomes in lanes 2, 3, and 4, panel C of Figure 11), it is not known if the variable elements are

haploid, diploid or polyploid for a particular homologue, or if different chromosomes of the same size are simply comigrating.

Salivarian trypanosomes are now known to be aneuploid organisms in which genetic reassortment may occur during cyclical transmission through the insect host (Sternberg et al. 1989). Although hybrid formation has not been demonstrated among the stercorarians, the questionable ploidy of each chromosome subsequently complicates a general estimate of genome complexity for the *T. cervi* isolates. Perhaps, since the values of the *T. cervi* molecular karyotype appear to be 'sandwiched' by those of *Leishmania* sp. and *T. cruzi*, an acceptable mean value can be derived from published values. The total genomic complexity of *T. cruzi* and *L. mexicana* is variable between isolates but has been maximally estimated as 1.85×10^8 bp (= 0.20 pg) and 1.34×10^8 bp (0.145 pg) respectively (Gibson and Miles, 1986; Galindo and Ochoa, 1989; Kooy et al. 1989). From these two values, an averaged molecular complexity of 1.60×10^8 bp (0.173 pg) is estimated for *T. cervi*. This estimation pertains to the nuclear DNA only and does not include the kinetoplast DNA which cannot migrate from the well because of its catenated structure.

CHAPTER IV

HIGHLY REPEATED DNA SEQUENCES AND MOLECULES REVEALED BY GENOMIC DIGEST

INTRODUCTION

Highly repetitive DNA sequences have recently been employed as molecular fingerprints to identify individuals or related organisms (Jeffreys et al. 1985). The 'fingerprints' are generated through the use of restriction endonucleases which recognize and cleave specific nucleic acid sequences. Although unprobed endonuclease digestions of trypanosomal whole cell DNA generally produces a continuous smear of fragment sizes, a reproducible electrophoretic pattern of discrete bands may also result. The bands represent the size-sorted fragments of repetitive nuclear DNA sequences or kinetoplast specific elements known as minicircles. Such features can be diagnostic or provide discriminating characteristics between species and/or strains (De Jonckheere, J.F. 1987). Presented in this chapter are the ethidium bromide stained gels of trypanosome whole cell restriction digests. The banding patterns are compared between the *T. cervi* isolates and contrasted with those of representative species.

Minicircles

All members of the order Kinetoplastida have a kinetoplast which is a DNA containing structure found at the base of the flagellum. From the kinetoplast, the single mitochondrion is generated. The kinetoplast of parasitic Kinetoplastids contains a unique network of thousands of topologically interlocked (catenated) double stranded DNA circular molecules (kDNA) which can account for 5 to more than 30% of total cellular DNA (Hoeijmakers, 1982).

Although examples of Kinetoplastids with noncatenated structures are known (Hajduk et al., 1986), all kDNA aggregates contain two types of DNA molecules, the minicircle and the maxicircle. These two components are categorized according to size, transcriptional activity and copy number, and appear to be coordinated in the post transcriptional regulation of stage specific mitochondrial gene expression (Simpson and Shaw, 1989).

While it was known that the mitochondrial genes were encoded in the maxicircle, the specific function of minicircles had long been the source of much speculation. Recently however, small transcripts that function in the process of RNA editing (Simpson and Shaw, 1989; Blum et al., 1990) were found encoded on minicircles (Strum et al., 1990, Bhat et al., 1990).

Minicircles are always present in high copy numbers ($>10^3$), but only a few maxicircles (25 to 50) are found in each kinetoplast. Collectively, the thousands of minicircles can represent 90 to 95% of the kDNA network and more than 10% of total cell DNA. Within a species, each molecular type is homogeneous in size but they may differ interspecifically in both size and organization. Minicircle size

generally ranges between 0.45 and 2.5 kb while maxicircle size can be 20 to 40 kb. Some examples of the diversity are listed in Table VII.

TABLE VII
KINETOPLAST DNA CHARACTERISTICS IN DIFFERENT SPECIES.

Species	Maxicircle (kb)	Minicircle (bp)	Minicircle complexity (kb)	Comments
<i>T. cervi</i>		2500		
<i>T. brucei</i>	20-22	1000	300	transposon-like components
<i>T. equiperdum</i>	variable	1000	1.0	transposon-like components
<i>T. evansi</i>	absent	1000	1.0	
<i>T. vivax</i>		465		
<i>T. mega</i>	24-26			10 different classes
<i>T. lewisi</i>		1020		dimeric minicircles
<i>T. cruzi</i>	38-39	1600		tetrameric minicircles
<i>L. tarentolae</i>	30	870	0.9-1.8	at least 10 sequence classes (a)
<i>L. tropica major</i>		700	2.3-5.7	
<i>Leptomonas pessoai</i>	31			
<i>L. gymnodactyli</i>	38			
<i>C. oncopeith</i>	24.5	2300		13 semi-homologous classes
<i>C. fasciculata</i> (b)		2500		dimeric minicircles almost homogeneous in one strain
<i>C. lucillae</i>	33			
<i>P. davidi</i>	36	1100	1.4	minor heterogeneities

Modified from Clayton, 1988.

Minicircle size is intraspecifically conserved but minicircle sequence may be homogeneous (i.e. *T. equiperdum*) or highly diverse between molecules of a single cell (Table VII). For example, the most sequentially diverse collection of minicircles has been found in *T. brucei*. *T. brucei* has over 200 sequence family types and a kinetic complexity which is 300 times the unit length (Jasmer et al., 1986, Clayton, 1988). While some researchers feel that the sequence heterogeneity of minicircles is of taxonomic value (Clayton, 1988), others contend that the extensive degree of variability found between

and even within a species renders mini-circles unsuitable for most taxonomic and diagnostic purposes (Hoeijmakers, 1982). However, a common feature of minicircle organization is the conservation of a short segment (about 130 bp) between all minicircle types within a cell, as well as subspecifically and among the general brucei group. A smaller segment (the universal minicircle sequence) of about 13 bp is contained within the 130 bp sequence and is conserved among the kinetoplastid genera. The universal minicircle sequence is thought to contain the origin of replication for the light strand of the minicircle and is of use in determining general taxonomic affinities (Jasmer et al., 1986).

Minicircle secondary structure can be super coiled, covalently closed (relaxed super coiled), nicked circular and/or linear. Even though all forms have the same number of nucleotides, the relative migration of these molecules through a gel depends upon their structure and the conditions of electrophoresis. The relative migrations of these molecular forms in agarose, with and without ethidium bromide, are diagrammed in Figure 12 (Maniatis et al., 1982).

In this case, all genomic digest gels were run with ethidium bromide. Although the identity of bands can not be resolved by observation of a single stained genomic digest, the location of minicircles may be deduced if the same sized bands appear throughout a battery of digests. Otherwise, only the application of specific probes may disclose bands which are minicircles. Both of these methods were used in this study.

Figure 12. Relative migration of DNA plasmid forms in an agarose gel.

METHODS

The ingredients of solutions and abbreviations used in this and following chapters are listed in the appendix.

Genomic DNA Isolation

All glassware was sterilized by baking at 400°F overnight. A one gram pellet of frozen cells (1×10^{10} cells) were crushed in liquid N₂ using a precooled mortar and pestle. The crushed cells were poured into an ice cold Potter-Elvehjem tissue homogenizer with 10 ml of ice-cold NET buffer and then dispersed with no more than 10 strokes. The cell suspension was transferred to a 125 ml flask and warmed to 65°C for 15 min. To this, 1/10 volume of prewarmed, 10% sodium dodecyl sulfate (SDS) was added. If the solution had not cleared after 15 min of incubation at 65°C, about 1/50 volume of 10% SDS was added and the

incubation continued for a few more minutes. After this treatment, lysis was considered total and Proteinase K was added to 100 $\mu\text{g}/\text{ml}$. The Proteinase K digestion was continued at 65°C for at least one hour after which the solution was transferred to a 50 ml Corex tube at room temperature and 4 M NaCl was added to a final concentration of 1 M. An equal volume of phenol saturated with 1 x SSC was added and the mixture was gently mixed by inverting for 10 min. An equal volume of chloroform:isoamyl alcohol (24:1) was then added and the mixture was gently mixed by inverting for another 10 min. The samples were centrifuged for 10 min at 15°C , $1500 \times g$ and the top (aqueous) phase removed to a 100 ml beaker with the wide mouth of a 10 ml pipette. Two volumes of cold 95% EtOH was layered onto the aqueous phase and the DNA slowly spooled out by mixing the two phases with a hooked Pasteur pipette. The DNA was resuspended overnight in 2 to 5 ml of cold TE buffer (pH 7.5) at 4°C in a 25 ml Corex tube. Two types of RNase, A and T1 were then added to 15 $\mu\text{g}/\text{ml}$ and 15 U/ml respectively, and the mixture was incubated for 30 min at 45°C . SDS (10%) was added to 1/100 of the digest volume. Proteinase K was then added to 50 $\mu\text{g}/\text{ml}$ and incubation continued for another 30 min. NaCl was added to 0.1 M and the sample gently extracted with an equal volume of SSC saturated phenol for 5 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the sample gently mixed by inverting for 5 min and then centrifuged at $1500 \times g$, 10 min, 15°C . The aqueous layer was drawn off to a 50 ml beaker and spooled as before with 2 x volumes of 95% EtOH. The DNA was resuspended in 2 to 3 ml of cold TE buffer at 4°C , overnight. The concentration of the DNA was determined with a spectrophotometer (one absorbance unit at 260 nm = 50 $\mu\text{g}/\text{ml}$ for double stranded DNA).

Restriction Digestion of Genomic DNA

Restriction digestion of the DNA samples was performed according to the recommendations of the manufacturing company (Bethesda Research Laboratories). Basically, 3 μ g of DNA was mixed to a final volume of 30 μ l with buffer and the appropriate number of enzyme units. This digestion mixture was allowed to incubate at 37°C for one hour before the sample was loaded into a 0.7% agarose gel (SeaKem ME in 1x TBE with 0.5 μ g ethidium bromide per ml) and submitted to electrophoresis.

Homogeneous Field Electrophoresis

These gels were run at 30V for about 40 hours as described by Maniatis et al. (1982). When the DNA was resolved, the molecular migrations were measured using computer aided analysis standardized against Hind III restriction enzyme digests of lambda phage C/ts857Sam7.

Southern Blotting

Southern blotting was performed according to the methods of Maniatis et al., (1982) as described in Chapter II.

Oligonucleotide Probes

Oligonucleotide probes are short, specific sequences of single stranded DNA which are chemically synthesized and end labeled with 32 P labeled γ -ATP by polynucleotide kinase. If properly applied, oligonucleotide probes produce highly specific results. The hybridization fluid (5x SSPE, 1% SDS) contains no ssDNA and the temperature of hybridization (T_m) and wash are determined on the basis of the oligo fragment nucleotide composition.

Computer Aided Data Analysis

Programs used in the analysis of REFLP migration relative to standardized markers include the DNASTAR system (DIGIGEL program), 1801 University Ave., Madison, WI, 53705 and the Least Squares Fit of DNA Length to Gel Migration from : Analytical Biochemistry (1981) 115:113-122.

RESULTS

Genomic Digest

Total cell (genomic) DNA samples were treated with six different restriction enzymes in single digestions. Presented here is a description of the resulting gels stained with ethidium bromide. The enzymes used were: EcoRI, PstI, DraI, SmaI, BglII and BamHI. A high molecular weight band due to incomplete digest and background smear are present in all sample lanes, as well as *T. brucei* and *T. cruzi* specific bands of approximately 1000 and 1500 bp, respectively. These bands are known to be the linearized minicircles. All are common features which will not be pointed out in the following description of each enzymatic digest.

EcoRI. The EcoRI digestion of *T. brucei* produced no notable features, however a collection of seven bands ranging in size from approximately 1500 bp to less than 550 bp can be seen in the *T. cruzi* lane (Figure 13). All of the *T. cervi* isolates digested with EcoRI produced a single common band at about 2460 bp except the analogous muledeer band which is slightly smaller with a second, less intense band above it. This muledeer doublet persists in the BamHI, BglII and DraI

digests as well. The 2460 bp band of New Mexican Rocky Mountain Elk also has a third band above the doublet.

Figure 13. Genomic digest with EcoRI and SmaI. 880722, 3 μ g, 0.7% + EtBr, 30V, 34hrs.

SmaI. The SmaI digestion produced no notable features in *T. brucei* and only a single band at approximately 3600 bp in the *T. cruzi* lane (Figure 13). This band is similar in size one of the common *T. cervi* bands. SmaI digestion of *T. cervi* resulted in the largest collection of bands (six) common to and uniform in all the isolates except muledeer. The muledeer array is missing the smallest band at approximately 900 bp, the 2460/2690 bp doublet and has an extra band slightly above the

doublet. The other three muledeer bands of approximately 6200, 6000 and 3600 bp are the same size as those of the other isolates.

Figure 14. Genomic digest with BamHI and BglIII. 880722, 3 μ g, 0.7% + EtBr, 30V, 34hrs.

BamHI. Except for a single 2000 bp *T. cruzi* band, the *T. brucei* and *T. cruzi* BamHI digests are generally featureless. The *T. cervi* digests all have a common 2460/2690 bp doublet except for the muledeer isolate, which appears to have a slightly smaller doublet. The only other detectable *T. cervi* bands were produced by the reindeer and muledeer digests. Both isolates have three extra bands, two of which

are the same size at approximately 2100 and 1800 bp. The third band is approximately 1650 and 1400 bp in reindeer and muledeer respectively.

BglIII. The *T. brucei* and *T. cruzi* BglIII digests are generally featureless except for a diffuse *T. brucei* band of approximately 3500 bp which is also present in the elk and antelope isolates. BglIII digestion of *T. cervi* again produced the 2460/2690 bp doublet as well as two other bands in all three elk isolates at approximately 4400 and 6560 bp. The 4400 bp band is also feightly present in the other *T. cervi* digests.

DraI. Both *T. brucei* and *T. cruzi* DraI digests are generally featureless and the 2460/2690 bp doublet is again a characteristic of all *T. cervi* digests. The only other *T. cervi* bands produced were three of approximately 2350, 2000 and 700 bp which are unique to the muledeer isolate and a single band of about 2900 bp in the moose and New Mexican Rocky Mountain elk digests.

PstI. Although PstI digestion of *T. brucei* provided no discriminating features, a diffuse band of approximately 2800 bp distinguishes the *T. cruzi* digest. The 2460/2690 bp doublet was produced in all except the muledeer *T. cervi* isolate. Only very light bands at approximately 2900 and 2690 bp can be seen in the muledeer digestion. An additional high molecular weight band of approximately 8000 bp is common in all elk samples.

Figure 15. Genomic digest with PstI and DraI. 880722, 3 μ g, 0.7% + EtBr, 30V, 34hrs.

Minicircle Probe

To confirm the identity of the 2460/2690 bp doublet common to the *T. cervi* genomic digests, a 39 bp oligonucleotide fragment containing the *T. brucei* 13 bp universal minicircle sequence was used to probe two BamHI digests of *T. cervi* isolates. Both the high and reduced stringency temperatures were used but only reduced stringency produced identifiable signal in *T. cervi* (Figure 16).

As in *T. brucei*, BamHI does not appear to restrict *T. cervi* minicircles. The three bands which hybridized with the probe correspond to the minicircle configurations mentioned earlier (Figure 12); nicked

and covalently closed (as a unit), linear and supercoiled. The center band is the linearized molecule. That reduced stringency had to be used to produce a signal in *T. cervi* indicates that the *T. brucei* universal minicircle sequence has limited homology with the *T. cervi* minicircles. The *T. cruzi* minicircles appear to have better homology with the *T. brucei* universal minicircle sequence.

Figure 16. BamHI digest of *T. cervi* isolates probed with the universal minicircle sequence. Samples include *T. brucei* (1), *T. cervi* isolates NM.R.Mt. elk (2), O.R.Mt. elk (3), Roosevelt elk (4), antelope (5), moose (6), reindeer (7), muledeer (8) and *T. cruzi* (9). Calculated $T_m(20GC, 19AT) = 98-118^{\circ}C$. Hybridization was at $65^{\circ}C$ with 1.3×10^6 counts per mil. (5xSSPE, 1% SDS). Wash was at $22^{\circ}C$ in 5xSSPE, 1% SDS for 1, 2, and 3 min. and at $42^{\circ}C$ for 2 min. in 1xSSPE, 1% SDS. Exposure was at $-80^{\circ}C$ with intensifying screen for 7 days.

Other than that they seem to be equal between isolates, no estimation of the numbers of minicircles in *T. cervi* can be made.

Except for the slightly smaller muledeer molecule, the size of the *T. cervi* minicircle appears uniform between isolates. The linearized *T. cervi* minicircle appears to be about 2.5 kb (2460 kb), which is one of the largest documented minicircles relative to the species documented in Table VI.

CHAPTER DISCUSSION

These results indicate that, as a group, *T. cervi* resembles neither of the subgeneric representatives but that differences do exist between some of the isolates. The most striking characteristic of the stained gels was the species specific consistency of the minicircle bands across the six enzyme treatments. These diagnostic features include single *T. brucei* and *T. cruzi* bands at about 1000 and 1500 bp respectively, and a *T. cervi* doublet of about 2690 and 2460 bp. The doublet generally persists in all *T. cervi* digestions but one. EcoRI eliminates the nicked and covalently closed molecules of the *T. cervi* doublet and produces a single band of about 2460 bp (approximately 2.5 kb). The *T. brucei* minicircle is also linearized by EcoRI (Simpson, 1986) and is known to be about 1.1 kb. These results indicate that of the enzymes applied, only EcoRI cuts *T. cervi* minicircles. Furthermore, EcoRI cuts at a single site, a characteristic common to both *T. brucei* and *T. cervi* minicircles. In addition, the apparent size of the linearized *T. cervi* minicircle (approximately 2.5 kb) is among the largest known (see Table VII, and per communication, Dr. D.S. Ray, University of California, Los Angeles, 1990).

Six of the seven isolates within the *T. cervi* group appear to be identical in the tested minicircle restriction features, the exceptional muledeer isolate consistently, though marginally differed from the overall *T. cervi* pattern. The most conspicuous difference was the slightly smaller size (about 115 bp) of the muledeer minicircle doublet in the *Dra*I, *Bam*HI, *Eco*RI and *Bgl*III digests. In addition, the muledeer minicircle differs from the other isolates in restriction sites. While it appears to lack the common *Eco*RI site, the muledeer minicircle doublet disappears altogether with *Pst*I and *Sma*I digestion. This result suggests that the muledeer minicircle has multiple minicircle restriction sites not found among the other isolates.

The banding patterns of highly repeated genomic sequences reveal inter- and intraspecific differences. Other than the minicircle, *T. brucei* digestions appear almost featureless. On the other hand, both *T. cruzi* and *T. cervi* produced distinct banding patterns, although not with the same enzymes. *Sma*I digestion produced the most uniform and complicated pattern which was characteristic in all *T. cervi* isolates. *Bam*HI revealed features unique to the reindeer and muledeer isolates while *Bgl*III and *Pst*I digestion produced bands peculiar to the three elk isolates. *Dra*I digestion revealed minor differences between the two Rocky Mountain elk isolates and produced the most extreme muledeer specific deviations (3 extra small bands) from the overall *T. cervi* pattern.

CHAPTER V

NUCLEAR CHARACTERISTICS

INTRODUCTION

Once localized on a gel, highly repeated DNA sequences can be isolated and used as probes. Restriction Enzyme Fragment Length Polymorphisms (REFLPs) are the banding pattern differences between digested samples revealed by probe application. REFLPs are important in many aspects of molecular biology and in this case, serve in inter and intraspecific discrimination.

Although the highly repeated sequences identified in Chapter IV were not subsequently used as probes, REFLP evaluation of the blotted genomic digests through the application of heterologous probes are presented in this chapter. The same probes were also applied to pulse field gels to distinguish the molecular karyotypes. The probes used in these applications were selected either because they represent genes that are extensively conserved throughout nature and found in high copy number within an individual genome or because they define discriminating taxonomic characteristics relative to this study. The results contribute towards the initial characterization and understanding of the molecular biology of *T. cervi*.

METHODS

Southern blots (Nytran membranes with bound DNA) of agarose PFG gels and genomic digests described in Chapters III and IV, were prehybridized and probed in "Seal-o-Meal" bags after overnight soaking at the desired temperature in prehybridization buffer. The volumes of both prehybridization and hybridization buffers were determined on the basis of surface area of the blots ($1 \text{ ml}/0.05\text{cm}^2$); however, the components of the hybridization buffer and specific wash treatments varied depending upon the type of probe being used and the degree of stringency desired. Refer to the appendix for a listing of the ingredients of solutions used in this and following chapters.

Nick Translated Probes

Nick translated probes are double stranded segments of DNA that have been radio-labelled using the activities of DNase I/Polymerase I (BRL). Because of the A-T rich (83%) nature of trypanosome DNA (Lake et al., 1988) and the 65% incorporation efficiency of the enzyme system, the most reactive probes used were those produced when the DNase I/Polymerase I system had been provided with ^{32}P labeled α -dATP (DuPont). The specific activity of the probe was determined by 10% trichloroacetic acid (TCA) precipitation (Maniatis et al., 1982). The specific activity of the probe usually approached 5×10^7 to 10^8 cts/min per μg DNA. After heating at 100°C for 10 min, the probes were applied to the blots at 5×10^5 to 1×10^6 counts per ml of hybridization fluid.

After hybridizing overnight, superfluous probe was washed from the blots with two, twenty minute washes in a solution of $1 \times$ SSPE and 0.1% SDS, followed by two more twenty minute washes in $0.1 \times$ SSPE and 0.01%

SDS. All washes were conducted in a temperature controlled shaking water bath at temperatures as specified for each probe. After washing, the blots were individually wrapped in plastic and film (XAR5, Kodak) was exposed directly to the blots with an intensifying screen at -80°C or room temperature.

RESULTS

The results obtained are the chromosomal locations of genes and restriction enzyme fragment length polymorphisms that exist between the *T. cervi* isolates, *Leishmania*, *T. brucei* and *T. cruzi*. The photographs of the pulse field gels are slightly larger (.03%) than those of the autoradiographs. In all cases, the molecular weights of the specific chromosomes were determined by comparative migration using species of known values (Scholler et al., 1986) and REFLPs have been approximated as described in Chapter III.

Alpha and Beta Tubulin

The primary components of microtubules are α - and β -tubulin subunits. Tubulin subunit genes are found in multiple copies in the nuclei of most organisms but are generally dispersed and variously arranged in the genome. In contrast, the subunit genes of the Trypanosomatidae are characteristically grouped in blocks of tandem repeats. In either case, each gene in a repeat block is separated from the next by an untranslated spacer region (Nagel et al., 1988).

Tubulin gene organization may vary within a repeat block and provides a distinguishing characteristic for generic differentiation within the Trypanosomatidae. Each gene block may be composed of a

single subunit repeat or organized with alternating α - and β -subunit genes. For example, the subunit genes of *Leishmania* are separated and tandemly repeated in disparate blocks, so a β -tubulin probe hybridizes to 3 separate chromosomes and an α -tubulin probe to a single but different element (see Figures 19, 20, 21; Scholler et al., 1986). In contrast, the gene blocks of *T. brucei* and all other known trypanosomes are composed of alternating α - and β -subunits. Consequently, both α - and β -tubulin probes hybridize to the same chromosomes (Esquenazi, et al., 1989).

T. brucei derived α - and β -tubulin cDNAs were used as probes to test for homology and to characterize the *T. cervi* tubulin subunit organization and distribution in the chromosome profile. Diagrammed in Figure 17 is a endonuclease restriction map of the *T. brucei* genomic tubulin repeat unit and the corresponding cDNAs (pTb α T-cl and pTb β T-cl) used for the tubulin probes.

Figure 17. Endonuclease restriction map of *T. brucei* genomic tubulin repeat unit and cDNA clones. From Thomashow et al. 1983. P=PstI, B=BamHI, E=EcoRI, H=HindIII, K=KpnI, L=BglIII, S=SalI. Fragment sizes are in base pairs.

pTb α T-cl and pTb β T-cl, are each contained in the PstI site of pBr322 and are 1.6 kb and 0.93 kb respectively.

Tubulin isotypes have been reported from a number of protozoans, some of which are post-transcriptionally controlled and stage regulated (Fong and Lee, 1988). Two allelic clusters of tubulin subunits are found in the genome of *T. brucei*, each being 3.6 or 3.7 kb and containing 14 to 17 copies of the tandem paired gene unit. It is not known if the probes used in this study represent a particular allelic sequence (Thomashow et al. 1983; Clayton, 1988).

Hybridization of the α -tubulin probe, pTb α T-cl with 42 sec. PFGE separated chromosomes resulted in the autoradiograph of Figure 18. The two defined species specific size markers present on the autoradiograph are the single leishmanial band (arrow) far to the left at 770kb and the 1.5 Mb *T. brucei* band to the far right (arrow) in the compression zone above the elk elements. At least two bands (850kb and 820kb) have been resolved in the other *T. cervi* isolates with intraspecific size variances, particularly among the elk isolates. The reindeer isolate produced a third (very faint) element at about 680kb. Under these parameters, the muledeer isolate produced a faint 820kb element (center arrow) and a single broad band in the compression zone.

Figure 18. Hybridization of α -tubulin gene probe with 42 sec. PFGE separated chromosomes. Polaroid and Southern blot autoradiograph of the 0.4% PFG 881031. *T. brucei* α -tubulin hybridized with DNA from *L. braziliensis braziliensis* (1), *T. cervi* isolates from; antelope (2), reindeer (3), muledeer (4), moose (5), NM.R.Mt. elk (6) O.R.Mt. elk (7), Roosevelt elk (8) and *T. brucei* (9). The DNA of PFG 881031 was separated in 37 hours with 300V and 60 sec. pulse frequency. Over night hybridization of the nick translated probe (specific activity = 7.9×10^7 cts/min/mgDNA) was at 65°C , 1.3×10^6 cts/ml. Exposure was over night at -80°C with an intensifying screen.

The result of probing the resolved compression zone with the α -tubulin probe is presented in Figure 19. The single muledeer band in the compression zone of Figure 18 has been resolved by PFG into multiple chromosomes that hybridize with the α -tubulin probe at about 820 kb, 975 kb and 1.2 Mb. In this gel, the smaller, α -tubulin bearing elements of reindeer have been compressed into a single broad band at about 820 kb.

Figure 19. Hybridization of α -tubulin gene probe with 60 sec. PFGE separated chromosomes. Polaroid and Southern blot autoradiograph of the 0.6% PFG MR6, 880724. *T. brucei* α -tubulin hybridized with DNA from *L. braziliensis* *braziliensis* (1), *T. cervi* isolates from; muledeer (2), reindeer (3) and *T. brucei* (4). (The DNA of MR6, 880724 was separated in 20 hours with 300V and 60 sec. pulse frequency. Over night hybridization of the nick translated probe was at 65°C, 1.3×10^6 cts/ml. Exposure was at -80°C, with an intensifying screen for 2 days.)

To test the subunit arrangement of the *T. cervi* tubulin repeat unit, a blot similar to blot MR6 was probed with the β -tubulin subunit probe pTb β Tc1, and is illustrated in Figure 20.

Figure 20. Hybridization of β -tubulin gene probe with 70 sec. PFGE separated chromosomes. Polaroid and Southern blot autoradiograph of the 0.6% PFG MR4, 880723. *T. brucei* β -tubulin hybridized with DNA from *L. braziliensis* *braziliensis* (1), *T. cervi* isolates from; muledeer (2), reindeer (3) and *T. brucei* (4). (The DNA of MR4, 880723 was separated in 21 hours with 300V and 70 sec. pulse frequency. Over night hybridization of the nick translated probe was at 65°C, with an undetermined number of counts. Exposure was at room temperature, with an intensifying screen for 17 days.)

Although *T. brucei* was not present on this blot, the three β -tubulin specific non-allelic elements of the leishmanial profile provide marker bands at 620 kb, 800 kb and 1500 kb. All three muledeer elements defined by the α -tubulin probe at 820 kb, 975 kb and 1.2 Mb also hybridize with β -tubulin. In addition, a fourth element at about 770kb is faintly present.

In Figure 21, the remaining *T. cervi* isolates (elk, moose and

Figure 21. Hybridization of β -tubulin gene probe with 60 and 70 sec. PFGE separated chromosomes. Southern blot autoradiographs of the 0.6% PFG E10, 880726 (left), and 0.5% PFG MA102, 880803 (right). *T. brucei* β -tubulin hybridized with DNA from (left); *L. braziliensis braziliensis* (1), *T. cervi* isolates from; O.R.Mt. elk (2), NM.R.Mt. elk (3) and Roosevelt elk (4).

(right); *T. cervi* isolates of; antelope (1), moose (2) and *L. braziliensis braziliensis* (3). (The DNA of both gels was separated in 20 hours with 300V and 70 sec. pulse frequency. Over night hybridization of the nick translated probe was at 65°C, with an undetermined number of counts. Exposure was at room temperature, with an intensifying screen for 17 days.)

antelope) have been probed with β -tubulin and appear uniform in the location of the compressed banding at about 820kb. Again, the three β -specific leishmanial elements at 620 kb, 800 kb and 1500 kb provide reference size markers.

In Figure 22, genomic digests of *T. cervi* were probed with the tubulin subunits which serves to characterize the restriction sites and fragment sizes of the *T. cervi* tubulin repeat unit.

DraI, 881208 β -tubulin. DraI does not cleave within the repeat block of *T. brucei* however, there are DraI sites in sequences flanking the tubulin gene clusters. When probed, a single, large band without partial bands results. These 'null' restrictions provide evidence of gene clustering.

Figure 22. Hybridization of tubulin gene probes with genomic digests DraI, PstI and HindIII. DraI hybridized with β -tubulin (left) includes *T. cervi* isolates from; O.R.Mt. elk (1), antelope (2), moose (3), reindeer (4) and muledeer (5). 1×10^6 cts/ml, exposed 5 d with an intensifying screen at room temperature. PstI hybridized with β -tubulin (center) includes *T. brucei* (1), *T. cervi* isolates of; NM.R.Mt. elk (2), O.R.Mt. elk (3), Roosevelt elk (4), antelope (5), moose (6), reindeer (7), muledeer (8) and *T. cruzi* (9). Exposed 5 d with an intensifying screen at -80°C . HindIII hybridized with α -tubulin (right) includes *L. donovani chagasi* (1), *L. braziliensis guyanensis* (2), *L. mexicana amazonensis* (3), *L. mexicana* (4), *T. cervi* isolates of; O.R.Mt. elk (5), antelope (6), moose (7), reindeer (8) and muledeer (9). Exposed 17 d at room temperature with an intensifying screen. All blots were hybridized and washed at 65°C . The gel conditions are described in Ch. III.

In all cases, probing *Dra*I digests of *T. cervi* with either α - or β -tubulin produced the same large molecular weight band (>23kb). In addition to the large band, the extra fragments that distinguish both reindeer and muledeer could represent internal *Dra*I sites and/or non-allelic tubulin clusters of other chromosomes. It is likely that the sequentially smaller one and three *Dra*I fragments of reindeer and moose (Figure 24) reflect reduced copy number of each subsequent non-allelic tubulin cluster (Figures 18, 19 and 20). Even though the tubulin clusters of O.R.Mt. elk, moose and probably antelope are located on at least two chromosomal elements, a single *Dra*I fragment resulted. This suggests that these non-allelic loci of the tubulin repeat blocks contain the same number of tubulin repeat units.

PstI 881208 top β -tubulin. According to the *T. brucei* map (Figure 17), *Pst*I cleaves twice within the β -subunit; at the 5' flank and about halfway through the gene. Consequently, two bands (about 750 bp and 2.8 kb) are produced from each *T. brucei* tandem repeat. However, because the β -tubulin probe used in this study is specific for the 3' end of the β -tubulin gene, both the α - and β -specific probes should hybridize only to the 2.8 kb *T. brucei* band.

Despite the inability to directly disclose any 5' β -tubulin fragments with this probe, they may be inferred from the partials that result from incomplete digestion. *Pst*I digests of *T. cervi* isolates which were probed with the β -tubulin gene (Figure 22) generally resulted in a common pattern of a single intense band of about 3175 bp in addition to several partials of about 4000, 5200 and 8420 bp, as well as a very light band at about 2300 bp. An 825 bp fragment is calculated from the difference between the 3175 bp terminal fragment and the 4000

bp partial common to all of the *T. cervi* isolates and the difference between the 8420 and 5200 bp fragments is approximately 3200 bp. If a 4000 bp tubulin repeat unit is accepted, then the 8420 bp PstI partial would represent 2.1 tubulin repeat units. The partial digest patterns suggest that, as in *T. brucei*, *T. cervi* tubulin repeat units have at least two PstI sites located within the β -tubulin subunit.

In some *T. brucei* strains there is an additional, very light β -specific band of about 3145 bp. Affolter et al. (1989) maintain that this faint band represents a truncated β -gene in some *T. brucei* strains and also in *T. rangeli* which flanks each tubulin repeat unit block (Esquenazi, 1989). When the same *T. cervi* PstI blot pictured in Figure 22 was probed with α -tubulin, the banding pattern was conserved except in the loss of the 2300 bp fragment (not shown). These results suggest that the *T. cervi* tubulin repeat blocks may also end with a truncated β -gene.

In addition to the conserved *T. cervi* PstI pattern, both reindeer and muledeer isolates demonstrate a more complicated partial digest pattern. The *T. cruzi* digest did not hybridize well and except for muledeer, appears to have produced the smallest fragments.

HindIII 881208, top α -tubulin. HindIII, BamHI and EcoRI each cleave once in the complete tubulin repeat units of *T. brucei* (Figure 17) and *T. rangeli*. Consequently, the results of these digests are considered to be indicative of the basic sizes of *T. brucei* and *T. rangeli* tubulin repeat units, which have been estimated as 3.65 and 3.8 kb, respectively (Thomashow et al., 1983; Esquenazi et al. 1989). To test the conservation of these sites in *T. cervi* and to aid in the

determination of the tubulin repeat unit size, HindIII and EcoRI genomic DNA digests were probed with the tubulin probes.

Except in the case of reindeer and muledeer, HindIII digests of *T. cervi* isolates probed with either α - or β -tubulin (Figure 22) resulted in a single, intense fragment of about 3.4 kb and a lighter partial fragment of about 4.2 kb which were common to all isolates. A larger partial band of about 9.3 kb was present in all tested isolates but muledeer. Although the actual size of these bands appears to differ slightly between isolates, the partial digest banding pattern suggest that, unlike *T. brucei*, *T. cervi* tubulin repeat units have at least two HindIII sites located in the β -tubulin subunit. An averaged fragment of about 840 bp is calculated from the difference between the 3390 bp terminal fragment and the 4230 bp partial. If a 4.2 kb tubulin repeat unit is accepted, then the 9.3 kb (9307 bp) HindIII partial would represent 2.2 tubulin repeat units.

A second intense band of about 4.5 kb is shared by both reindeer and muledeer. The actual size of the second band differed slightly between the two isolates and in addition to the 4.5 and 3.4 kb fragments, a third intense band of about 3.0 kb was unique to the muledeer digestion. Smaller β -tubulin specific bands (not shown) of about 2.4 and 2.6 kb can barely be seen in the reindeer and muledeer digestions, respectively. HindIII digests of the *Leishmania* sp. probed with α or β -tubulin resulted in terminal fragments of about 2.5 kb (2360 bp) which generally agrees with known values for Leishmania (Spithill and Samaras, 1987).

EcoRI 881208, top β -tubulin. As this photocopy demonstrates (Figure 23), EcoRI digests of *T. cervi* which are hybridized with β -tubulin also generally produce a single intense 'terminal' band of about

Figure 23. EcoRI digest of *T. cervi* isolates probed with β -tubulin. Isolates include muledeer (1), reindeer (2), moose (3), antelope (4) and O.R.Mt. elk (5). Exposed 12 d with an intensifying screen at -80°C . The blot was hybridized and washed at 65°C .

3.5 kb (3563 bp), as well as a collection of partial fragments.

Averaged values of 3826, 5245 and 5814 bp were derived for the three common partial fragments in the last three lanes. The differences between these partials results in calculated fragments of 569, 1419 and 297 bp. The calculated total of these fragments is 2249 bp. When this

figure is added to the 5814 bp partial band, a calculated partial of about 8064 results. Although this particular digest appears too complete to generally produce this calculated partial band, it could represent two tubulin repeat units of about 4032 bp.

As with HindIII digests, the muledeer and reindeer EcoRI results differ from the other *T. cervi* isolates in having a more complicated partialling pattern with additional (fainter) fragments smaller than the 3.5 kb unit size fragments. Muledeer produced two fragments of about 2.3 and 1.9 kb, while reindeer produced three fragments of approximately 2.8, 3.4 and 3.7 kb. The Oregon Rocky Mountain elk isolate also produced a single smaller fragment of about 2.2 kb.

Ribosomal RNA

Although the process of protein translation is still not fully resolved, it is generally accepted that ribosomal RNA (rRNA) may have a catalytic function that is structurally dependent upon highly conserved folding patterns (Figure 24). Thus, the gene sequences that code for

Figure 24. Model demonstrating secondary structure of *E. coli* large subunit (23S) rRNA. From Spencer et al. 1987.

the folded, double stranded portion of the rRNAs tend to diverge very slowly and are a good source of comparison between distantly related taxa.

In contrast, the single stranded loop portions and nontranscribed spacer sequences tend to evolve more rapidly. Their variation in size and number can provide a reference for comparison between closely related species and strains (Lane et al., 1985; Spencer et al. 1987; Dahlberg, 1989, Hide et al. 1990). Within a single gene unit, is the resource for the evaluation of phylogenetic relationships as well as specific affinities.

The cytoplasmic rRNA genes of all eukaryote organisms are highly repeated within the genome and functionally similar. Each eukaryote rRNA repeat unit is transcribed as a precursor molecule which is post transcriptionally processed into at least two high-molecular-weight rRNAs, the large subunit (l.s. subunit) and small subunit (s.s. subunit). Homologous l.s. subunit rRNAs of eukaryote organisms range in size between 25-28S while s.s. subunit rRNAs are 17-19S. Generally, two other subunits are also encoded in the rRNA repeat unit, the 5.8S and 5S rRNAs. Trypanosomatids differ considerably in that four extra rRNAs are produced from the rRNA repeat unit and are the smallest rRNAs know. In addition, the 5S rRNA is arranged in it's own block of repeat units and is not associated with the polycistronic rRNA repeat block. The discontinuous nature of trypanosomatid rRNA is thought to be a very ancient pattern that has been lost in many higher eukaryotes (Cordingley 1985; Spencer et al. 1987; Vieira de Arruda et al., 1990). Despite these differences, the basic organization of each *T. brucei* polycistronic repeat unit has been found to parallel the observed

pattern of other eukaryotes and is diagrammed in Figure 25, with the two rRNA genomic probes derived from *T. brucei* (Hasan et al. 1982; Spencer et al. 1987).

The basic rRNA cistron gene features include: 1) a nontranscribed spacer region; 2) the sequence encoding the small subunit RNA (s.s. RNA = 18S); 3) an internal transcribed spacer that contains the sequence encoding the 5.8S rRNA common to all eukaryotes; 4) the sequence encoding the large subunit rRNA (l.s. RNA = 28S); 5) an internal transcribed spacer (Hasan et al. 1982).

Figure 25. Restriction map and subclones of the *T. brucei* rRNA genes. From Hasan, et al., 1982. E=EcoRI, B=BglIII, H=HindIII, B=BamHI.

Normally, the transcribed *T. brucei* l.s. rRNA (= 28S) is 4.1 kb and further fragmented into two 'large' (2.3 kb and 1.8 kb) and four 'small' rRNAs, which range in size from 215 to 70 bp. One of the small rRNAs

comes from between the two larger products and the other three come from the 3' end of the 1.8 kb molecule.

The two subclones diagrammed in Figure 25 (pGH18 and pGH174) were produced through the work of Hasan, et al., (1982) and were used to explore the rRNA genes of *T. cervi* isolates. pGH18 is a 3 kb BglII genomic fragment that includes a small part of the 2.3 kb l.s. segment, the internal small rRNA, the complete 1.8kb l.s. segment, the three other small rRNAs as well as some spacing sequence of the *T. brucei* rRNA transcription unit. pGH174 is a 5.0kb BglII genomic fragment of *T. brucei* MITat 1.6 which contains the rest of the rRNA cistron including all of the 3000 bp s.s. rRNA, the 5.8S rRNA and most of the 2.3 kb l.s. rRNA.

The cytoplasmic rRNA genes of all eukaryote organisms are highly repeated within the eukaryotic genome but they are variably distributed and arranged. The rRNA repeat units of *T. brucei* alternate with spacer sequences and are organized in large blocks which are typically located among several of the larger chromosomes (Van der Ploeg et al., 1984). To determine the chromosomal location of the *T. cervi* rRNA repeat unit blocks, the following blots (Figure 26) were probed with pGH18. The results indicate that *T. cervi* rRNA repeat unit blocks are intraspecifically variable in chromosome distribution. Three basic patterns are apparent; (1) rRNA blocks located on two elements on either side of the 850 to 1050 kb break. This pattern is seen in reindeer, NM.R.Mt. elk and O.R.Mt. elk. (2) rRNA blocks located on two elements above the 850 to 1050 kb break. This pattern is characteristic of Roosevelt elk, antelope and moose (antelope and moose not shown). (3) a single rRNA block located on a single element above the 850 to 1050 kb

break. This pattern is characteristic of the muledeer isolate.

Extended exposure of this blot (not shown) reveals one and possibly two additional muledeer elements which are located below the 850 to 1050 kb break. The very weak hybridization of the probe indicates that the rRNA repeat units of these elements are in low copy number or have low homology to the probe.

Figure 26. Hybridization of a rRNA gene probe pGH18 with 55 sec. PFGE separated chromosomes. Polaroid and Southern blot autoradiographs of the 0.6% PFG MR5, 880723 (A) and E11, 880727 (B). Samples include: (A) *L. braziliensis* *braziliensis* (1), *T. cervi* isolates from; muledeer (2) and reindeer (3); (B) *L. braziliensis* *braziliensis* (1), *T. cervi* isolates from; O.R.Mt. elk (2), NM.R.Mt. elk (3) and Roosevelt elk (4). The DNA was resolved in 20 hours with 300V. 3.9×10^5 cts/ml, 2.8×10^7 cts/ μ gDNA. Blots were hybridized and washed at 65°C.

To characterize the restriction sites and fragment sizes of the transcribed portion of the *T. cervi* rRNA repeat unit, Southern blots of the BglII, EcoRI and HindIII *T. cervi* genomic DNA digests were probed

with either of the two rRNA probes, pGH174 or pGH18. Perhaps *Leishmania* sp. did not hybridize well with the probe pGH18 because the majority of pGH18 codes for many of the small subunits which are unique to *T. brucei*. These results are presented in Figure 27. The basic organization of rRNA genes is very similar in all eukaryotes but due to

Figure 27. Hybridization of rRNA gene probes with genomic digests BglII, HindIII and EcoRI. Southern blot autoradiographs of BglII/pGH174 (left) includes *T. brucei* (1), *T. cervi* isolates from; NM.R.Mt. elk (2), O.R.Mt. elk (3), Roosevelt elk (4), antelope (5), moose (6), reindeer (7), muledeer (8) and *T. cruzi* (9). 12 d exposure at RT. HindIII/pGH18 (center) includes *L. donovani chagasi* (1), *L. braziliensis guyanensis* (2), *L. mexicana amazonensis* (3), *L. mexicana* (4), *T. cervi* isolates from; O.R.Mt. elk (5), antelope (6), moose (7), reindeer (8) and muledeer (9). EcoRI/pGH18 (right) includes *T. cervi* isolates from; O.R.Mt. elk (1), antelope (2), moose (3), reindeer (4) and muledeer (5). 2.8×10^7 cts/ μ g, 3 d exposure at -80°C with and intensifying screen All blots were hybridized and washed at 65°C . The probe was applied to the *T. cervi* blot at a concentration of 1.7×10^6 counts per ml, . Exposure was at -80°C with and intensifying screen for one day.

differences in the nontranscribed spacing sequence, the size of the actual repeat unit can be very diverse. For example, from the results

of EcoRI, HindIII and SalI digests, Hasan et al., (1982) estimate that the repeat unit of *T. brucei* is at least 21 kb and that the transcribed portion of the unit is about 18 kb. The Leishmania repeat unit is thought to be about 17 kb, of which 8 kb is transcribed (Ramirez et al. 1987). The repeat unit of *T. cruzi* is now thought to be about 30 kb (de Arruda et al. 1990).

BglII 880722, pGH174 (left panel): When probed with pGH174, BglII digested *T. brucei* produced two partials of about 20 and 10 kb and a single intense band at about 5.4 kb (Figure 27), which is in approximate agreement with the value (5 kb) obtained by Hasan et al. (1982). No BglII values have been published for *T. cruzi* but a single fragment of about 6.5 kb resulted with no partials. The *T. cervi* isolates were uniformly represented by a single band at about 5.0 kb and a partial band of about 19 kb. Probing the same blot with pGH18 (not shown) produced a common *T. cervi* band of about 7 kb and the same high molecular weight partial. Neither of the other two species produced bands with this probe except for the signal above 23.1kb which was common to both probings of this blot and is probably undigested sequences. The overall results of this blot suggest that there are at least three BglII sites in the *T. cervi* rRNA gene repeat and demonstrates the uniform conservation of these sites between *T. cervi* isolates. Summation of the probed fragments suggests a transcribed sequence unit of no more than 12 kb. If this value is subtracted from the 19 kb partial fragment, a 7 kb nontranscribed spacer sequence is calculated.

HindIII 881208, pGH18 (central): In addition to a 6.5 and 19 kb partial, a common 4.8 kb band was produced when *T. cervi* HindIII digests

were probed with pGH18, although muledeer produced a slightly smaller fragment. These results suggest that there are at least three HindIII sites in the *T. cervi* rRNA cistron. The pGH174 probe was not used on this blot. HindIII sites were conserved between *Leishmania* species as well but a smaller 3.25 kb band resulted.

EcoRI 881208 pGH18 (right): *T. cervi* isolates digested with EcoRI and probed with either pGH18 or pGH174 produced identical results. However, diverging banding patterns demonstrate differences in the *T. cervi* group. A single intense band of about 8 kb and a 19 kb partial were common to the elk, moose and antelope isolates but reindeer and muledeer patterns lacked the intense 8 kb terminal band and were composed of a series of smaller and less intense bands. Both the reindeer and muledeer digests produced as many as 6 bands ranging from 8 to 2.0 kb and distributed in a reproducible but uneven pattern. While the muledeer array contained the most fragments and a more intense terminal band of about 3.2 kb, reindeer produced no intense 'terminal' band and the smallest fragment, about 2.0kb.

T. brucei 5' VSG 76 bp barren region repeat

Successive waves of *T. brucei* parasitism are possible only through access and expression of new variant surface glycoprotein (VSG) genes in the telemetric expression site. Although expression of a mosaic VSG is possible through recombination within the VSG gene, total gene conversion is critical for the expression of unique VSGs. The significance of the 76 bp repeat (probe = pGE117.6) lies in its 5' telemetric and internal position to VSG genes. Because of these locations, the 76 bp repeat provides sites for recombination and

functions in gene conversion through crossover mediated transposition events (see figure 28).

Figure 28. Location and telemetric interaction of the *T. brucei* 76 bp barren region repeat. From Campbell et al., 1984 and Van der Werf et al., 1990.

Although the results of this experiment were too dark to reproduce, the 76bp repeat probe did hybridize convincingly to a large fragment (>23kb) of *T. brucei* and all *T. cervi* isolates digested with *Sma*I (881208). However, none of the *Leishmania* sp. or *T. cruzi* samples hybridized with the 76bp repeat probe. On the basis of these results, it can be stated that *T. cervi* has sequences similar to the 76bp repeat probe. In addition, since neither *Leishmania* or *T. cruzi* are known to be capable of antigenic variation (Campbell et al., 1984) it is not surprising that there was no hybridization to *Leishmania* and *T. cruzi*. These results tend to support the importance of the 76bp repeat region in gene conversion events and suggest that *T. cervi* might be at least

partially equipped with the means. The probe was applied to the *T. cervi* blot at a concentration of 2×10^6 counts per ml, 2.8×10^7 cts/ μ g DNA. The blot was hybridized and washed at 42°C and 47°C respectively. Exposure was at -80°C with an intensifying screen for one day.

T. brucei 5' INGI/RIME retroposon sequence

Many of the abundant repetitive sequences of *T. brucei* are found in blocks of tandem arrays (76bp repeats, mini-exon, satellite DNA, etc.). However, this probe (pLY6HRv) contains a sequence which represents two of the most abundant dispersed repetitive sequences in the *T. brucei* genome. The importance of repeated elements lies in the fluid dispersion and rearrangement of genetic information. Their dispersed nature provides an abundance of recombinational 'hot spots' for mutation or superinfection that could result in rearrangement of flanking sequences or act in trans to promote other genes (Smiley et al., 1990). Ingi means 'many' in Swahili and is a 5.2kb element thought to have older origins than the 0.5kb RIME (Ribosomal Mobile Element). Both elements form short flanking insertion site duplications derived from sequences at their site of insertion. Other structural and transcriptional characteristics suggest that the elements belong to a family of retroposons (Long Interspersed Nuclear Elements) found in high copy number in the mammalian genome. It is thought that *T. brucei* may have acquired the element from either its mammalian or insect host (Hasan et al., 1984; Murphy et al., 1987, Kimmel et al., 1987). In this probe, (probe = pLY6HRv) one mobile element (RIME) appears to have

inserted into the other (Ingi). This provides a probe for both sequences (pLY6HRv) which was used on the blot represented in Figure 29.

Figure 29. Hybridization of the *T. brucei* 5' INGI/RIME retroposon sequence with 42 sec. CHEF separated chromosomes.

Leishmania sp. and *T. cervi* isolates did not hybridize with this probe (Figure 29). Perhaps the lack of these elements helps to explain the apparent limited antigenic variability of *T. cervi* and other stercorarians.

CHAPTER DISCUSSION

The results of both tubulin and rRNA analysis are based on the use of heterologous probes which represent only a portion of the respective repeat units. The missing sequences are the 3' end of the β -tubulin subunit and the nontranscribed spacing portion of the rRNA repeat unit. Consequently, any restriction sites which map within these omitted regions will not be specifically defined by these results. Indirect evidence of these sites is the benefit of incomplete digestion of the genomic DNAs. The result is a lighter pattern of bands which are characteristically larger than the intensely hybridizing terminal fragment. These lighter bands are commonly called partial products and reveal the intermediate breakdown products of the digestion. The 'partials' provide many of the conclusions presented here.

Tubulin: Tubulin analysis of the combined REFLP and PFG results substantiate the *Trypanosoma* taxon designation for the general *cervi* group and distinguishes *T. cervi* from other genera and species. The conclusion that a standard tubulin repeat unit size is generally conserved among all *T. cervi* isolates and is organized in blocks of alternating subunits is based on the following line of evidence. The subunits are known to be located on the same chromosomes because both tubulin subunit probes hybridize to the same element (Figures 19 and 20). *Dra*I ('null') restriction established clustering of the genes in large blocks. The alternating subunit pattern was established by REFLP results in which both probes hybridize to the same terminal fragments (Figure 22).

The *T. cervi* tubulin repeat unit size of 4.1-4.2 kb was estimated by adding the calculated differences of partial fragments. This value differs from those of representative genera and species and probably reflects variability in the nontranscribed spacing region (see Table VIII, Chapter VI). Considering the highly conserved nature of the transcribed tubulin genes, it would not be surprising if the distribution and relative orientation of both HindIII and EcoRI restriction sites within the tubulin repeat unit (one per unit) was an ancestral motif which is generally maintained throughout the Trypanosomatidae. According to the literature, this appears to be true in the case of *T. brucei*, *Leishmania* sp. and *T. rangeli*. This is not the conclusion for *T. cervi* in which at least two fragments are evolved in each of these digestions.

Divergence of the muledeer and reindeer isolates from the *T. cervi* pattern has again been demonstrated in this investigation. Their relative genetic complexity is immediately reflected in the karyotype hybridization profile. Except in the case of the muledeer isolate, *T. cervi* tubulin genes are clustered on at least one of several elements which are generally located below the 850 kb chromosome profile break (Figure 18). Within this pattern, most of the *T. cervi* isolates differ only in the size and/or number of tubulin bearing elements. The muledeer isolate is sharply unique in having at least four tubulin bearing elements, three of which are located at or above the 850/1050 kb profile break. The reindeer isolate holds to the *T. cervi* pattern but has a third tubulin bearing element which is located above the primary elements on the lower margin of the 850 kb break.

In addition to the generally conserved *T. cervi* tubulin repeat unit 'allele' of 4.1-4.2 kb, REFLP analysis discloses at least one other tubulin unit isotype in muledeer and reindeer isolates. HindIII digests of both muledeer and reindeer isolates which were probed with the tubulin subunits share an intense terminal band of about 4.35 kb. In the case of muledeer, a third, equally intense band of about 3.3 kb also results (Figure 22) and the band total, including partials is six or exactly twice that of the conserved pattern. EcoRI digestions probed with β -tubulin completely eliminated the 3.3 kb band in muledeer and only the 'conserved' 3.5 kb fragment persists in all isolates with equal intensity. While the 4.35 kb band common to both muledeer and reindeer isolates is present on the EcoRI autoradiograph, it is somewhat reduced in intensity and several smaller fragments have been added to the array (Muledeer = 2.3 and 1.9 kb; Reindeer = 3.3, 3.2 and 2.5 kb). In contrast with the conserved EcoRI pattern, these results suggest the release of terminal truncated genes and/or flanking regions from the 4.35 kb fragment and the recombinant composition of the 3.3 kb fragment. It is not known if these divergent tubulin sequences have different developmentally regulated functions and/or are mutated pseudogenes. Probing the EcoRI blot with α -tubulin would Perhaps determine the composition of the smaller EcoRI generated fragments and help resolve these questions.

The combined results of muledeer karyotype pattern, PFG signal intensity and DraI ('null') restriction suggest that the tubulin unit cluster blocks are arranged in loci of progressively smaller, non-allelic elements which decline in repeat copy number and perhaps homology to the probes as well (Figures 20 and 22). It is possible that

the muledeer tubulin isotypes might have been generated and distributed through a combination of chromosome deletion, translocation, breakage and/or unequal crossover events. These possibilities could be investigated by isolating the tubulin isogene fragments and subsequently sequencing and using them as probes. This would also help clarify the identity of the elements which carry the conserved allele block or the isogenes. This approach would be helpful because the number of hybridizing elements within a profile and the intensity of the hybridization signal of a particular probe do not necessarily provide a reliable indication of isogene diversity within a genome. This is an issue because trypanosomes are known to be anuploid and in some cases, the intensity of a signal might be largely due to the non-stoichiometric representation of a particular element within a profile. Consequently, a sequence of low homology with the probe but magnified by chromosome polyploidy might dominate the signal of a highly homologous sequence located on a haploid element. For this reason, signal intensity can be misleading with respect to probe homology. For example, the electrokaryotypes of both moose and antelope (Figure 18) contain at least two elements bearing tubulin blocks of equal intensity. REFLP analysis reveals only one tubulin unit type and the single band produced by *Dra*I ('null') restriction suggests that the number of repeat units is the same on each chromosome. Thus, in this case, the equivalent intensity of the hybridizing elements is substantiated by the cumulative results.

rRNA: It is difficult to come to any conclusions concerning the characterization of the complete *T. cervi* rRNA repeat unit because the probes used were homologous to the coding region only. Complete

analysis would require more digestions and the use of a probe specific for or including the nontranscribed region of the rRNA repeat unit. Based on the evidence provided by terminal and partial fragments, it appears that the rRNA cistron unit size is uniform between isolates and is approximately 19 kb. The authors (Vieira de Arruda et al., 1990) feel that the single *T. cruzi* rRNA cistron may be 30 kb while Hasan et al., (1982) postulate a 21 kb rRNA cistron for *T. brucei*.

Examination of several *T. brucei* serodemes with pGH174 by Hasan et al., (1982) suggests an invariant arrangement of rRNA genes between the majority if *T. brucei* clones. In general, probed EcoRI digests of *T. brucei* that have been probed with pGH174 result in a strong 8 kb band as well as faint hybridization to a 7 kb band. pGH18 hybridizes to the 7 kb band. Only one EcoRI site has been mapped in *T. cruzi* and results in a fragment larger than 20 kb. EcoRI digested *T. cervi* that has been so probed (Figure 27) results in a pattern that is uniform in most isolates tested and similar to that seen in *T. brucei* except that both probes hybridize to the same 8 kb band. If it is assumed that there are actually two comigrating 8 kb bands, then a 16 kb transcribed region may be calculated. On the other hand, combined BglII results provides 5 and 7 kb bands, totalling a 12 kb transcribed region. The smaller value is probably closer to the actual size of the transcribed region, which would correspond to a 7 kb nontranscribed region in a 19 kb rRNA cistron. It appears that the rRNA cistron and most of the tested restriction sites are conserved in all *T. cervi* but both muledeer and reindeer appear to deviate when digested with EcoRI (Figure 27).

Reindeer and particularly muledeer are unique among the *T. cervi* isolates in the divergence and diversification of both tubulin and rRNA

genes. Perhaps the multiple unlinked chromosomal loci of the muledeer and reindeer tubulin gene families were generated through simple chromosome breakage but the apparent diversification of tubulin and rRNA isogenes reflects recombinational events. Even though *T. cervi* does not contain the *T. brucei* INGI/RIME retroposon sequence (but does have the 76 bp repeats), the results presented here suggest that other means exist for generating the recombinant diversification demonstrated in such highly conserved sequences. The added genomic complexity may have been generated by one or several mutagenic events within the tandem array blocks and subsequent recombination mediated dissemination of the isogene sequences.

CHAPTER VI

FINAL DISCUSSION

To my knowledge, this work presents original information which serves to support the classification of and further characterize the stercorearian *Trypanosoma cervi*. The general *T. cervi* motif of chromosome profile, homologous elements, minicircle size, REFLPs and highly repeated sequences are approximate values which are based on those of the Rocky Mountain elk isolates. All isolates tested including the antelope trypanosome, generally conform to this pattern of characteristics which differ from those of other genera and species. Many of these features are presented in Table VIII in comparison with published features of other Trypanosomatidae. Similar studies of stercorearian species include those of Morel et al. 1980; Van der Ploeg et al. 1984a; Aymerich and Goldenberg, 1989 and Esquenazi et al. 1989.

While the *T. cervi* motif is generally conserved throughout the group, this study does demonstrate a range of intraspecific variability, which extends even to isolates of discrete populations of the same host species; Wyoming Rocky Mountain elk transplants of Oregon and New Mexico. The transplant stocks were taken from the Wyoming Jackson Hole herd at least 30 years ago. In all cases, the most highly conserved characteristics were minicircle size and REFLPs, both of which may prove to be diagnostic characteristics of *T. cervi*. On the other hand, specific numbers and sizes of elements within the chromosome profile, size and number of homologous elements and pattern of highly repeated

DNA sequences are variable characteristics which may be used to determine intraspecific affinities.

TABLE VIII
COMPARISON OF CHARACTERISTICS IN DIFFERENT TRYPANOSOMATIDAE

FEATURE	<i>L. species</i>	<i>T. cervi</i>	<i>T. brucei</i>	<i>T. lewisi</i>	<i>T. cruzi</i>
CHROMOSOME PROFILE	^c 22-33: 200-2200 kb	19-25: 275-2200 kb	3(+): 1.4-4.7 Mb 1(+): 350 kb many: 50-150 kb	3(+): 1.4-4.7 Mb 8(+): 150-450 kb many: 25-100 kb	^d 15-18: 500-1500kb
MINICIRCLE SIZE	^f ~870 bp	^e 2500 bp	^e 1000 bp	^e 1020 bp	^e 1600 bp
TUB. UNIT ORGANIZ.	^a subunits separate	subunits alternate	^g subunits alternate	ND	^b subunit alternate
TUB. UNIT SIZE	^a 2.5 kb= α 3.9 kb= β	4.1-4.2 kb	^g 3.6-3.7 kb	ND	^b 4.3 kb
rRNA UNIT SIZE	^h 17 kb 8 kb trans.	19 kb 12kb trans.	ⁱ 21 kb 18kb trans.	ND	^j 30 kb
76 bp REPEAT	-	+	+	ND	-
INGI/RIME SEQUENCE	-	-	+	ND	ND

trans.= transcribed, TUB.= tubulin, ORGANIZ.= organization, ND = not done.

^a Spithill and Samaras, 1987.

^b Soares et al., 1989.

^c Samaras and Spithill, 1987; and Scholler et al., 1986.

^d Aymerich and Goldenberg, 1989.

^e Clayton, 1988.

^f Simpson, 1986.

^g Thomashow et al. 1983.

^h Ramirez and Guevara, 1987.

ⁱ Hasan et al., 1982.

^j Vieira de Arruda et al., 1990.

The collective data indicate that the muledeer isolate in particular differs from the other tested *T. cervi* strains. Support for this observation is based on the following criteria:

1. Minicircle size and restriction sites. The *T. cervi* minicircle (Figure 16) is approximately 2500 bp but that of the muledeer isolate is at least 115 bp smaller. Of the six enzymes used, only EcoRI cuts

(linearizes) the *T. cervi* minicircle. The muledeer isolate minicircle lacks all *EcoRI* sites and appears to have several of each *PstI* and *SmaI* restriction sites.

2. Highly repeated sequences demonstrated in genomic digests. The muledeer isolate consistently differed in all genomic digests demonstrating specific features. *BamHI* digestion revealed bands common to only muledeer and reindeer isolates and *DraI* produced the most unique muledeer deviations from the *T. cervi* pattern.

3. Chromosomal distribution of tubulin genes. The tubulin genes of all isolates but muledeer are clustered on chromosomes located below the 850 kb profile break (Figure 18). The muledeer isolate has at least four tubulin bearing elements, three of which are located above the 850/1050 kb profile break (Figures 18 and 20). *T. cervi* rRNA gene loci are generally clustered on at least two chromosomes which are variously distributed in the profile. The rRNA genes of the muledeer isolate are located on a single element (Figure 26).

4. Restriction site differentiation of rRNA and tubulin genes. The *DraI* ('null') digestions (Figure 22) indicate that the muledeer isolate tubulin repeat unit either contains internal *DraI* sites or tubulin cluster loci with progressively fewer repeat units. While the muledeer isolate appears to have the *T. cervi* common 4.1-4.2 kb tubulin repeat unit allele, it also has at least one other allelic type or pseudogene (Figure 22). The majority of rRNA probed digestions again demonstrate that the muledeer conforms to the *T. cervi* pattern but the *EcoRI* digestion (Figure 27) indicates that differences exist between the *T. cervi* rRNA allele and the additional muledeer rRNA isogenes.

The *T. cervi* reindeer strain also differs to a lesser degree with respect to tubulin and rRNA gene polymorphic sites and chromosomal distribution however, unlike muledeer, the reindeer minicircle conforms to the *T. cervi* standard. The observed divergence of both reindeer and muledeer appear to represent a gradient of variability which may ultimately result in separate specific designations, especially in the case of the muledeer trypanosome. To justify such action, further characterization and sequence analysis would be required.

There is value in the characterization of low risk trypanosomes in addition to those that are eminently lethal. In many long standing host/parasite associations, coevolution has culminated in compromised commensalism and ultimately, mutualism. Many well adapted parasitic associations may be easily tolerated by the host but when combined with the stress of overcrowding, malnutrition or coinfection with other organisms, death can result (Schmidt and Roberts, 1985). This appears to be the trend of stercorarian trypanosomes and in this case, *T. cervi*. The importance of such a relationship does not lie in the threat of pathogenicity, rather in the potential for clinical application. Although the process would require extensive study and experimentation, such a cosmopolitan parasite could be engineered for the purposes of gene therapy.

In contrast, the subgenus Trypanozoon is currently undergoing a period of rapid evolution which may culminate in the development of a life cycle with a single vertebrate host (Hasan et al., 1984). This evolutionary burst may be partially due to infection with mobile genetic elements. The salivarian specific collection of mobile elements may be unique to the African continent, and because of their lack of sequence

divergence, it is thought that they may have very recently contributed to trypanosome evolution (Weiner et al., 1986 and Murphy et al., 1987). Man's infection with trypanosomes is also a recent phenomenon, one which is still evolving. This is true of both stercorearian and salivarian trypanosome infections and although it would seem that the subgenus *Trypanozoon* has been provided with a powerful driving force in evolution, it could well be one that ultimately leads to its own extinction. On the other hand, the benign nature of stercorearian trypanosomes may in the future prove to be the vehicle of prevention and cure.

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APPENDIX A

SOLUTIONS

APPENDIX A

SOLUTIONS

Unless otherwise stated, all listed preparations are water based solutions used in the techniques described throughout this paper. The solutions are listed in alphabetical order.

DENATURING BUFFER: 0.5 M NaOH, 1.5 M NaCl.

HYBRIDIZATION BUFFER: The hybridization buffer for nick translated probes was composed of 5x SSPE, 1% SDS and 50 $\mu\text{g/ml}$ salmon sperm DNA heated at 100°C, 10 min.

NET BUFFER for genomic DNA: 100 mM NaCl, 200 mM EDTA, 50 mM Tris, pH 7.5, autoclave.

NET BUFFER for kinetoplast DNA: 100 mM NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 7.5, autoclave.

PHOSPHATE BUFFERED SALINE (0.06M PBS): 0.057 M Na_2HPO_4 , 0.0037 M NaH_2PO_4 , 0.0436 M NaCl, pH 8.0, autoclave.

PHOSPHATE BUFFERED SALINE GLUCOSE (PBSG): PBS + sterile 10% glucose to 1% (w/v) final concentration.

PREHYBRIDIZATION BUFFER: 5x SSPE, 1% SDS, 100 $\mu\text{g/ml}$ salmon sperm DNA denatured at 100°C, 10 min.

PRIMARY LYSIS BUFFER: 0.5 M EDTA (pH 9.5), 1% SDS and 2 mg/ml

Proteinase K (Bethesda Research Labs).

SECONDARY LYSIS BUFFER: 0.5 M EDTA (pH 9.5), 1% SDS and 1 mg/ml

Proteinase K (Bethesda Research Labs).

SSC BUFFER (20x): 3 M NaCl, 0.3 M Na₃Citrate, pH 7.5, autoclave.

SSPE BUFFER (20x): 0.16 M NaOH, 0.2 M NaH₂PO₄, 20 mM Na₂EDTA, pH 7.0,
3.6 M NaCl, autoclave.

TBE BUFFER (10x): 10X TBE = 108gm Tris-base, 55gm boric acid 9.3 gm
Na₂EDTA, to 1 liter.

TE BUFFER: 10 mM Tris, 1 mM EDTA, pH 8, autoclave.

TRIS BUFFER FOR PHENOL SATURATION, K DNA: 50 mM Tris HCL, 0.1 M NaCl,
pH 7.5, autoclave.

TRYPANOSOMA DILUTION BUFFER: 5 mM KCl, 80 mM NaCl, 1 mM MgSO₄, 20 mM
Na₂HPO₄, 2 mM NaH₂PO₄, 10 mM glucose, pH 7.5, filter sterilize.

VERSENE SALINE, 5X HEPES-NaOH BUFFERED, pH 7.5: 17.88 gm HEPES, 3.60 gm
glucose, 1.10 gm KCl, 37.95 gm NaCl, 0.70 gm Na₂HPO₄, 1.00 gm EDTA
disodium salt, dihydrate, 20 ml Phenol red (0.5%), 800 ml ddH₂O, adjust
pH to 7.5 with NaOH (approximately 60 'beans'), adjust to 1000 ml,
filter sterilize.

APPENDIX B

ABBREVIATIONS

APPENDIX B

ABBREVIATIONS

°C - degrees Centigrade

cpm - counts per minute

DNA - deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

EtBr - ethidium bromide

EtOH - ethanol

FCS - fetal calf serum

DMSO - dimethyl sulfoxide

ddH₂O - double distilled water

g - gravity

gm - grams

MEM - minimal essential medium

mg/ml - milligrams per milliliter

min - minutes

ml - milliliters

M - molar

mM - millimolar

MW - molecular weight

NET - NaCl, EDTA, tris

PBS - phosphate buffered saline

PBSG - Phosphate Buffered Saline Glucose

PFGE - pulse field gel electrophoresis

SDS - sodium dodecyl sulfate

SSC - sodium chloride, sodium citrate

SSPE - sodium hydroxide, sodium phosphate, EDTA

TBE - tris, boric acid, EDTA

TDB - trypanosoma dilution buffer

TE - tris, EDTA

TCA - trichloroacetic acid

Tris - tris (hydroxymethyl) aminomethane

U/ml - units per milliliter

μg - micrograms

$\mu\text{g/ml}$ - micrograms per milliliter

μm - microns

V - volts

v/v - volume per volume

w/v - weight per volume