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GENE EXPRESSION ASSOCIATED WITH VIRULENCE IN THE AMOEBA NAEGLERIA FOWLERI

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GENE EXPRESSION ASSOCIATED WITH VIRULENCE IN THE AMOEBA *NAEGLERIA FOWLERI*

By

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ABSTRACT

GENE EXPRESSION ASSOCIATED WITH VIRULENCE
IN THE AMOEBA NAEGLERIA FOWLERI

By

Wang-nan Hu

Naegleria fowleri is an opportunistic pathogen causing primary amoebic meningoencephalitis (PAM) in humans. Protein synthesis patterns of low-virulence amoebae from axenic culture, after mouse brain passage to increase virulence, and after growth on bacteria were analyzed by two-dimensional gel electrophoresis. Comparisons of accumulated proteins, in vivo-synthesized proteins, and in vitro-synthesized proteins translated from poly(A)+ mRNA were made. Differences between amoebae from the different treatments were noted. The increase in virulence was correlated with numerous specific changes in protein synthesis. To identify transcripts differentially expressed in high virulent versus weakly virulent amoebae, a cDNA library was made using mRNA from amoebae recovered from mouse brains. By differential screening the cDNA library with probes made from total RNA from axenic amoebae and from high virulence amoebae, three cDNA clones were isolated. The transcripts homologous to clone Nf314 and Nf116 were preferentially expressed in high virulent cells whereas the transcript homologous to clone Nf435 was preferentially expressed in weakly virulent cells. Further sequence

analysis showed that Nf314 cDNA had an open reading frame for a 53-kDa protein 94% similar and 19% identical over 194 amino acid residues to serine carboxypeptidase from yeast cells, barley, and wheat. Southern blot analysis of Nf314 suggested that there was a single gene in the genome with unknown copy numbers. The increased Nf314 transcript levels were present in cells fed on mouse brain, liver, or NIH3T3 fibroblasts but not in cells fed on bacteria or in axenic culture. Thus, the inducer of the increased gene expression correlated with the use of mammalian cells as a food source without regard to the level of virulence. Clone Nf435 had a short open reading frame encoding 107 amino acid residues. This sequence was 34% identical to troponin C in 47 amino acids. Clone Nf116 had an open reading frame of 375 amino acid residues encoding actin. These clones Nf116, Nf314, and Nf435 were associated with virulence but not responsible for virulence. Amoebae fed on dissociated mouse brain cells expressed the above genes although they were weakly virulent.

In addition, a new cDNA cloning method was described. In this method, adding adapters, linkers or enzyme digestion are no longer necessary after cDNA synthesis.

DEDICATION

To

My grand parents,

Dad and Mom,

Jimei and Jin

ACKNOWLEDGEMENTS

I am most grateful to thank Dr. R. Neal Band, my major professor, for his guidance, encouragement and support for the successful completion of my research. I would like to thank Dr. Will Kopachik for his guidance, allowing me to work in his Lab, teaching me molecular biology techniques and data analysis. I also would like to thank my other committee members: Dr. R. Brubaker, Dr. T. Friedman and Dr. D. Jump for their excellent guidance and assistance. Special thanks to Mrs. Band for her kindly help to me and my family.

I would like to thank my grand parents, Xiang-gai Hu and Jian Wang, who I regret passed away before they could see me to earn my doctorate; my parents, Bin Hu and Xian-ying Song, my uncles Bun-choi Wu, Bill Pu-choi Woo and their wives, who made my dream come true to study in the United State of America and for their love, encouragement and support during my study. I would like to thank my wife, Jimei and my daughter, Jin for their love, understanding and support. I would like to thank my cousin W. Wayne Wu and his wife for their great help during my first two years in the Michigan State University. Without all of people I have mentioned, the work would be impossible to complete.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Introduction

Chapter One

Chapter Two

Chapter Three

INTRODUCTION

The small, free living amoebae, that are pathogenic to man, belong to two common genera, Naegleria and Acanthamoeba (Page, 1988). They occur world-wide and have been isolated from a variety of habitats. Both are regarded by clinicians and microbiologists as exotic organisms in infections such as primary amoebic meningoencephalitis (PAM) (N. fowleri), granulomatous amoebic encephalitis (GAE) and non-fatal but painful, infections of the human cornea, keratitis (Acanthamoeba spp.) (Ma et al., 1990, Lambert, 1991, Visvesvara, 1990). Although both infect the central nervous system, the routes of invasion and penetration by amoebae are different. Pre-acquisition of most human infections of PAM are aquatic-related due to free-living amoebae or cysts entering the nasal cavity. Invasion and penetration in the case of GAE appears to be hematogenous, probably from a primary focus in the lower respiratory tract or the skin (Ma, et al., 1990).

The genus Naegleria (Sarcodina, Vahlkampfiidae) consists of free-living amoebaflagellates widely distributed in soil and freshwater where they feed on bacteria and fungi (Page, 1988). The life cycle includes three stages: trophozoite, flagellate and cyst (Fig. 1). Naegleria can transform from trophozoite to flagellate (Fulton, 1970, Schuster, 1979) while in contrast Acanthamoeba has two

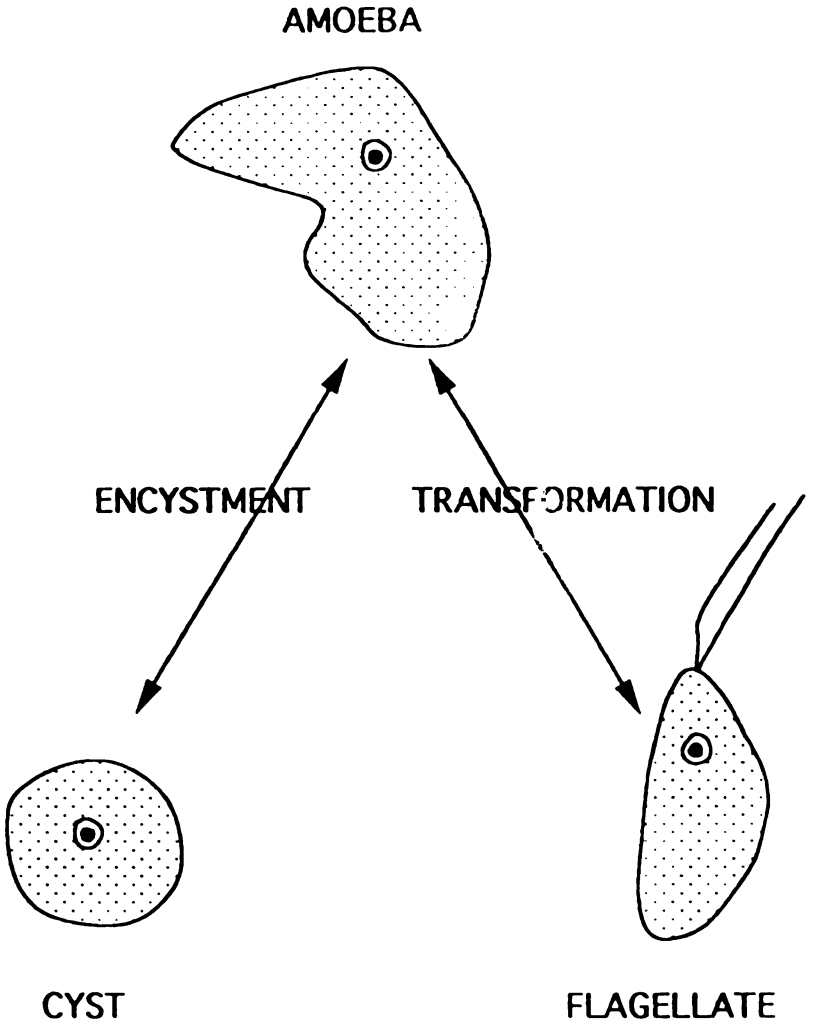
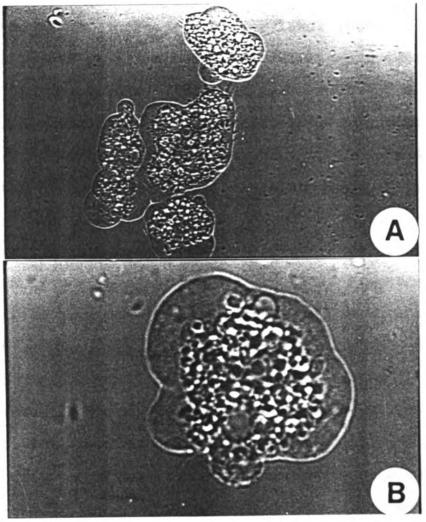


Figure 1. Three stage life cycle of Naegleria

stages and cannot transform to a flagellate stage. There are six species of Naegleria: N. australiensis, N. fowleri, N. gruberi, N. lovaniensis, N. jadini and N. thorntoni (Marciano-Cabral, 1988). Naegleria are studied because the flagellate transformation serves as a model system in gene regulation and developmental biology while some species are of medical importance as facultative pathogens.

Species N. fowleri (Fig. 2) is an opportunistic pathogen, causing PAM, a rapidly fatal disease of the central nervous system (CNS). The disease was detected in man in Australia (Fowler et al., 1965). The amoeba was named in honor of Dr. Malcom Fowler who first recognized the disease. The term "primary amoebic meningoencephalitis" was used to distinguish infection of the CNS in humans from the rare invasion of the brain by the intestinal amoeba Entamoeba histolytica (Duma, 1991; Marciano-Cabral, 1988) or by Acanthamoeba. The conditions under which N. fowleri becomes an opportunistic pathogen of men are not known. The disease usually occurs in healthy children and young adults with a history of swimming in freshwater lakes or ponds (John, 1982). The infection possibly results from the introduction of water containing amoebae into the nasal cavity and subsequently amoebae penetrate the nasal mucosa to the CNS via the olfactory nerves. The disease is rapidly fatal, usually producing death within 72 hr after the onset of symptoms (John, 1982) and to date, an effective treatment

Figure 2. Amoebae of Naegleria fowleri LEE strain in axenic culture viewed with Ziess microscope. A. X 2,000 B. X 5,000


A
B

is still lacking (Ma et al., 1990). Although it is a rare disease, PAM has been reported in many countries. Human PAM infections have resulted from contact with amoebae from a variety of environmental sources such as swimming pools (Cerva and Novak, 1968), freshwater lakes (Butt et al., 1968; Callicott et al., 1968), thermal streams (Cursons et al., 1976), mud puddles (Apley et al., 1970) and a household water supply (Anderson and Jamieson, 1972). Isolation of N. fowleri from thermal effluents, hot springs, and waters with naturally or artificially elevated temperatures up to 42°C are common. Since the first report of PAM, there have been more than 140 cases reported (Visvesvara, 1990).

Identification of N. fowleri isolated from fresh water has utilized a variety of methods. For example, after presumptive Naegleria spp. are identified by characteristic morphology and flagellate formation, the next step is to grow the sample in enrichment culture medium (Aufy, et al., 1986) at 43°C. Testing the pathogenicity with mice is a another critical step. If the isolate can kill mice, it probably is N. fowleri. Other methods can be added as an aid for further identification, such as isoenzyme analysis (Adams, et al., 1989; De Jonckheere, 1982; Kilvington, et al., 1985), restriction fragment length polymorphism of genomic DNA (McLaughlin, 1988) or mitochondria DNA (Milligan, 1988) and electron microscopy (Stevens, et al., 1980).

Unlike the obligate parasite E. histolytica, N. fowleri is a free-living amoeba. Therefore, what makes N. fowleri pathogenic, what triggers the switch from a free-living amoeba to an invader in this species but not in other species such as N. gruberi, and what molecules are key to the process are of major interest in this field. A clear-cut answer to these problems are not only of great biologic significance, but also have public health and economic consequences. A variety of approaches, such as cytological and biochemical methods have been applied to the problem of understanding the capacity of N. fowleri to become pathogenic either in vivo or in vitro. The cytopathic effects of N. fowleri might be due to release of cytolytic substances by amoebae (Chang, 1978) or the result of a combination of phagocytosis and the cytolytic action (Visvesvara and Callaway, 1974). Phospholipase A enzyme was suggested as one of the cytolytic factors. Higher levels of this enzyme were produced by pathogenic strains compared with low levels of the enzyme in non-pathogenic strains. Reduction of cytolytic activity was observed in the presence of the inhibitor (Rosenthal's reagent or dimethyl-DL-2,3,-α-distearoyloxypropyl-2'-hydroxyethylammonium acetate) (Fulford, et al., 1987). Recent studies (Marciano-Cabral, 1988) indicated a different mechanism of cytopathogenicity between axenically cultivated, low virulent N. fowleri LEE strain and mouse brain passaged amoebae. Axenic cultured

amoebae ingested nerve cells mediated by an apparatus called an amoebaestome. In contrast, the highly virulent amoebae lysed cells by cell-cell contact without cytolytic factors secreted into the culture medium. The ability of the amoebae to attach to and penetrate the nasal mucosa or to exhibit an increased rate of locomotion or both might be determinative parameters (Marciano-Cabral, 1988). However the precise nature of the mechanism(s) employed by N. fowleri to penetrate the nasal mucosa and to invade the CNS is still little known. In contrast to previous works which concentrated in cytology and biochemistry, my research focused on epigenetic changes in virulence through molecular and genetic techniques to investigate the basic mechanisms accounting for PAM.

LEE strain of N. fowleri was used in this study because of its experimental advantage for molecular studies. The virulence[1] of N. fowleri LEE strain can be varied (Wong et al., 1977). When amoebae are axenically cultured for a long period, virulence is attenuated but can be restored by serial mouse brain passages (John et al., 1985, Marciano-Cabral, 1988). The ability to manipulate virulence makes it

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[1] It is necessary to distinguish between the terms pathogenicity and virulence. I will use the term "pathogenicity" to mean the capacity to cause disease by a given strain of amoeba; therefore there are two types of amoebae, pathogenic and non-pathogenic. On the other hand, "virulence" reflects the degree to which pathogenicity is expressed. The grade of virulence may depend on many factors.

a good model to study the molecular mechanisms of virulence in parasitic protozoa.

Cell interactions between N. fowleri amoebae and host cells are complex and involve an unusually effective cytolysis and phagocytosis which may involve with the expression of many genes. Because N. fowleri is always pathogenic but virulence can be easily varied in the LEE strain, my research emphasizes a study of epigenetic changes in virulence and not differences in pathogenesis between species or strains. To begin a molecular and genetic analysis I first assessed the protein synthesis patterns and identified proteins whose abundance was altered during the transition of amoebae from low to high virulence, by two dimensional gel electrophoresis. This tested the hypothesis that the increased virulence was a result of altered gene expression in response to contact with the nasal-mucosa epithelium and invade the CNS. The next step was to clone these cDNAs encoding these proteins. The study of eukaryotic gene structure and expression relies on the availability of probes. The successful strategy toward isolating a particular eukaryotic gene is first to isolate a DNA copy (cDNA clone) of the mRNA encoded by that gene. The cDNA clone also offers a direct rout towards cloning the corresponding chromosome gene. In order to do that, a cDNA library was constructed from mRNAs. The reverse transcriptase (RNA-dependent DNA polymerase) was then used

to reverse transcribe mRNAs isolated from mouse brain passaged amoebae into cDNA. This pool of cDNAs was inserted into an appropriate cloning vector, then transformed into an appropriate bacterium strain. A differential screening method was used to screen the cDNA library to identify possible cDNA clones (Fig. 3). After a few rounds of isolation, we picked up a few cDNA clones. The cloned cDNAs was used as probes to hybridize with Northern blots to further confirm the specific mRNAs differentially expressed in highly versus weakly virulent amoebae. Sequencing the cloned cDNAs and comparing the deduced amino acid sequence to the Swiss Protein Database was used to search for clues as to the possible role of the protein.

In the following chapters, I describe: changes of protein synthesis pattern and the isolation and characterization of three cloned cDNAs whose corresponding transcript levels were altered when the virulence was changed. In addition, a new cDNA library construction method was presented.

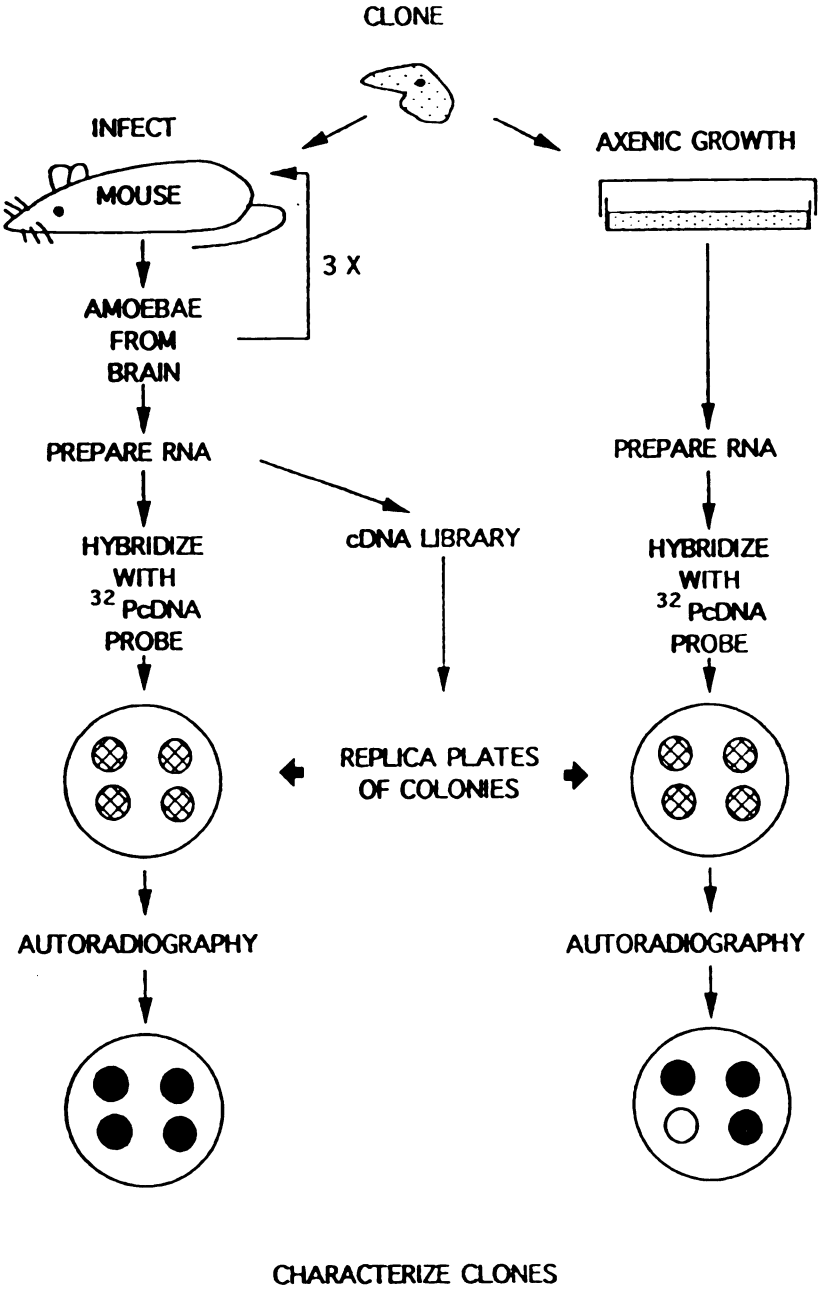

Figure 3. Strategy of differential screening.

CHAPTER ONE: Virulence-related protein synthesis in *Naegleria fowleri*[1]

A. Introduction

The free living amoeboflagellate *Naegleria fowleri* is a virulent pathogen of man, causing primary amebic meningoencephalitis (PAM) (Fowler, *et al.*, 1965). Studies on the pathogenesis of *N. fowleri* have been done in an attempt to discover the determinants of pathogenesis (John, 1982, Marciano-Cabral, 1988). The mouse is a useful model to investigate PAM, because basic features of the disease are the same for mouse and man (John, 1982).

Several proteins, such as phospholipase (Fulford, *et al.*, 1986), membrane-associated pore-forming protein (N-PFP) (Young, *et al.*, 1989) and *Naegleria* amoeba cytopathogenic material (NACM) (Dunnbacke, *et al.*, 1989), may play some role in pathogenesis of *Naegleria*. However, none of these proteins are unique to high virulence amoebae of *N. fowleri*. Therefore, virulence of *N. fowleri* may be a result of numerous protein changes not yet identified. Virulence varies in some strains of *N. fowleri* (Wong, *et al.*, 1977). The LEE strain of *N. fowleri* isolated from human cerebrospinal fluid, loses virulence when cultured axenically for extended periods of time. However, virulence can be restored by serial mouse brain passage (John, *et al.*,

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[1]Chapter one was published by W. Hu, R. N. Band and W. Kopachik in Infect. & Immun. 59:4278-4282 in 1991.

1985, Marciano-Cabral, 1988). Virulence persisted for approximately one month in axenic culture (Marciano-Cabral, et al., 1990).

We used two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to observe global changes in protein synthesis by the LEE strain of N. fowleri. Comparisons of low and high virulent amoebae were made with silver stained, in vivo synthesized and in vitro translated protein. Protein patterns were done after growth on Escherichia coli K-12 to test the effect of diet on virulence. To test the stability of the pathogenic phenotype, protein patterns were analyzed between the LEE strain after mouse brain passages to increase virulence, and after axenic cultivation for 6 months.

Low virulence amoebae, LEE strain of N. fowleri were obtained from Dr. David J. John, grown in H4 liquid medium (peptone medium supplemented with hemin) (Band, et al., 1974) at 37°C and recloned before use. For a clone isolation, we used migration on H4-agar plates at 37°C. High virulence (LEEmp) amoebae were obtained by serial inoculation of the LEE strain into mouse brains for four consecutive passages. Ten, three to four week old male mice (Mus musculus strain: BALB/C) were used for each passage. Amoebae were harvested and washed by centrifugation three times with LS saline (50 mM NaCl, 4.6 mM $MgSO_4$ and 0.36 mM $CaCl_2$)(Band, et al. 1969). A series of 2 μl of LS saline

containing N. fowleri (10^6 amoebae were used for the first passage and 10^4 for subsequent passages) were applied into the nares of each mouse, using a narrow bore 0-200 µl microcapillary pipet tip (Western Coast Scientific, Inc.). Within 4-6 hr after death pieces of brains were placed in 250 ml tissue culture flasks (Corning Science Products) containing 10 ml H4 medium with Penicillin-G (1000 units/ml), Streptomycin (100 units/ml) and incubated at 37^0C. Two hr later, mouse brain tissues were removed and fresh H4 medium without antibiotics was added to the residual amoebae. The amoebae were then cultured for 24 to 36 hr before protein analysis. Amoebae were also fed with E. coli (K-12) (1 mg/ml LS saline) and incubated at 37^0C (LEE.b). Before protein analysis, the amoebae were cultured in LS saline overnight without bacteria.

To radiolabel cellular proteins, 300 µCi L-^{35}S-methionine (>1000 Ci/mmol) (Amersham) was added to each culture (10 ml) for 6-8 hr at 37^0C before the cells were harvested. About 10^7 amoebae for each sample were harvested and washed three times with LS saline, and prepared by the methods described by Kopachik (Kopachik, etc., 1985) for 2-D PAGE analysis (See Appendix 3 for detailed protocol). Total cellular RNA from 10^8 amoebae was prepared according to the methods of Chirgwin (Chirgwin, et al., 1979), and Kopachik (Kopachik, et al., 1985) (See Appendix 3 for detailed protocol). Poly A (+) mRNA was selected from 100 µg total RNA with HYBOND-mAP

paper (Amersham) following the manufacture's instruction. Then mRNAs were translated *in vitro* in a rabbit reticulocyte cell-free system (Amersham) with 40 μCi L-^{35}S-methionine for 1 hr at 37^{0}C.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed according to the methods of Garrels (Garrels, 1979) and modified by Kopachik (Kopachik, *et al.*, 1985) (See Appendix 3 for detailed protocol). The first dimension gel contained the ampholyte (Pharmacia LKB) of pH range 5 to 7 for *in vitro* translation gels and pH 3.5 to 10 for other gels. The second dimension gel was a uniform 11% polyacrylamide with sodium dodecyl sulfate (SDS). Proteins (200 μg) were stained with silver by the methods described by Morrissey (Morrissey, 1981) (See Appendix 3 for detailed protocol). Gels with samples labelled by ^{35}S-methionine were fixed and soaked in EN^{3}HANCE (New England Nuclear) for 60 min. Dried gels were exposed at -70^{0}C to Kodak XAR-5 film. To eliminate inconsistencies, each experiment was repeated three times under the same conditions and three replicas were done with each experiment. Only the proteins that qualitatively changed and were consistantly viewed on gels were labelled.

To compare the virulence of LEEmp after 6 months growth in axenic culture versus the virulence of LEE amoebae grown either in axenic or bacterized (LEE.b) cultures, 7 mice per group were inoculated with 10^6 amoebae. A completely random

block design (Gill, 1978) was used to minimize the variation between different litters of mice.

B. Result and discussion

The steady state level of proteins present in LEE low virulence amoebae, in comparison to LEEmp, high virulence amoebae, was analyzed by silver staining of accumulated proteins. We sought both quantitative and qualitative differences (Fig. 1). Ten proteins were detectable only in the LEEmp whereas twelve proteins were detectable only in LEE cells (Table 1). Quantitative differences were also observed, such as the protein just above spot #6 which had a lower level in LEEmp versus LEE amoebae (Fig. 1). The protein spot numbers are only comparable between similar treatment. Thus numbers in Fig. 1, 2 and 3 cannot be compared.

We analyzed the incorporation of L-^{35}S-methionine during growth in axenic broth. Here again numerous proteins showed qualitative and quantitative differences (Fig. 2). Some of these proteins were preferentially to LEEmp or to the LEE cells (Table 1). In addition, many proteins were synthesized at a lower rate in the LEEmp versus the LEE amoebae. This suggests that the synthesis as well as accumulation, of many proteins is affected by differences in pathogenesis. Changes in protein synthesis or accumulation could be the result of corresponding changes in mRNA transcripts encoding these proteins. To determine if transcript abundance changes can

Figure 1. Silver-stained electropherograms of accumulated proteins extracted from low-virulence LEE amoebae (A) and from high-virulence LEEmp amoebae (B). The proteins which differed between them were identified by numbers.

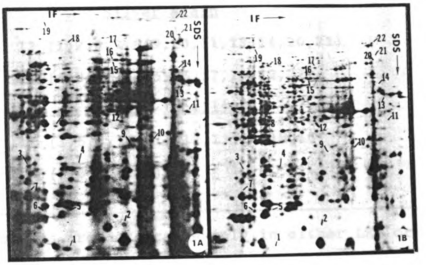
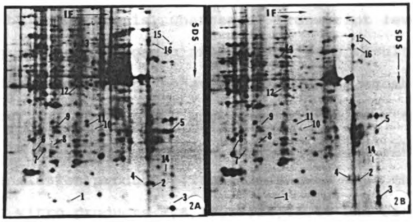

IF
SDS
1A
IF
SDS
1B

Table 1. Differences in the expression of protein at accumulated level (silver stain) and *in vivo* synthesized (L-^{35}S-methionine incorporation) level between LEE and LEEmp amoebae.

Cell type	Qualitative changes of Proteins[a]	Fig. No.
	Silver stain	
LEE	12 (1,2,3,7,8,9,10,11,13,14,20,21)	1A
LEEmp	10 (4,5,6,12,15,16,17,18,19,22)	1B
	In vivo labelling	
LEE	11 (1,5,6,7,9,11,12,13,14,15,16)	2A
LEEmp	5 (2,3,4,8,10)	2B

[a]The proteins are detectable only in either LEE or LEEmp.

Fig. 2. Autofluorograms of two-dimensional gel of proteins radiolabelled <u>in vivo</u> with L-[^{35}S]-methionine from low virulent LEE amoebae (2A) and from high virulent LEEmp amoebae (2B). The proteins which differed were identified by numbers.


1E
SDS
15
16
12
9
11
10
5
14
4
2
3
1
2A
1F
SDS
15
16
14
12
9
11
10
5
4
2
3
1
2B

be detected, poly A (+) mRNA was extracted from LEE and LEEmp cells and translated in vitro in rabbit reticulocytes (Fig. 3). We found that eleven translation products were detectable only when LEEmp mRNA was used: this suggests that higher levels of some transcripts appeared in virulent amoebae (Table 2, LEE vs. LEEmp amoebae). The change from the LEE to the LEEmp pathogenic state also included a changein diet from the axenic culture to a diet of cells ingested by phagocytosis. Changes of transcript level occurring in the pathogenic amoebae may be a result of altered diet and feeding behavior. Therefore, amoebae fed with bacteria (LEE.b) might show some of the same changes apparent in pathogenic amoebae. A comparison of LEEmp to LEE.b translation products (Table 2) showed that this did occur: in vitro products 1,8,10,12,14,15,19 and 25 were translated from LEEmp and LEE.b mRNAs but not from LEE mRNAs (Fig. 3). There were in spite of these similarities extensive differences both qualitatively and quantitatively between the LEEmp and LEE.b translation products (Table 2 and Fig. 3).

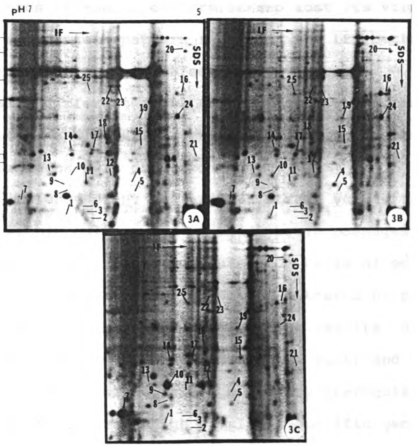
Twenty-five of the main characteristic, in vitro gene products of LEE, LEEmp and LEE.b transcripts are shown with apparent molecular weights and isoelectric points (Table 3). There is a correlation between the numerous changes in the accumulation and synthesis of proteins observed and the susceptibility of mice to infection. The virulence of LEEmp

Table 2. Differences in the expression of protein *in vitro* translation from poly A (+) mRNA among LEE, LEEmp and LEE.b.

Cell type		Qualitative changes of proteins[a]	Fig. No.
		LEE vs LEEmp	
LEE	6	(2,3,6,13,17,21)	3A
LEEmp	11	(1,4,8,10,12,14,15,16,19,20,25)	3B
		LEE vs LEE.b	
LEE	10	(3,6,11,13,17,18,21,22,23,24)	3A
LEE.b	11	(1,5,7,8,9,10,12,14,15,19,25)	3C
		LEEmp vs LEE.b	
LEEmp	7	(4,11,16,18,20,22,23)	3B
LEE.b	4	(2,5,7,9)	3C

[a]The proteins detectable only in either LEE, LEEmp or LEE.b.

Fig. 3. Autofluorograms from two-dimensional gel electrophoresis of proteins translated *in vitro*. Poly A (+) mRNA from low virulent LEE amoebae (3A), high virulent LEEmp amoebae (3B) and LEE amoebae after growth on bacteria (3C) was translated in rabbit reticulocytes with L-[^{35}S]-methionine. The proteins which differed were identified by numbers. Approximate molecular weight range (vertical axis) and the pH range were used to construct Table 3.


pH 7
5
SDS
3A
3B
3C

amoebae increased after the amoebae were passaged through mouse brain three times. About 10^4 LEEmp cells killed a mouse in approximately 5 days after three passages, while 10^6 axenic cultured LEE cells were required to kill a mouse in about 10 days in the first mouse brain passage. However, after 6 months in axenic culture, LEEmp lost its virulent state and its protein pattern resembled the LEE patterns; the time required to kill infected mice was not statistically different from LEE or LEEmp after 6 months in axenic culture and on silver-stained gels none of the LEEmp-specific proteins were observed (Fig. 4).

The virulence of N. fowleri LEE strain varies in prolonged axenic culture and by mouse brain passages. It is this unique feature that constituted the basis of our study so we could investigate the regulation patterns of protein synthesis during this transformation. The results, different patterns of protein synthesis between low (LEE) and high virulence (LEEmp) amoebae, are a necessary prerequisite for future attempts to clone high virulence-specific genes by differential screening of a cDNA library made from LEEmp amoebae. The partial characterization of the gene products in this experiment (Table 3) may facilitate the correlation of cloned genes to characterize translation products. Numerous changes in protein synthesis occur during the transformation of LEE strain from low to high virulence. Some of the changes in protein synthesis might be directly

Fig. 4. Silver-stained electropherograms of accumulated proteins extracted from low virulent LEE amoebae (4A) and from high virulent LEEmp amoebae after 6 month removal from mouse brain (4B).

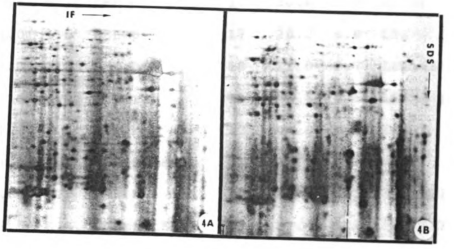

IF
SDS
4A
4B

Table 3. Partial characterization of some proteins translated *in vitro* from poly A(+) mRNA.

Protein #[a]	Estimated M.W.[b]	pI[c]	Present-in	Protein #	Estimated M.W.	pI	Present-in
1	10.5	6.7	LEEmp,LEE.b	14	20.0	6.3	LEEmp,LEE.b
2	10.0	6.0	LEE,LEE.b	15	21.5	5.4	LEEmp,LEE.b
3	10.6	6.1	LEE	16	30.0	5.2	LEEmp
4	12.0	5.5	LEEmp	17	20.0	6.0	LEE
5	11.5	5.5	LEE.b	18	21.0	5.7	LEE,LEEmp
6	10.6	6.2	LEE	19	23.0	5.5	LEEmp,LEE.
7	11.0	6.8	LEE.b	20	44.0	5.2	LEEmp
8	12.0	6.3	LEEmp,LEE.b	21	17.0	5.0	LEE
9	11.5	6.3	LEE.b	22	32.0	5.6	LEE,LEEmp
10	12.5	6.3	LEEmp,LEE.b	23	32.0	5.6	LEE,LEEmp
11	11.5	6.1	LEE,LEEmp	24	23.0	5.2	LEE,LEEmp
12	13.0	5.6	LEEmp,LEE.b	25	30.0	6.0	LEEmp,LEE.b
13	14.0	6.5	LEE				

[a]See Fig. 3A, 3B and 3C and table 2 for spot numbers.
[b]M.W., Molecular weight ($x10^{-3}$Da).
[c]pI, isoelectric point.

responsible for virulence while others could reflect indirect relationships to virulence. For example, carbohydrate moieties of surface glycoproteins have been implicated in pathogenesis of Entamoeba histolytica (Petri, et al., 1990). Many proteins were lost in the LEEmp amoebae; the decreases were qualitative (#6 in Fig. 1) and quantitative (the protein above #6 for example). Similarly the loss of proteins may be important in converting a cell into a more virulent state. Several low virulent and avirulent amoebae from various genera will kill mice when directly injected into the brain (John, 1982). Penetration of the nasal mucosa and related barriers is necessary under natural conditions for invasion of the central nervous system. Thus, in addition to interaction with brain tissue, other factors related to invading the central nervous system may be inducers of increased virulence in the amoebae.

CHAPTER TWO: Cloning and characterization of transcripts showing virulence-related gene expression in *Naegleria fowleri*[1]

A. INTRODUCTION

Naegleria fowleri is a free-living amoeba of soil and freshwater which feeds on bacteria present in these environments. It also exists as a virulent pathogen causing primary amoebic meningoencephalitis in humans, mice and other mammals (Fowler, *et al.*, 1965; John, 1982). Infection of the brain occurs after amoebae reach the nasal cavity, attach to and invade the nasal mucosa and olfactory nerve. Invasive amoebae able to enter the nervous system digest neuronal tissue and other mammalian cells by unusually effective cytolysis and phagocytosis as observed in culture or in sections of infected brain tissue (Marciano-Cabral, 1988, 1990). Highly virulent amoebae exhibit faster movement *in vitro* than do weakly virulent amoebae (Cline, *et al.*, 1986, Thong, *et al.*, 1986). A mechanism to evade the host immune system defenses has also been suggested as a necessary determinative change in the pathogenic form (Marciano-Cabral, 1988). However, the molecular mechanisms responsible for the capacity to be pathogenic are still poorly understood.

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[1]Chapter Two was published by Wang-nan Hu, Will Kopachik and Neal Band in Infect. & Immun. 60:2418-2424 in 1992.

Four proteins, a phospholipase (Fulford, et al., 1986), a neuraminidase (Elsen, et al., 1987), cytopathogenic material (Dunnebacke, et al., 1989) and a membrane-associated pore-forming protein (Young, et al., 1989) have been implicated in pathogenesis but the exact role that these markers play is unknown since they are also found in non-pathogenic species. Recently, this transition from the free-living to the highly virulent state was found to be associated with many additional quantitative as well as previously unknown qualitative changes. Increases and decreases in protein synthesis or stability were found by two dimensional gel electrophoresis (Hu, et al., 1991).

The LEE strain has experimental advantages for molecular studies of the transition from free-living to highly virulent forms. Virulence can be varied in this strain (Wong, et al., 1977) because it loses virulence when cultured axenically. Virulence can be restored by serial mouse brain passages. The ability to manipulate virulence by serial mouse brain passage made it possible to examine gene expression during the transition from low to high virulence. The changes in protein synthesis could arise by transcriptional or post-transcriptional regulatory mechanisms. Changes in the levels of specific transcripts were observed by examination of the translation products from poly (A)+ mRNA in a rabbit reticulocyte extract (Hu, et al., 1991). This suggested that clones of the genes for

these regulated mRNAs might be found by differential screening of a cDNA library made to virulent cell mRNA.

Here we describe the isolation and characterization of two cloned cDNAs whose corresponding transcript levels are altered between weakly and highly virulent amoebae. Having such clones allowed an examination of the possible role of the genes in pathogenesis through DNA sequence analysis. The nature of the inducer of the gene expression changes could be sought by examination of mRNA levels in amoebae incubated in culture with mammalian cells.

B. RESULTS

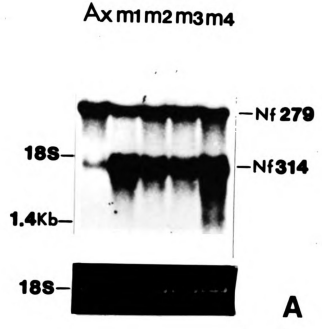
Cloning of transcript sequences preferentially expressed in highly virulent amoebae. A cDNA library of 1,000 clones in the vector pUC18 was made from poly (A)+ mRNA from LEEmp amoebae which had been isolated from the brains of infected mice. Initial screening attempts by methods in which bacterial colonies (10^5) on replica filters were lysed gave variable results. Consequently, dot blots of the plasmid DNA ($\approx$ 1 μg prepared by a rapid preparation method) were made on replica filters in order to increase the sensitivity of detection and make sure that equal amounts of the plasmid DNA were immobilized on the replica filters. One set of filters were hybridized with single stranded cDNA probe made from 25 μg of oligo dT-primed total RNA of highly virulent amoebae and the other was hybridized

with a comparable probe prepared from weakly virulent amoebae grown axenically. The autoradiographs of the filters were examined to find cDNA clones which differed in quantity of hybridization between the two probes. Only two clones exhibited reproducible and significant differential hybridization signals by visual screening. Clone Nf314 exhibited higher hybridization to the cDNA probe made to highly virulent amoebae RNA whereas clone Nf435 exhibited lower hybridization to that probe. The other 998 clones showed either negligible hybridization (≈ 10% of the library) at or near background levels or approximately equal hybridization (≈ 90%) with the two cDNA probes. We analyzed a randomly chosen set of 15 clones and found that all had inserts: these inserts ranged in size from ≈ 100 bp to over 2 kb (data not shown). We concluded that moderate to high abundance transcripts representing ≈ 90% of the library of 900 clones had only 2 differentially hybridizing clones. The other 10% of the clones without a signal above background probably contained inserts homologous to low abundance transcripts such that they are below the level of detection by this differential screening method.

Expression of transcripts in weakly and highly virulent amoebae. We used Northern blot analysis to confirm the apparent differential hybridization of the clones and to determine when during the transition of the amoebae from low to high virulence the steady state transcript levels change.

Inserts prepared from the three plasmids were labeled by random priming. The probes were hybridized to gel blots of total RNA from axenically growing amoebae and from amoebae taken from brains of mice after one, two, three or four serial infections of mice. The results (Fig. 1 A) show that although equal amounts of intact RNA were isolated in the five samples as assessed by equal ethidium bromide staining of 18 S ribosomal RNA, there is a strong differential expression of clone Nf314. The Nf314 transcript levels dramatically increase to high levels after only one passage in the mouse. Quantitative analysis of transcript levels with a Betascope showed that the induced levels of Nf314 mRNA are seven times the basal level found in axenic cells. The levels stay high after subsequent passages (compare m1 RNA to m2, m3 and m4 RNAs). This autoradiograph was overexposed to show the minor transcript levels hybridizing to the Nf314 probe in axenically growing cells. RNA size markers run on the same gel as the Nf314 transcript allowed us to estimate the single transcript size as 1.7 kb; this length was confirmed by primer extension of the mRNA (data not shown). As a further control an insert from one of the 998 unregulated clones from the library, designated Nf279, was used to probe the same blot. As shown the Nf279 transcript is present at about the same level in all cells so the reduction in the Nf314 transcript level is neither the result of blotting artifacts nor degradation of the RNA

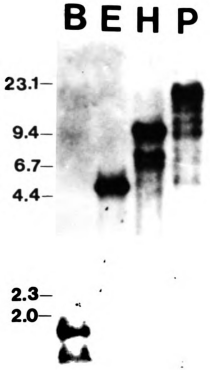
Figure 1. Autoradiograph of Northern blot of RNA from axenically grown amoebae and amoebae from mouse brain. Total RNA (10 μg) was electrophoretically size separated on an agarose gel containing formaldehyde. An ethidium bromide stained gel shows that the 18S rRNA is intact and appears to be of similar intensity in all of the samples: Ax (axenic amoebae); m1,m2,m3 and m4 amoebae after 1,2,3 and 4 passages of infection in mouse brains. A. The blot of the gel was probed with the cDNA inserts from the Nf314 and Nf279 clones. The location of hybridizing mRNA is shown. Size markers on the left side are from endogenous rRNA and *in vitro* synthesized transcript size markers. B. An identical blot was probed with an ^{32}P-end labeled oligonucleotide homologous to a actin mRNA sequence.


Ax m1 m2 m3 m4
Nf 279
18S
Nf 314
1.4Kb
18S
A
Ax m1 m2 m3 m4
18S
Nf Actin1
B

in the Ax lane. Since this method of mRNA analysis allows detection of differentially expressed mRNA of unknown function we wanted to confirm the apparent difference by using an actin gene as a probe. An oligonucleotide was made to a conserved region of a N. fowleri actin cDNA clone and end radiolabelled for use as a hybridization probe. As mentioned high virulence amoebae exhibit increased motility (Cline, et al., 1986; Thong, et al., 1986). Interestingly, actin transcript levels were slightly higher in high virulence versus low virulence amoebae (Fig. 1 B). Thus actin mRNA levels which might be expected to increase, did increase, albeit to a much lesser extent than do Nf314 transcript levels. As will be reported (Fig. 5) the Nf435 clone exhibits dramatically reduced hybridization to RNA isolated from LEEmp.

Southern blot and sequence analysis of Nf314. We chose to examine clone Nf314 in more detail because of the interesting regulation of the transcript. Southern blot analysis was performed to determine the number of copies of this gene and to determine if other closely related genes were present in the genome. Total nuclear DNA was digested individually with four different restriction enzymes and the digestion products size separated by agarose gel electrophoresis. The blot of the gel was probed with radiolabelled Nf314 insert at moderate stringency. From the autoradiograph (Fig. 2) we conclude that Nf314 is probably a

Figure 2. Autoradiograph of Southern blot probed with the Nf314 cDNA. Genomic DNA (10 μg) digested with BamH I (B), EcoR I (E), Hind III (H) and Pst I (P) restriction enzymes was electrophoretically size separated, blotted onto Gene Screen and hybridized to radiolabelled Nf314 insert. Size markers on the left were from lambda DNA digested with Hind III.


B E H P
23.1
9.4
6.7
4.4
2.3
2.0

single copy gene because there is only one band of EcoR I digested DNA of 5 kb hybridizing with the probe. We have not excluded the possibility that two tandem copies are present on this 5 kb fragment. The results also show that at least one BamH I and Hind III site are present in the gene. Even at a reduced stringency no other DNA hybridized to the probe. We conclude that the 1.7 kb transcript detected by Northern blot analysis is transcribed from a single gene. The low steady state level of transcripts in axenic cells is from the same gene transcribed to give a high level of transcripts in the highly virulent cells.

The insert for clone Nf314 was determined to be approximately 1.6 kb by agarose gel electrophoresis. Since this insert was too long to sequence and obtain unambiguous base determination we made subclones of the insert by taking advantage of the restriction enzyme sites which the cDNA shares with the gene. Sequence data was obtained both from single and double stranded templates of an overlapping set of subclones from both strands: the cDNA insert sequence of 1584 bp is shown (Fig. 3). Only one long open reading frame was present extending 1449 bases from three ATG putative start codons (nucleotides 34-42) to a TAA stop codon (nucleotides 1480-1482); two additional possible stop codons are at nucleotides 1492-1494 and 1510-1512. A possible poly A addition signal is underlined 26 bases after the stop codon. A polypeptide of 53 kDa with a isoelectric

Figure 3. DNA sequence of the insert to clone Nf314. The sequences of both strands of the insert were determined by use of universal primers and specific internal oligonucleotide primers. An open reading frame starting from three ATG codons to a single TAA codon is shown. A potential poly(A) addition sequence is underlined.

```
                                                  M  M  M  R  C  V  N  G  Q
GTC TTT GTC TCT CTC TGG TTG GTT TCC TTC ATC ATG ATG ATG AGA TGC GTG AAT GGA CAA 60

 T  A  Q  D  H  L  V  T  Q  L  P  G  L  S  G  N  I  G  V  K
ACT GCT CAA GAT CAT TTA GTG ACT CAA CTT CCT GGT TTG AGT GGA AAC ATC GGA GTG AAA 120

 S  Y  T  G  Y  L  L  A  N  A  T  R  G  R  Y  L  F  Y  W  F
TCC TAC ACG GGA TAT TTG TTG GCA AAT GCC ACT CGA GGT CGA TAC TTG TTT TAT TGG TTT 180

 F  E  S  M  R  N  P  S  Q  D  P  L  V  M  W  T  N  G  G  P
TTT GAA TCG ATG AGA AAT CCA TCT CAG GAC CCA CTT GTC ATG TGG ACC AAT GGT GGA CCT 240

 G  C  S  S  L  G  G  E  A  S  E  H  G  L  F  L  V  N  A  D
GGA TGC AGT AGT TTG GGC GGT GAA GCC AGT GAA CAT GGT TTA TTC CTT GTC AAT GCA GAT 300

 G  A  T  I  T  R  N  P  Y  S  W  N  R  V  S  N  I  L  Y  I
GGT GCA ACC ATT ACA AGA AAT CCC TAT TCT TGG AAT CGT GTT TCT AAT ATT CTC TAT ATT 360

 E  Q  P  V  G  V  G  F  S  Y  S  N  S  T  D  D  Y  Q  N  L
GAA CAA CCA GTG GGT GTT GGA TTT TCA TAT TCC AAT TCG ACC GAT GAT TAT CAA AAT CTC 420

 N  D  V  Q  A  A  S  D  M  N  N  A  L  R  D  F  L  T  R  F
AAT GAT GTA CAA GCT GCT TCT GAT ATG AAT AAT GCA TTG AGA GAT TTC TTG ACT CGA TTT 480

 P  Q  F  I  G  R  E  T  Y  L  A  G  E  S  Y  G  G  V  Y  V
CCT CAA TTT ATT GGA AGA GAA ACC TAT TTG GCA GGT GAA TCG TAT GGT GGA GTT TAT GTT 540

 P  T  T  A  Y  N  I  V  E  G  N  G  K  G  Q  Q  P  Y  V  N
CCA ACA ACG GCT TAC AAC ATT GTT GAA GGA AAT GGA AAG GGA CAA CAA CCC TAT GTG AAT 600

 L  V  G  I  L  V  G  N  G  V  T  D  A  E  A  D  S  N  S  I
TTA GTG GGT ATT TTA GTT GGT AAT GGT GTG ACT GAT GCT GAA GCG GAT AGT AAT AGT ATT 660

 P  P  M  M  K  Y  H  S  L  I  S  I  K  Y  Y  E  E  G  Y  K
CCA CCA ATG ATG AAA TAT CAC AGT TTG ATT TCT ATC AAG TAT TAT GAA GAG GGA TAT AAG 720

 A  C  Q  G  D  F  Y  A  N  Q  N  L  P  A  C  Q  K  F  L  T
GCA TGT CAA GGT GAT TTT TAT GCG AAT CAG AAT TTG CCA GCT TGT CAA AAA TTT TTG ACA 780

 D  S  S  N  A  M  G  N  I  N  P  Y  Y  I  Y  D  S  C  P  W
GAT AGT AGT AAT GCC ATG GGA AAT ATT AAT CCC TAT TAT ATT TAT GAT TCG TGT CCA TGG 840

 L  G  I  N  L  Q  Q  K  L  K  T  T  Q  E  M  T  F  Q  V  L
TTA GGA ATC AAC TTG CAA CAA AAA CTC AAA ACA ACA CAA GAA ATG ACA TTC CAA GTG TTG 900

 D  P  K  T  Q  Q  P  V  K  I  H  P  L  F  Q  M  Y  K  H  G
GAT CCA AAG ACT CAA CAA CCT GTC AAA ATT CAT CCA CTC TTC CAA ATG TAT AAA CAT GGT 960

 G  W  S  K  R  V  A  N  E  R  N  F  A  P  R  F  E  T  D  A
GGA TGG AGT AAG AGA GTT GCC AAT GAA AGA AAC TTT GCA CCT CGT TTC GAA ACT GAT GCT 1020

 P  C  V  P  N  Q  S  I  A  K  Y  F  R  R  L  D  V  Q  Q  A
CCT TGC GTT CCA AAT CAA TCC ATT GCA AAG TAT TTC CGT AGA TTG GAC GTC CAA CAA GCA 1080

 L  G  V  R  R  K  T  A  D  P  N  G  W  N  I  C  T  G  I  I
TTA GGA GTG AGA AGA AAG ACT GCC GAT CCA AAT GGA TGG AAT ATT TGT ACA GGA ATT ATT 1140

 N  Y  T  Q  V  Y  S  T  I  L  P  F  Y  A  K  L  L  P  H  I
AAC TAC ACT CAG GTG TAT TCC ACT ATT TTA CCT TTC TAT GCC AAG TTA TTG CCT CAT ATT 1200

 R  I  L  V  Y  S  G  D  T  D  M  V  V  N  G  L  G  T  Q  A
CGT ATT CTT GTC TAC TCG GGA GAT ACT GAC ATG GTT GTT AAT GGT CTT GGA ACA CAA GCT 1260

 A  I  D  K  L  Q  L  Q  E  T  S  S  W  R  T  W  E  F  D  S
GCC ATT GAC AAG TTG CAA TTA CAA GAA ACT TCA TCT TGG AGA ACT TGG GAA TTT GAT TCA 1320

 A  L  G  T  V  V  G  G  Y  I  R  K  F  E  K  S  G  K  G  L
GCC TTA GGA ACT GTT GTT GGA GGA TAT ATT CGT AAA TTT GAA AAG AGT GGA AAG GGG TTG 1380
```

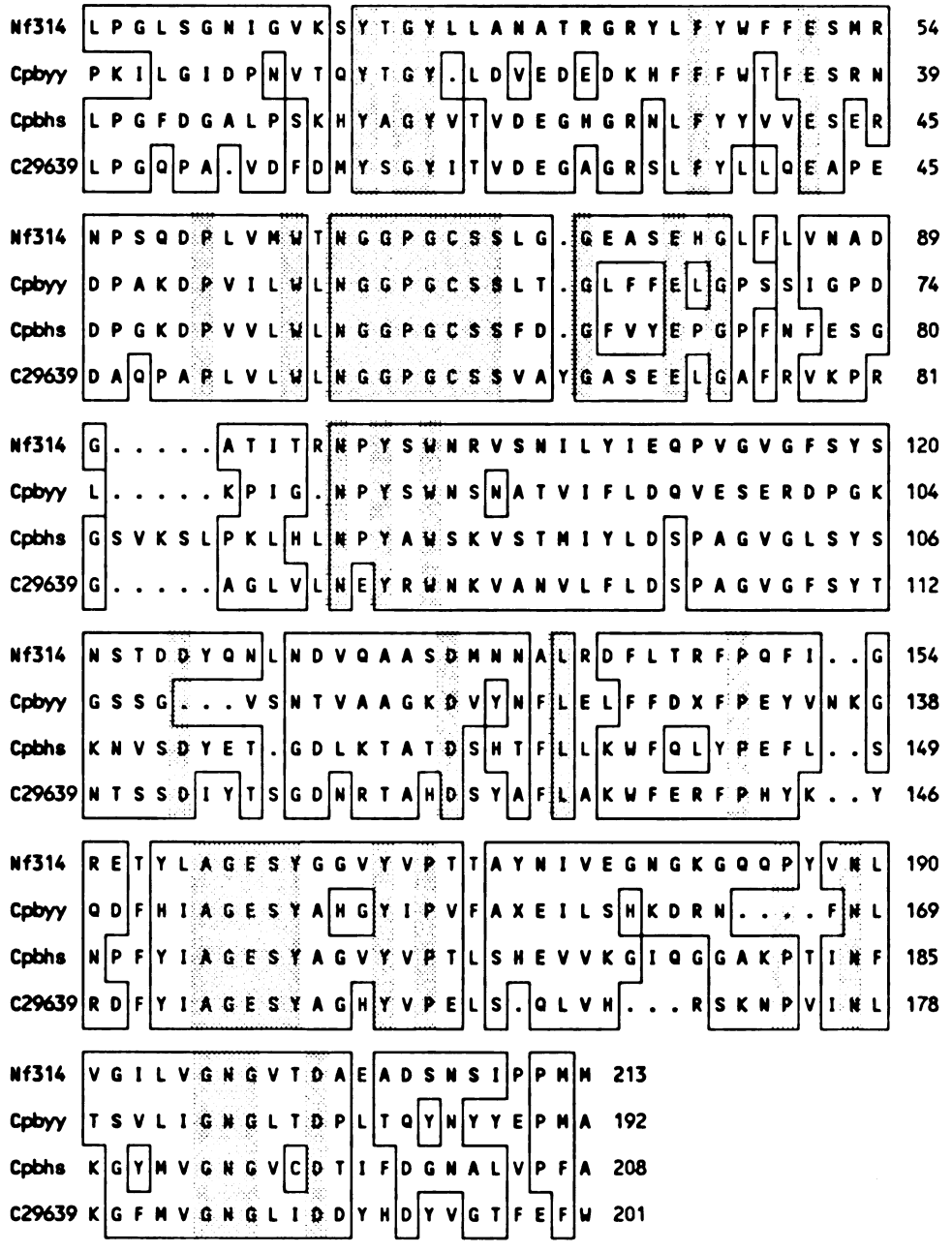
Figure 3. cont'd

```
 T   F   I   T   V   R   G   A   G   H   M   V   P   L   V   K   P   D   S   A
ACC TTT ATA ACT GTT CGT GGA GCA GGT CAC ATG GTG CCT CTT GTT AAG CCT GAT AGT GCC 1440

 F   Y   M   F   K   N   F   I   D   G   T   C   C  End
TTT TAC ATG TTT AAG AAT TTC ATT GAT GGA ACA TGT TGT TAA GAGAATGCATAAATGTTCATTCC 1505

AAAATAAAATCACACAGGAATTCACACTGTGATTTTATTTTGGAATTTATCACAGTGTGAATTCATTTGTCAAAAAAAA 1584

AAAAAAAAAAAAAAA
```

point of pH 7.8 was predicted from the cDNA sequence. Comparison of the predicted amino acid sequence to the GENEMBL database sequences resulted in identification of possible related peptide sequences (Fig. 4). The Nf314 sequence is shown in comparison to serine carboxypeptidase sequences from yeast (carboxypeptidase Y; Svendsen, et al., 1982), barley (carboxypeptidase I; Sorensen, 1986) and wheat (carboxypeptidase II; Breddam, et al., 1987): within 194 amino acids (starting at residue 19) the Nf314 sequence is 94 % similar and 19 % identical to the other sequences. Outside of this area of high sequence homology, amino acid residues 1 through 18 and 214 to the carboxyl terminal end there is negligible homology of sequences (not shown). The active site of the yeast carboxypeptidase Y has been determined to be at serine 146 (Svendsen, et al., 1982). There are five invariant amino acids in a peptide domain including the serine at the yeast residue 146, A-G-E-S-Y, that are shared by all four sequences. A carboxypeptidase role for the Nf314 gene product is conceivable since there are probably different proteins ingested by amoebae invading the nasal mucosa, olfactory nerve or brain of the host animal in comparison to amoebae growing axenically.

Induction of Nf314 gene expression by mammalian cells in culture. The Nf314 gene expression gene product might play a role in phagocytosis and feeding on the host cells or for invasion of the host or evasion of the host immune

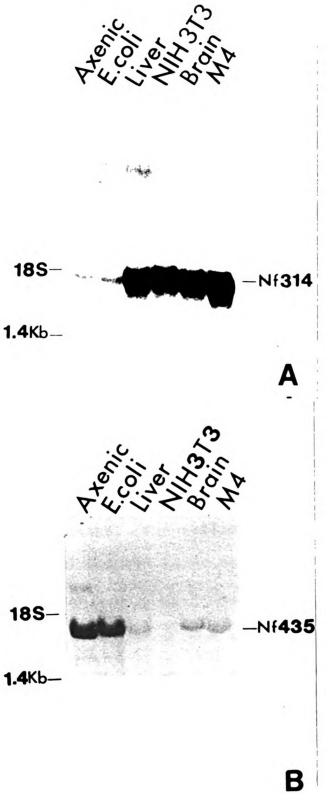
Figure 4. Comparison of the predicted Nf314 amino acid sequence (amino acid residues 19 to 213) optimally aligned by use of the FASTP program (7) with the carboxypeptidase sequences of yeast, Cpbyy (25), barley, Cpbhs (24) and wheat, C29639 (4). The amino acid residues that are identical in all four sequences are shaded. The amino acid residues of the Nf314 sequence that are similar (identical or with conservative amino acid replacements) to any of the other sequences are in boxed areas.


Nf314 LPGLSGNIGVKSYTGYLLANATRGRYLFYWFFESMR 54
Cpbyy PKILGIDPNVTQYTGY.LDVEDEDKHFFFWTFESRN 39
Cpbhs LPGFDGALPSKHYAGYVTVDEGHGRNLFYYVVESER 45
C29639 LPGQPA.VDFDMYSGYITVDEGAGRSLFYLLQEAPE 45
Nf314 NPSQDPLVMWTNGGPGCSSLG.GEASEHGLFLVNAD 89
Cpbyy DPAKDPVILWLNGGPGCSSLT.GLFFELGPSSIGPD 74
Cpbhs DPGKDPVVLWLNGGPGCSSFD.GFVYEPGPFNFESG 80
C29639 DAQPAPLVLWLNGGPGCSSVAYGASEELGAFRVKPR 81
Nf314 G.....ATITRNPYSWNRVSNILYIEQPVGVGFSYS 120
Cpbyy L.....KPIG.NPYSWNSNATVIFLDQVESERDPGK 104
Cpbhs GSVKSLPKLHLNPYAWSKVSTMIYLDSPAGVGLSYS 106
C29639 G.....AGLVLNEYRWNKVANVLFLDSPAGVGFSYT 112
Nf314 NSTDDYQNLNDVQAASDMNNALRDFLTRFPQFI..G 154
Cpbyy GSSG...VSNTVAAGKDVYNFLELFFDXFPEYVNKG 138
Cpbhs KNVSDYET.GDLKTATDSHTFLLKWFQLYPEFL..S 149
C29639 NTSSDIYTSGDNRTAHDSYAFLAKWFERFPHYK..Y 146
Nf314 RETYLAGESYGGVYVPTTAYNIVEGNGKGQQPYVNL 190
Cpbyy QDFHIAGESYAHGYIPVFAXEILSHKDRN....FNL 169
Cpbhs NPFYIAGESYAGVYVPTLSHEVVKGIQGGAKPTINF 185
C29639 RDFYIAGESYAGHYVPELS.QLVH...RSKNPVINL 178
Nf314 VGILVGNGVTDAEADSNSIPPMM 213
Cpbyy TSVLIGNGLTDPLTQYNYYEPMA 192
Cpbhs KGYMVGNGVCDTIFDGNALVPFA 208
C29639 KGFMVGNGLIDDYHDYVGTFEFW 201

defenses. To determine what might be the inducer of increased Nf314 gene expression in amoebae we incubated amoebae with mammalian cells in cell culture. In this experiment amoebae might be exposed to an inducer of increased gene expression even though the amoebae have not been in contact with the nasal mucosa. Northern blot analysis of RNA showed that amoebae incubated with mouse brain cells, NIH 3T3 fibroblasts, or liver cells, but not bacteria, E. coli or Bacillus subtilis (not shown), had a dramatic increase in gene expression (Fig. 5A). The increase was of the same magnitude to that induced in fourth passage amoebae isolated from mice brain. Thus all of the induction can be accounted by some interaction of amoebae with several types of mammalian cells in vitro. This induction does not correlate with a simple change in nutrients or ingestion of particulate foodstuffs as the inducer of the gene expression since amoebae fed on gram negative bacteria, E. coli, or on gram positive bacteria, B. subtilis, showed no elevation in Nf314 transcript levels.

The Nf435 transcript levels, in contrast, showed a completely different response to co-culturing amoebae with mammalian or bacterial cells. For Nf435 gene expression axenic cell culture or culture with E. coli provided an inductive stimulus to maintain high steady state transcript levels. In contrast the interaction of amoebae with any of the three types of mammalian cells caused a large decrease,

Figure 5. A. Autoradiograph of a Northern blot of RNA from amoebae in cell culture. RNA was analyzed as described in the legend to Fig. 1: The source of the RNA was axenically growing cells (Axenic), amoebae fed with bacteria (*E. coli*), mouse liver (liver), 3T3 fibroblasts (NIH 3T3), dissociated mouse brain (brain) and amoebae from brains after the fourth infection of mice (M4). The blot was probed with the cDNA insert to clone Nf314. B. An identical blot probed with the cDNA insert to clone Nf435.


Axenic
E.coli
Liver
NIH 3T3
Brain
M4
18S
Nf314
1.4Kb
A
Axenic
E.coli
Liver
NIH3T3
Brain
M4
18S
Nf435
1.4Kb
B

not an increase, in transcript levels (Fig. 5B). Here too all of the change in level could be entirely explained by the interaction with mammalian cells in culture. Transcripts from Nf435 are also not affected by a simple change from axenic culture conditions because a substantial change in diet, growth on bacteria, does not affect levels. As a further control the Nf279 constitutively expressed transcript levels did not vary with any of the conditions which affect Nf314 or Nf435 transcript levels (data not shown).

Sequence analysis of clone Nf435. Gene Nf435 was differentially expressed during transition from low to high virulence. Northern blots showed that the estimated size of this transcript was about 1.5 kbp. However, sequence analysis showed that this cDNA insert was incomplete and only contained 324 base pairs and one open reading frame of 107 amino acids was present. The following is the nucleotide sequence and deduced amino acid sequence.

```
     N   S   Q   Q   D   E   I   R   I   K   R   R   K   I   K   E  16
GA AAT TCA CAG CAA GAT GAA ATT CGA ATC AAG CGG AGA AAA ATT AAG GAG  50

 K   M   M   S   S   I   D   D   I   I   Q   K   T   D   L   D   G  33
AAA ATG ATG TCT TCT ATT GAC GAT ATT ATT CAA AAA ACA GAT CTC GAT GGT 101

 D   G   N   I   N   Y   Q   E   F   L   E   F   M   K   N   F   D  50
GAT GGT AAT ATT AAT TAT CAA GAA TTT TTA GAA TTT ATG AAA AAC TTT GAT 152

 R   S   E   M   L   T   S   I   E   P   S   Q   V   N   Q   I   L  67
CGA AGT GAA ATG TTG ACG AGT ATT GAA CCT TCT CAA GTC AAT CAA ATC TTG 203

 S   Q   P   F   N   L   L   E   E   D   L   S   I   D   N   S   T  84
TCA CAA CCT TTT AAT CTT TTA GAG GAG GAT TTA TCT ATT GAT AAT TCA ACA 254

 I   S   E   L   T   N   E   S   N   R   N   Y   F   K   F   S   H 101
ATT TCA GAA CTT ACA AAT GAA TCA AAT CGC AAT TAC TTC AAG TTC TCT CAC 305

 N   L   H   L   Q   I                                             107
AAT CTG CAT CTC CAG ATT T                                          324
```

Comparison of the predicted amino acid sequence to the Swiss protein database resulted in the following possible related peptide sequences:

1. Of 40 best matched scores 17 are troponin C, e.g. from human, pig, chicken, rabbit, mouse ranging from 22% to 36% identical in 38 to 82 amino acid residues (Table 1). The following is the comparison of the predicted Nf435 amino acid sequence aligned with troponin C sequences from slow skeletal and cardiac muscles of rabbit, human, chicken and mouse. Asterisks(*) indicate positions of calcium-ligating structures. Calcium binding domain III of troponin C is highly conserved in rabbit, chicken, mouse and human and located from 140aa to 153aa. Calcium is bound in the domain by 6 amino acid, 141(D), 143(N), 145(D), 147(R), 149(D), 152(E) and 153(F). Alignment showed that Nf435 has high similarity in this calcium binding region (77%). It has an identical match at the positions 30(D) vs. 141(D), 34(D) vs. 145(D), 41(E) vs. 152(E) and 42(F) vs. 153(F), and similar (with conservative amino acid replacements) at 32(D)(Asp) vs. 143(N)(Asn), 36(N)(Asn) vs. 147(R)(Arg) and 38(N)(Asn) vs. 149(D)(Asp). We noted that there was a segment of 7 amino acids E-F-L-E-F-M-K outside of the calcium-binding region, which was identical with all of the troponin C segments.

```
                10          20          30          40          50
Nf435    NSQQDEIRIKRRKIKEKMM-SSIDDIIQKTDLDGDGNINYQEFLEFMKNFD
                                                                
Tpcc_R   YIDLDELKIMLQATGETITEDDIEELMKDGDKNNDGRIDYDEFLEFMKGVE
                120         130         140* * * * *  **    160
Tpcc_H       EELKIMLQATGETITEDDIEELMKDGDKNNDGRIDYDEFLEFMKGVE
Tpcc_C       EELKIMLQATGETITEDDIEELMKDGDKNNDGRIDYDEFLEFMKGVE
Tpcc_M       DELKMMLQATGETITEDDIEELMKDGDKNNDGRIDYDEFLEFMKGVE
```

Table 1. Comparison of predicted Nf435 amino acid sequence with troponin C from various species.

Species	Accs.#	Troponin C in	Identity(%)	Overlap(aa)	In Nf435
Rabbit	P02591	SLOW SKELETAL AND CARDIAC MUSCLES	36.2	47	4-50
Human	P02590	SLOW SKELETAL AND CARDIAC MUSCLES	34.0	47	5-50
Mouse	P19123	SLOW SKELETAL AND CARDIAC MUSCLES	34.0	47	5-50
Turkey	P10246	SKELETAL MUSCLE	34.2	38	9-46
Chick	P02588	SLOW SKELETAL MUSCLE	34.0	47	5-50
Barnacle	P21797	Troponin C, isoform	27.6	58	20-77
Pig	P02587	SKELETAL MUSCLE	22.0	82	9-88

2. Calmodulin from D. discoideum (P02599): 40% identical in 42 amino acid residues ranges from 4 to 46 aa of Nf435 verses 106 to 147 aa of calmodulin. Following is the comparison of the predicted Nf435 amino acid sequence aligned with calmodulin sequence of D. discoideum (Marshak, et al., 1984). The calmodulin molecule contains four structural domains that are homologous to each other and to the four domains of troponin C (Watterson et al., 1980). Calcium binding domain IV (Asterisks (*) represent the positions of calcium-ligating bonds) is located from 129aa to 140aa of calmodulin and binding bond at positions of

129(D), 131(D), 133(D), 135(Q), 137(N) and 140(E), which are identical to Nf435 at the position of 30(D), 32(D), 34(D), 38(N) and 41(E), similar at 36(N)(Asn) vs. 135(Q)(Gln). It is interesting that the possible calcium ligating site in the product of Nf435 matched with calmodulin of Dictyostelium is the same as the site matched with troponin C.

```
                10        20        30        40
435f.P      DEIRIKRRKIKEKMMS-SIDDIIQKTDLDGDGNINYQEFLEFM
            |:|  : :: ||: : ::|::|:::|||||||::||:||:::|
Calm_D      AELRHVMTSLGEKLTNEEVDEMIREADLDGDGQVNYDEFVKMM
                110       120       * * * * *  *  145
```

3. Also, there was a match with another calcium-binding protein (SM20) from Schistosoma mansoni (P15845): 44.4% identity in 27aa overlap ranges from 22 to 48aa of Nf435.

All of these genes encode a protein that binds calcium. Calcium binding proteins, such as calmodulin are important regulatory proteins. With bound calcium, they can then bind to and regulate the activity of many different cell proteins. This includes calcium-calmodulin-dependent protein kinase, which, in turn, phosphorylates target proteins and alters their activity levels. The function of troponin C in non-muscle cells is unknown. It is interesting that repression of transcript encoded the calcium binding protein was related with high virulence of amoebae.

The above alignment analysis suggested that there was a possible calcium binding function in the product of gene Nf435. Northern blot analysis showed that the transcript of gene Nf435 was repressed during the transition from low to high virulence. However, since the expression also was inhibited when amoebae were fed with mammalian cells, like gene Nf314, the altered expression of this gene was related to high virulence but not related excluding to virulence.

C. DISCUSSION

Complex changes occur in N. fowleri amoebae when these cells undergo a transition from low to high virulence. One approach to try and determine the molecular basis of this transition has been to compare high and low virulent amoebae by cytological or biochemical methods and to search for differences. In most studies negligible differences have been noted but highly virulent LEE strain differ from weakly virulent amoebae in relative absence of phagocytotic food cups (John, et al., 1985), in more rapid movement in vitro (Cline, et al., 1986, Thong, et al., 1986) and in contact-mediated lysis of cultured mammalian cells. Specific proteins, a phospholipase (Fulford, et al., 1986), a neuraminidase (Elsen, et al., 1987), cytopathogenic material (Dunnebacke, et al., 1989) and a membrane-associated pore-forming protein (Young, et al., 1989) may play a role in establishing degrees of virulence. Whether these quantitative changes are necessary and sufficient to convert

amoebae has been difficult to determine.

A second approach using molecular and genetic techniques can be complementary to the cytological and biochemical work. To begin a molecular and genetic analysis we assessed the protein changes which occur during the transition from low to highly virulent LEE strain by two dimensional gel electrophoresis (Hu, et al., 1991). Many more quantitative and most importantly new qualitative changes in protein accumulation and synthesis were found than had been previously thought to exist. The mechanism for increasing or decreasing protein levels in the cells was in part ascribed to changes in levels of specific transcripts. In vitro translation of poly (A)+ mRNA in rabbit reticulocyte extracts revealed many changes in steady state levels of individual transcripts (Hu, et al., 1991). We have now extended our results based on the previous knowledge of variable transcript levels by isolating cDNA clones of some of the differentially expressed mRNAs. Clones representing increases (Nf314) and decreases (Nf435) in levels have been found but as expected from the previous work most clones are constitutively expressed transcripts (Nf279 for example).

Here we have concentrated on the analysis of the Nf314 clone. The deduced sequence of the amino acid residues shows it to be related to a carboxypeptidase from yeast, barley and wheat. This role for the gene product would be

consistent with the previously noted importance of digestive enzymes, elastase (Ferrante, et al., 1988) or hydrolytic enzymes or cytolytic factors (Fulford, et al., 1986; Lowrey, et al., 1985) for the increased virulence. The overall similarity of identical and conservative changes of amino acid residues is high when the FASTP program is used for alignment of the sequences (Devereux, et al., 1984). A 194 amino acid section of the predicted amino acid sequence is 94 % similar to the known serine carboxypeptidase sequences. Little information is currently available to assess whether this percentage is within the range expected for comparisons of the sequences of N. fowleri. In Dictyostelium amoebae similarity to related proteins of the yeast Saccharomyces cerevisiae is not common even for highly conserved ribosomal proteins (Kopachik, unpublished). Data on the active site sequences of carboxypeptidases suggests that the serine 146 of yeast carboxypeptidase Y is at or near the active site. It is perhaps therefore significant that the deduced Nf314 gene product shares the high level of identical or conserved amino acids found in all three carboxypeptidases in the putative active site. If a transformation vector can be developed further knowledge about the function of the Nf314 gene product might be obtained by over-expression of the gene product in N. fowleri. Increased levels of carboxypeptidase in the expressing cells would be consistent with the role of Nf314 gene product predicted from sequence

analysis.

The current work showed that Nf314 transcript levels are regulated by mammalian cells in culture. The inducer for maximum increased mRNA levels was determined to be mammalian and not bacterial cells. This finding is not consistent with an inductive signal associated with invasiveness of nasal mucosa or response of amoebae to the host soluble or cellular immune defense mechanisms. Macrophages can target and kill amoebae in culture and antibodies are present against N. fowleri in many individuals (Fisher-Stenger, et al., 1990; Marciano-Cabral, 1988). It is conceivable that these immune responses could induce changes in amoeba gene expression but we have not found this to be the case for any of the two regulated genes cloned. Given this background the biochemical nature of the inducer of increased Nf314 gene expression remains to be determined but we have shown that the inducer is not unique to nerve cells. The induction also occurred when liver or fibroblast cells were used. It will be interesting to determine if transcripts from other genes are coordinately induced along with Nf314 gene expression. We expect this might occur because the synthesis of numerous proteins is coordinately increased or decreased (Hu, et al., 1991). The Nf435 transcript levels are oppositely regulated to Nf314 transcript levels: when Nf314 transcript levels are high, Nf435 transcript levels are low and when Nf314 transcript

levels are low Nf435 transcript levels are high. It is conceivable that the same induction signal from mammalian cells is transduced in cells by a common second messenger pathway, but the signal is interpreted differently to result in either increases or decreases of mRNA levels. Having found regulated genes we are interested to know if the mechanism of response to increase Nf314 mRNA levels includes increased transcription rates and increased mRNA stability, or both. Transcriptional regulation of gene expression can be determined by nuclear run-off experiments and post-transcription regulation could be assessed by examination of mRNA half lives.

A variety of approaches, cytological, biochemical and molecular-genetic have now been applied to the problem of understanding the capacity of N. fowleri to become pathogenic. All of these approaches are rapidly increasing our knowledge of this complex change and interaction of the amoebae with host cells. With more information on the biology of Naegleria improved strategies for prevention and treatment of primary amebic meningoencephalitis should emerge.

CHAPTER THREE: A simple, efficient method to create a cDNA library[1]

A. INTRODUCTION

Making a successful cDNA library is the one of the key steps in molecular cloning mRNA expressed from the eukaryotic genome. Currently, two popular techniques are used. The first "Classic method" (3) uses oligo(dT) while the second uses a "vector-primer" (Okayama-Berg method) (1,2), with an oligo(dT) at one end to prime poly (A)+ mRNA for cDNA synthesis. Although these two methods have been widely used, the chief drawbacks are the large amounts of poly (A) + mRNA necessary and the number of steps needed. In the "Classic method" either adapters or linkers are added to cDNA ends before inserting into an appropriate vector. The cDNAs must be separated from the excess unattached adapters which results in loss of cDNA. In the Okayama-Berg method, much less poly (A) + mRNA is needed but an oligo(dC) tail has to be added. Second, if the first strand cDNA is not completely synthesized a recessed 3′ end will be difficult to tail and some potential inserts will be lost. Third, a Hind III enzyme has to be used to remove one oligo(dC) tail, but incomplete enzyme digestion may be a problem. Fourth,

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[1]Chapter 3 has been accepted for publication in BioTechinique, December issue of 1992. (Hu, W., W. Kopachik, and R. Neal Band. 1992. A simple, efficient method to create a cDNA library. BioTechinique.)

the oligo (dC) tail of the non-cDNA end must be removed to prevent interference with ligation. The extra steps and additional manipulations taken increase the chances of losing samples and making errors. Therefore, even though large amounts of mRNAs may be available the yield of clones is sometimes low. This may be part of the reason why many beginners often fail to make a good cDNA library. Here we describe a quick simplified "All In One Tube" procedure to construct a cDNA library.

B. MATERIALS AND METHODS

Vector-Primer Preparation (Fig. 1). We used pBluescript II KS plasmid (Stratagene) to create a vector-primer. In the poly-cloning site, following complete digestion of 10 μg of the vector to form a blunt end with the restriction enzyme EcoR V (GAT'ATC), we added oligo(dT) tails to both ends of the vector with terminal deoxynucleotidyl transferase (Pharmacia) according to the Okayama and Berg method (1,2). The sample was extracted with phenol-chloroform and precipitated with ethanol and then digested with Sma I (CCC'GGG). The vector-primer was removed from the small part of the other oligo(dT) end by electrophoresis on a GTG SeaPlaque low melting agarose gel(1%) (FMC) and electroelution. After ethanol precipitation, the vector-primer was dissolved in 40 μl 10 mM Tris (pH 8) buffer.

cDNA Cloning (Fig. 2). Vector-primer 0.5 μg was mixed with the poly(A)+ mRNA selected with an oligo(dT) cellulose column (Pharmacia) from 200 μg total cellular RNA of soil amoebae Naegleria fowleri and heated 2 min. at 65°C. It was slowly cooled to room temperature to allow annealing. The first and second strand cDNA was synthesized using the "cDNA synthesis system plus" kit (Amersham) following the manufacturer's procedures. The second strand cDNA synthesis was labelled with 1 μl (10 μCi α-^{32}P-dCTP) (3000 Ci/mmol) (DuPont/NEN) and used as a tracer. After double-stranded cDNA synthesis, the sample was extracted with phenol-chloroform and ethanol precipitated overnight. The next step was self-ligation of the two intra-molecular blunt end arms. The DNA was resuspended in 28 μl ligation buffer with 2 μl (20 units) ligase (Boehringer Mannheim) and incubated overnight at 15°C (Fig. 2). DH5α cells (BRL) were transformed with the re-circularized recombinants following the manufacturer's directions.

C. RESULT AND DISCUSSION

The cDNA library made by this method contains about $2x10^5$ clones/μg vector and has 85% white colonies. Ten white colonies were selected and plasmids prepared (3). After digestion with restriction enzymes BamH I and Hind III the DNA was separated on an agarose gel (1.2%) (Fig.3). The cDNA insert sizes ranged from 200 bp to 2,000 bp, and are similar

in size to the inserts synthesized when using oligo(dT) as a primer (data not shown). This cDNA insert size range is representative of the mRNA size range of N. fowleri amoebae. Double-stranded DNA sequencing of one plasmid (4) confirmed its cDNA insert was correctly connected to the vector EcoR V site (Fig. 4).

The cDNA orientation can easily be constructed in the opposite direction if in the "vector-primer" preparation the restriction enzyme Sma I is first used,

The procedure described here has the following improvements over the two other methods:

1) It has the advantages of vector-primed double-stranded cDNA synthesis which include the cDNA inserts with a known orientation and low background of plasmids without inserts.

2) It is simpler because addition of linkers, adapters and C-tails is not necessary. It is therefore quicker and especially useful when only small amounts or valuable mRNA samples are available. The vector-primer synthesized cDNA needs only to be extracted with phenol-chloroform, ethanol precipitated and self-ligated.

3) This method overcomes the disadvantages of bi-molecular blunt end ligation while adding linkers or adapters in the "Classic method". Here a more efficient intramolecular ligation occurs.

4) In addition, the cDNA clones are already in pBluescript II KS with T3 and T7 promoter sites flanking the

insert. Once cloned, RNA transcripts can be generated directly by in vitro transcription systems.

A limitation of this method is that it misses the genes whose transcripts do not have poly(A) tails, such as histone gene.

Recommended Protocol:

Part I. Vector-Primer Preparation (Fig. 1):

1. Completely digest 10 μg pBluescript KS with EcoR V in a 100 μl volume.

2. Extract the DNA with phenol-chloroform once and chloroform once. Add 1/10 vol 3 M NaAc and 2 vol cold ethanol to precipitate the DNA on ice for at least 0.5 hr. Spin down at 13,000 rpm for 15 min at 4°C, rinse with 70% ethanol, dry and dissolve in 16 μl H_2O.

3. Add poly(dT) tails to both ends with terminal transferase (TdT) (1,2). Add 2 μl 10x TdT buffer, 1 μl of 5 mM dTTP and 40 units of TdT at 37°C for 15 min. The DNA is purified as in step 2.

4. Digest the poly(dT) tailed DNA with Sma I in a 100 μl volume.

5. Remove the vector-primer from the small dT tailed partial cloning site by running the DNA in a 1% GTG SeaPlaque low melting gel (FMC) at 4°C following the manufacturer's protocol.

6. The vector-primer is electro-eluted in a dialysis bag at 4°C and repurified as in step 2 and dissolved in 40 μl TE buffer.

Part II. cDNA cloning (Fig. 2): Adapted from the Amersham's cDNA synthesis Kit procedure.

7. For first strand cDNA synthesis: Mix 2 μl (0.5 μg) vector-primer with 9 μl poly (A)+ mRNA (~1 μg) at 65°C for 2 min and let tube cool down for 15 min at room temperature; add 4 μl 5x reaction buffer, 1 μl Sodium pyrophosphate, 1 μl RNAse inhibitor, 2 μl dNTPs (10mM) and 1.5 μl (30 units) reverse transcriptase and incubate for 1 hr. at 42°C. Keep on ice.

8. For second strand cDNA synthesis: Add 37.5 μl second strand synthesis buffer, 1 μl E. coli RNAse H (0.8 unit), 7 μl E. coli DNA polymerase I (24.5 units) and 34.5 μl H_2O (total 100 μl) and incubate at 12°C for 1 hr (optional: add 1 μl ^{32}P-dCTP for tracer) and then at 22°C for 1 hr; transfer to 70°C for 10 min. After spinning down for 15 sec. in a microcentrifuge, add 1 μl T4 DNA polymerase (4 units) at 37°C for 10-15 min.

9. Purify the DNA as in step 2. Add an equal vol. of 4 M ammonium acetate and 2 vol. of ethanol to precipitate overnight at -20°C. Spin down at 13,000 rpm at 4°C for 15 min. Aspirate the supernatant, rinse the cDNA with 70% ethanol, dry and dissolve in 25 μl H_2O.

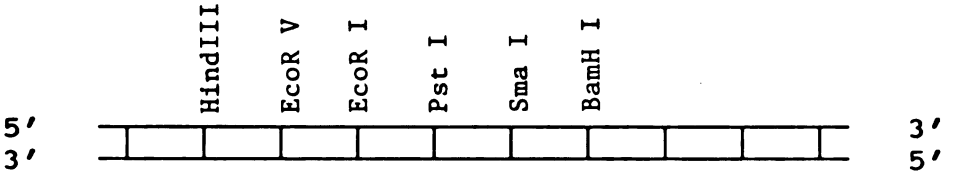
10. Blunt end ligation. Add 3 μl 10x ligation buffer

and 2 μl T4 DNA ligase (20 units, Boehringer Mannheim) and incubate at 15°C overnight.

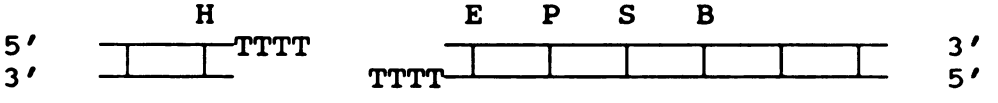
Part III. Transformation:

11. Take 5 μl of the ligation mix and add to 100-200 μl DH5α competent cells (BRL) following the manufacturer's procedure.

a. Partial Multiple Cloning Sites of Plasmid pBluescript II KS (Stratagene)



b. Eco RV digestion & Oligo(dT) tail



c. Sma I digestion & purification

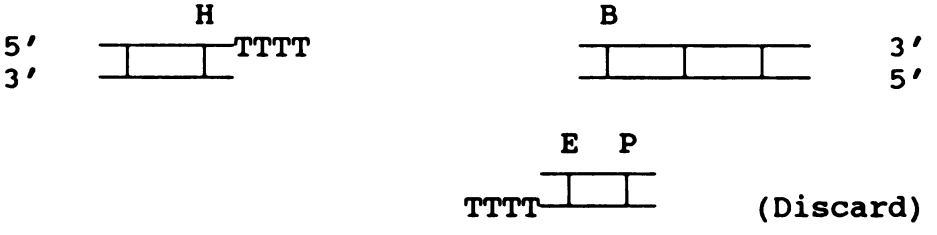


Figure 1. Diagram of vector-primer preparation. Abbreviations: H: Hind III; E: EcoR I; P: Pst I; S: Sma I; B: BamH I.

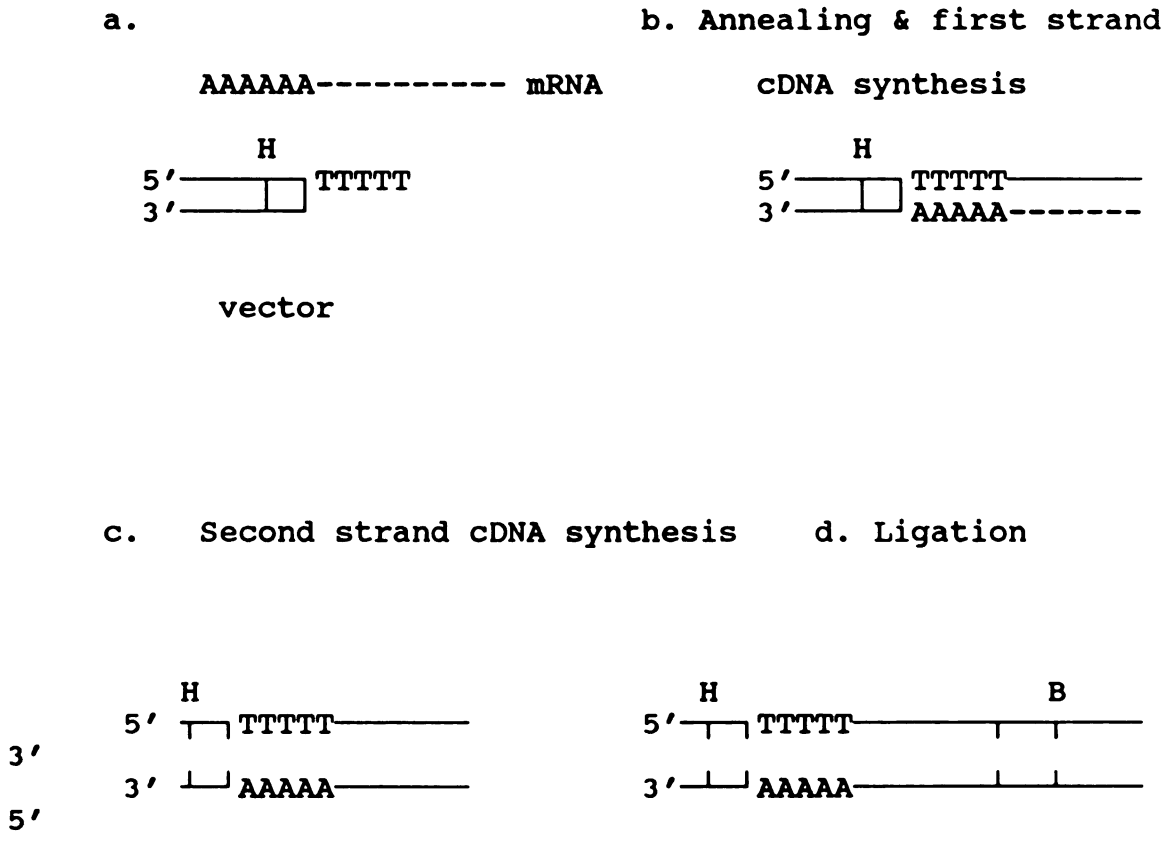


Figure 2. Diagram of cDNA recombinant construction.

Figure 3. Mini-prep analysis of Naegleria fowleri cDNAs. cDNA inserts were released with double-digestion of restriction enzymes BamH I and Hind III and electrophoresed on 1.2% agarose gel. Lanes 1 to 10 were cDNA clones; M, size markers.

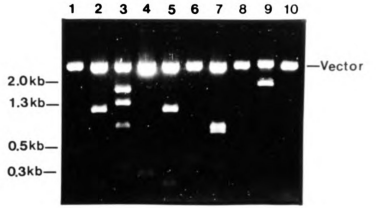
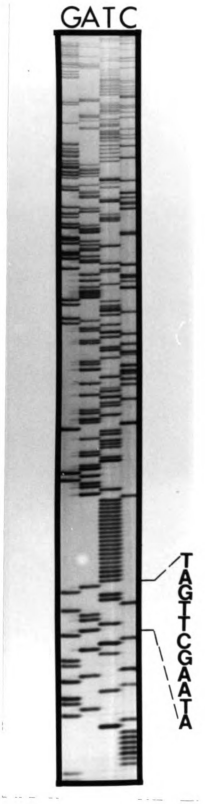

1 2 3 4 5 6 7 8 9 10
Vector
2.0kb
1.3kb
0.5kb
0.3kb

Figure 4. Double-stranded DNA sequencing of a randomly selected cDNA clone to show that cDNA insert was connected at the Eco RV site. Sequencing was performed with Sequenase kit Version 2.0 (United State Biochemical) according manufacture's procedure and labelled with ^{35}S-dATP (DuPont).


GATC
TAGTTCGAATA

SUMMARY AND DISCUSSION

My research emphasized a study of epigenetic changes in virulence of *N. fowleri* and not differences in pathogenesis between species or strains among *Naegleria*. This contrasts with the work on pathogenesis in *E. histolytica*. Much of the work on *E. histolytica* now focuses on distinguishing pathogenic from non-pathogenic isolates (Ravdin, 1989). As mentioned, *N. fowleri* is always pathogenic but virulence can be altered. Therefore, I focused on differential expression of genes responsible for virulence changes during the transition of LEE amoebae from axenic growth to growth in the mouse brain. The goal of this work was an initial step to uncover the molecular signals of high virulence by *N. fowleri*. By inoculating long term, axenically cultured LEE strain amoebae into the mouse brain to trigger high virulence, I was able to investigate the mechanism of the process. Changes of gene expression of amoebae were observed by two-dimensional gel electrophoresis analysis during the transition of amoebae from low to high virulence. To identify the transcripts differentially expressed in highly virulent versus weakly virulent amoebae, a cDNA library was made to highly virulent amoebae. Three cDNA clones were isolated by differential screening of the cDNA library. The transcripts homologous to Nf314 and Nf116 were preferentially expressed in highly virulent cells whereas the transcript homologous to Nf435 was preferentially

expressed in low virulent amoebae. The results were important because mRNA levels of these genes correlated with changes in virulence. However, because the Nf116 (actin), Nf314 and Nf435 gene expression correlated with feeding on mammalian cells as a food source, these genes might be required but are not sufficient for increased virulence.

We eliminated the possibility that the initial low virulence of N. fowleri was due to a mixture of low or non-virulent species or variants. We used a cloned amoeba of N. fowleri for our experiment so that this potential problem was not possible.

Is it possible that the induced gene expression responsible for high virulence is terminated after amoebae entered the brain? I believe this is unlikely since the virulence of amoebae after brain passage has been increased which indicates the gene(s) are still being expressed. However, it is possible that transient gene expression is needed to penetrate host barriers (e.g. the nasal mucosa) and these would not be detected in virulent amoebae isolated from the brain.

Could the changes in protein synthesis be due to stress proteins? Stress protein synthesis inhibits other protein synthesis and cell growth (Atkinson, et al., 1985). LEEmp amoebae in mouse brain and in axenic culture grow very well. The changes associated with virulence noted in two-dimensional gels, persisted for weeks or for months and

conserved patterns of stress proteins was not observed in two-dimensional gels. Therefore, there is no reason to believe the induced proteins are simply stress proteins.

Protein synthesis changes could have arisen by transcriptional and by post-transcriptional regulatory mechanisms. My research mainly focused on the changes in transcript levels. Therefore, what genes would I miss by technical limitations or by the way I screened for the cDNA library? In the current cDNA screen method, I only detect relatively high abundance transcripts at about 5%. I will miss some epigenetic changes in gene expression. Here are representative examples. We would not detect a change: if the mRNA level does not change, but the translatability does; if the protein abundance does not change, but the protein is post-translationally activated or inactivated and undetectable on two-dimensional gels; if a change in glycosylation of a glycoprotein or glycolipid is important for increasing virulence; if membrane surface rearrangements increase virulence; if physiological(e.g. chemotaxis, chemokinesis, phagocytosis) and metabolic changes are important for virulence and do not result in protein or mRNA change. Fortunately other investigators using immunological, biochemical and other cell biological methods will address some of what we miss.

We knew that the changes in gene expression during transition from low to high virulence were complex (Hu, et

al., 1991). To simplify the complexity and to clarify the future work, it is necessary to make a classification of genes in order to focus on those regulated genes and gene products which are related to virulence. Genes whose expression is altered in response to feeding on mammalian cells and tissues without significantly increasing virulence are referred to as LEE:CELL genes. We will refer to those genes, whose expression is changed due to introducing amoebae intranasally in mice and coinciding with increased virulence, as LEE:MP (mouse brain passage) genes. LEE:MP gene products could be used to overcome host barriers and participate in the pathogenic action of amoebae on brain tissues together with LEE:CELL gene products.

To clone LEE:MP genes, we will use the cDNAs made from mRNA of amoebae fed with mouse brain cells (LEE:CELL) as probes, instead of using cDNAs from mRNA of axenic amoebae (LEE:AX). The other probe will be cDNAs from mRNA of mouse brain passaged amoebae (LEE:MP). This will permit us to isolate the virulence-specific genes. As an alternative, we could also construct a subtracted cDNA library from LEE:MP mRNA. We would isolate LEE:CELL mRNA and bind them onto a poly(T) column. First strand LEE:MP cDNAs would be passed through the poly(T) column a few times to hybridize with homologous mRNAs. The un-hybridized cDNAs will be used to construct a cDNA library. With small amounts of mRNA, we can use the method described in Chapter three to make and then

differentially screen the library.

Increased virulence could be a result of either increased or decreased gene expression in response to contact with the nasal-mucosa epithelial matrix and cells. To clone transcripts present at a lower level when amoebae enter the mouse brain, we could make a cDNA library from axenic cultured amoeba LEE:AX mRNAs. Those low level transcripts could be isolated by differential screening the cDNA library with cDNAs made from LEE:MP and LEE:AX mRNAs.

The increased protein synthesis changes may be due to transcripts regulated to a high level. To clone those genes, we could run two-dimensional gels using protein samples prepared from LEE:MP and LEE:CELL amoebae to observe changes in protein synthesis. The negative control would be to run a two-dimensional gel with a protein sample made from mouse brain cells to eliminate the possibility of contamination. We can then, isolate the specific spots and micro-sequence the protein (Matsudaira, 1987). Oligonucleotides based on that sequence could be used to isolate the cDNA by screening of a cDNA library.

Many micro-organisms such as viruses, bacteria, fungi and protozoa can cause CNS diseases. The routes of infection are diverse depending on the microbes. For example, bacteria may gain access to the subarachnoid space via hematological invasion or local spread. Virus-induced diseases are mostly due to infection of skin, usually by the

bite of insects, or via the exposed mucous membranes of the respiratory, gastrointestinal or urogenital tracts (Adams, et al., 1988, Chadwick, et al., 1989). Once penetration occurs, viruses may reach the CNS by direct neural spread or by hematogenous or lymphatic spread to cause disease. Fungi may give rise to a variety of neurological syndromes causing a subacute meningitis. Some diseases of the CNS are also caused by amoebae such as Naegleria fowleri or Acanthamoeba spp. The port of entry may be different between them. Infection from N. fowleri is via swimming in warm polluted lakes or pond, whereas Acanthamoeba spp. may gain entry by lungs, urogenital tract, or skin (Ma, et al., 1990, Lambert, 1991). However, the precise nature of the mechanisms employed by many micro-organisms to gain an entry is still not well known. Factors involved in parasite entry may be mediated by one major protein or multiple gene products. The molecular and genetic study to understand the precise nature how N. fowleri gains the entry into the host is the area of the present research. However, to date, none of those genes have been identified.

The host-parasite interaction is a very complex process. Signaling between microbe and host involves reciprocal interaction. For example, virulence modulation occurs for poliovirus serotype 3 in host cells (Wick, et al., 1991). It is neurotropic but is attenuated after passage through cell cultures and monkeys. The genetic basis

of viral modulation is several nucleotide differences in the region upstream of the poly-protein encoded by the viral RNA. Position 472 in the 5' untranslated region of the viral RNA is particularly related to neuro-virulence. Virulence correlates with Cytosine phosphate-472 whereas attenuation correlates with Uridine phosphate-472. This was supported by site mutagenesis (Wick, et al., 1991). We do not know whether there is a similar process involved in the amoebae, but it does serve as one example of virulence modification at the molecular level.

One essential factor for infection by a particular microbe is that the host cell must have specific receptors on its plasma membrane. For several bacterial genera, specific proteins encoded by the microbe appear to play a critical role in their ability to invade eukaryotic cells (Falkow, 1991). The bacterial pathogen Yersinia pseudotuberculosis is a well-studied example of an invasion process mediated by a membrane protein encoded by a so-called "invasion gene" (Miller, 1992). This invasin protein interacts with integrins (host surface receptor) to mediate the bacterial entry of the host.

Entamoeba histolytica is an obligate human gut parasite. The invasive amoebae penetrating the intestinal mucosa and phagocytosis of host tissues is a multifactorial process. The first step is surface contact between trophozoites and the target cells. This interaction is

believed to be mediated by specific molecules present in the surface of the parasite. The adherence is mediated with a galactose-specific cell surface lectin (Tachibana, et al. 1991). It is not known if the invasion and penetration of the nasal mucosa requires a similar receptor-ligand binding mechanism in N. fowleri.

In the future, with LEE:MP genes which are likely to play a role in virulence, the question may be: what is the biological function of the gene(s)? In our case, a cloned gene may be of interest because of its differential expression at a specific developmental stage (necessary and specific for high virulence). To test possible roles of cloned LEE:MP genes, a method of "Reverse genetics" may be used. The logic is simple in principle: isolate a gene, then test the gene's role by molecular genetic techniques. One of the advantages of this method is that without knowing the role of a particular DNA sequence beforehand or reference to a specific protein, we could determine its function or developmental significance by "reverse genetics" according to an altered developmental program. For example, the experimenter can place a gene under the control of regulatory elements that will alter the temporal or spatial regulation of gene expression. The resulting phenotype may suggest how the gene product functions in normal development. This technique has been widely and successfully used in analyzing the functions for many genes in many

organisms such as human genetic diseases (Orkin, 1986), mouse (Landel, et al., 1990), Drosophila (Ballinger and Benzer, 1989) and yeast (Campbell, 1988). Here is an example of "reverse genetics" in human genetic disease. Transgenic animals that display phenotypes similar to human disorders have been useful for defining gene function. The generation of animal models for human disease has used the insertion of genes encoding mouse homologue of human dominant mutation into the mouse germline in attempts to create a mouse analogue of a human disorder. Osteogenesis imperfecta (OI) type 2 is a autosomal dominant disorder caused by the substitution of a single glycine residue in the triple helix of the α1-procollagen gene COLIAI. This substitution in a repeating Gly-X-Y structure alters the molecule so that collagen assembly is affected and results in abnormalities of bone formation. A similar phenotype was induced in a transgenic mouse strain by performing in vitro mutagenesis on the mouse homolog of the human α1-procollagen gene to induce the same amino acid substitution. The mutated gene was then used to produce transgenic mice by micro-injection into isolated mouse embryos. All of the resulting transgenic animals died shortly after birth due to severe developmental defects in the formation of the skeleton. Analysis of these mice for the mutant procollagen demonstrated that expression of the mutation gene at levels as low as 10% of normal was sufficient to disrupt bone

formation and lead to death. This suggests that the defective protein inhibits collagen assembly rather than forming a less stable molecule that is susceptible to degradation or altered secretion (Landel, et al., 1990).

In our case, we would develop a transformation expression vector carrying the cloned LEE:MP gene and re-introduce this into weakly virulent amoebae. The gene can be over-expressed at the transcriptional level under the control of a strong promoter (e.g. actin gene). Transformants will then be tested for virulence. Site-directed or deletion mutagenesis could also be used to define the bases or sequences necessary for regulation of transcription.

One limitation to study the function of introduced gene is the presence of the wild counterpart of the gene in the cell. This requires that both alleles be inactivated prior to testing. To overcome this limitation, "gene targeting or homologous recombination" could be used, by which the endogenous functional gene can be precisely replaced with an engineered derivative. This is a very powerful method for altering and testing gene functions. This has been successful in Dictyostelium (Witke, et al., 1987; De Lozanne, et al., 1987; Manstein, et al., 1989), yeast and mice (Bollag, et al., 1989, Capecchi, 1989). In diploid organisms with a sexual cycle, a single heterozygous replacement is first obtained, and then rendered homozygous

by sexual crossing. However, many diploid unicellular organisms, or cultured mammalian cells lack a sexual cycle and they offer superb systems for studying biological phenomena not readily accessible in whole organisms. Gene sequences in mammalian cells have been targeted by homologous recombination. This is a strategy to obtain a double knockout of the 2 copies of a targeted gene, creating null mutants (Riele et al., 1990, Mortensen, et al., 1991). It has also been used in the asexual protozoan parasite of Leishmania major (Cruz, et al., 1991). In this method, homologous recombination was used to sequentially inactivate both alleles of an endogenous gene. Two markers (neomycin phosphotransferase [NEO] and hygromycin phosphotransferase [HYG]) were used in their studies. Briefly, a targeting vector that contains the targeting gene interrupted by the marker gene (NEO), was constructed and introduced into cells by electroporation. Stable transformants (+/neo) were then selected with G418. The remaining normal allele was inactivated by a similar process. The same vector containing the target gene but interrupted by a different marker gene (HYG) was used to knock out the second allele. The transfection and selection were the same as for the +/neo except hygromycin B was substituted for G418. Both Southern blot analysis and/or PCR were used for analysis of targeted event. In the PCR approach, two primers were synthesized: one was designed to anneal and prime at the site of the

target gene and the other at the site of G418 resistant gene. With non-homologous recombination, no fragment was amplified. In targeted insertion, the primers were annealed to each other and produced an amplified fragment at a predicted size.

Naegleria fowleri is at least a diploid organism. This is strongly suggested by the occurrence of heterozygous electrophoretic patterns of many isozymes (Pernin, et al., 1985, Cariou, et al., 1987 and Adams, et al., 1989). Sexual recombination in this organism is unknown. Therefore, the above strategy could be used to inactivate both alleles of the endogenous gene (LEE:MP) before testing its function. Availability of antibiotic markers is another important issue. Resistance of N. fowleri LEE strain to G418 has been tested previously in our laboratory. The LEE strain is not sensitive to this drug. Resistance to Hygromycin-B and some other antibiotics such as Bleomycin could be tested in the future.

Other methods could also be used to obtain homozygous mutant lines from a heterozygous parent in the absence of a sexual cycle. Parasexual crossing has been utilized in Dictyostelium, but has not been demonstrated in Naegleria. Another approach may be the use of radiation or other agents to induce mutations. The disadvantage of this method is that radiation or mutagenic agents may create many unpredictable mutations which are not easy to analyze.

An alternative approach for shutting off the functional existing gene is called the "antisense" method. The idea is to target the gene's mRNA rather than the gene itself. We could transfect amoebae with an expression vector that carries a portion of the target gene (LEE:MP) downstream of a strong promoter. But the orientation of the target gene in the vector would be backward, so that the RNA transcribed from the vector is complementary in sequence to the mRNA transcribed from the corresponding cellular gene. The antisense RNA is present in large excess, its bases pair to the functional mRNA to form a double-stranded RNA that cannot be translated into protein. Then the transformed amoebae would be tested for virulence.

Much of the above depends on the availability of a transfection vector. Therefore, constructing a transfection vector used for above tasks will be another important project in the future. This vector will have an E. coli ori and an antibiotic (e.g. ampicillin) resistance gene for propagation in bacteria. A strong expression promoter (e.g. actin gene) would be included to overexpress the transgene in a sense or antisense configuration. Multiple cloning sites with three translational reading frames would be used to insert into a gene of interest in a proper position. Finally, translation stop codons in all three reading frames and a transcription termination signal (AUAAA) segment would be also included in the vector.

There are several things we could do in the analysis of gene Nf314. First, to further understand more about the structure, function and possible regulatory mechanism of the gene Nf314, a genomic clone of Nf314 is needed. A genomic library could be constructed by using bacteriophage lambda Zap II (Stratagene) vector. The cDNA insert of Nf314 would be used as a probe to screen a genomic DNA library of N. fowleri to obtain the genomic clone of gene Nf314.

To further verify that a serine carboxypeptidase is the product of gene Nf314, an oligo-peptide from the deduced amino acid of gene Nf314 could be synthesized and used to generate an antisera in rabbits (Reilly, et al., 1991). The immunoglobin fraction is precipitated from the sera, dialyzed and redissolved in a proper buffer. The antibodies would be then used for immuno binding to: a) a known carboxypeptidase; b) the amoeba extract fractionated on SDS/PAGE gel and transferred to a nylon-membrane (Western blot analysis). An additional control would use a known carboxypeptidase antibody generated from another species (e.g. from mosquito, Cho, et al., 1991) to hybridize with the blot of amoeba extract. We expect that the hybridization band in b) experiment would be the same as that of control experiment if the product of Nf314 is a serine carboxypeptidase.

A binding analysis to an inhibitor could also be performed to determine the molecular size and amounts of

serine carboxypeptidase between LEE:AX and LEE:MP amoebae. Amoeba extract would be incubated with a labelled serine protease inhibitor, [^{3}H]-diisopropyl fluorophosphate (DFP) which binds to the active site of serine protease (Cho et al., 1991) as a marker. The mixture would then be separated by SDS/PAGE. The gel would then be dried and exposed to a X-ray film. We expect to see thicker and darker band in LEE:MP comparing with LEE:AX amoeba extract line.

Serine carboxypeptidase purification for partial sequencing compared with the deduced amino acid sequence of Nf314 may be another way to verify the gene Nf314 product. The purification of the enzyme could be performed according to the methods described by Cho, et al.(1991). Partial amino acid sequencing could be done by using the protein sequencer from the Department of Biochemistry, Michigan State University.

We know that the increased Nf314 expression was induced by co-culturing amoebae with mammalian cells. However, we do not know where the inducer is located. We could incubate amoebae with mammalian cell surface membrane or cytoplasm individually (Clarke, et al., 1988). Then RNA would be extracted from amoebae and Northern blot analysis used to see the response of Nf314 transcript to the different feeding studies. After determining the possible location of the inducer, its ultimate identification would be possible.

There are still many other questions to be answered

such as how many LEE:MP genes are really involved in the amoebae's virulence? how are those genes expression regulated? what is the inducer of the virulence? How do the signal transmitted from environment to genes? what are the immune functions of the host to the microbe? why amoebae fed by an mouse brain cells (LEE:CELL amoebae) did not increase in virulence? We are just beginning to explore the nature of the molecular mechanism of virulence. With more information available, better methods for prevention and treatment of PAM may be revealed.

Appendix 1.

Electrophoretic Karyotype of Naegleria fowleri

The analysis of chromosomal organization has been difficult in Naegleria. The exact number and size of chromosomes are not known. This controversy is due to the difficulty in counting the chromosomes cytologically since they are small and closely packed to accurately enumerate even with electron microscopy (Fulton, 1970).

Pulsed-field gel electrophoresis (PFGE) allows separation of chromosome-sized DNAs. Previous PFGE in N. fowleri (De Jonckheere, 1989) did not give a good separation pattern. The bands were smeared and blurry. It was reported that between 15 to 23 bands resolved in size ranged from a few hundred kb to about 1.5 Mbp.

I attempted to establish an electrophoretic karyotype for N. fowleri. It would be possible to localize the cloned genes onto specific chromosomes, to study linkages with other genes of this organism.

Materials and Methods

1. Equipment for PFGE: CHEF-DR II Megabase DNA pulsed field electrophoresis system from BioRad was used.

2. Preparation of Chromosome Sample for PFGE: 10^8 cells were harvested and embedded in 1% low melting agarose gel (Sea Plaque) in the mold on ice for 1 hr. The agarose blocks were incubated in lysis buffer (1% SDS, 0.5 M EDTA pH 9.5, and 2 mg/ml proteinase K) at 51°C for 3 days. Blocks

were stored in the same buffer at 4°C until electrophoresis.

3. Pulsed Field Gel Electrophoresis: The gel was run in 1% SeaKem Gold agarose (FMC Inc.) in 0.5 X TBE buffer at 180 V with pulse times of 80 to 100 sec. for 24 hr along with the standard DNA molecular weight marker (Lambda ladder and Yeast chromosomes).

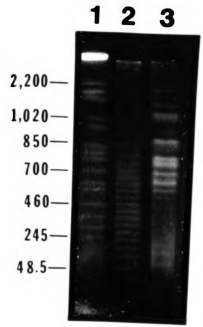
Results and Discussion

The gel resolved about 14 bands and 15 Yeast chromosomes (Fig.1). Bands having stronger intensity may contain more than one chromosome with similar molecular weights. The size ranged from about 50 Kbp to more than 1.6 Mbp.

In comparing my result to De Jonckheere's work, a clearer banding pattern was observed. This can be seen by the sharp resolution of yeast chromosomal and lamba markers.

This was a preliminary experiment. Many factors such as different pulsed times, voltages, concentration of agarose and temperature affect the migration and resolution of banding pattern (Lai, et al, 1989). Further improvement could be done by adjusting the above factors to achieve the best resolution. For example, using a lower range of pulsed time (50 to 70 sec. instead of 80 to 100 sec.) to increase the mobility of low molecular weight chromosomes and repress larger ones to get a better resolution of smaller sized chromosomes.

Figure 1. Electrokaryotype of N. fowleri LEE strain. Yeast (Saccharomyces cerevisiae) chromosomes (line 1) and lambda DNA (Line 2) were used as size markers (Kbp). Line 3 is N. fowleri karyotype pattern.


1 2 3
2,200
1,020
850
700
460
245
48.5

Appendix 2. Sequence analysis of an actin cDNA insert[1].

The rapid penetration of amoebae into the brain implies that actin, one of the components governing cell mobility, may play an important function for pathogenesis of N. fowleri, in addition to participating in maintenance of cell structure and cytokinesis. A study of four species of Naegleria under agarose showed that pathogenic spp. are more motile (Thong and Ferrante, 1986). N. fowleri and N. australiensis (pathogenic to mice) exhibited more rapid locomotion than nonpathogenic N. lovaniensis and N. gruberi (Marciano-Cabral, 1988). The species differences in motility were also observed when these four species were placed in proximity to nerve cells (Marciano-Cabral, 1988). Highly virulent amoebae of N. fowleri LEE strain (LEE:MP), but not axenic cultured, weakly virulent amoebae (LEE:AX) were chemotaxic in vitro to neuroblastoma cells extract. (Brinkley and Marciano-Cabral, 1992). Northern blot analysis showed that the level of actin transcripts in LEEmp amoebae increased at least 3 fold over the LEE amoebae (Hu, et al., 1991). An increase of actin transcripts and motility might be associated with enhanced amoeba virulence. We isolated a few positive clones from the cDNA library by hybridization with a 20mer oligo-nucleotide (5'-TAGAAGCATTTTCTGTGCAC-3'), which was complementary to the sense strand near the

C G G G

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[1]This work was in part of Jonghee Ahn's master thesis.

carboxyl terminus end of actin genes according to a consensus sequence from various species. The longest insert 1220 bp was sequenced and had an open reading frame of 375 amino acids (Jonghee Ahn, 1992, M.S. thesis, unpublished). Sequence analysis comparison revealed a difference in genetic codon usage between N. fowleri and A. castellanii (Table 1.). For example, N. fowleri (actin and carboxypeptidase) uses only TAA as the stop codon, but TGA and TAA were both used in A. castellanii. Table 1 illustrated that CGT (30%) and AGA (59%) were the preferred codons for arginine in N. fowleri, while CGC, CGG and AGG were not used. In contrast, CGC (54%) was used by A. castellanii. In the case of leucine, TTG (63%) was the dominant codon in N. fowleri but CTC (56%) and CTG (36%) were used by A. castellanii. Other comparisons included glutamine in which N. fowleri used CAA (92%) and GAA (89%) for glutamic acid, instead of CAG (90%) and GAC (95%) in A. castellanii. We compared the third-position nucleotide frequency codon usage for actin cDNA with A. castellanii and several other species (Table 2). Interestingly, there is a significant difference in G+C usage between these two species. Actin cDNA in N. fowleri used (40%) G+C; which was comparable to other eukaryotic microorganisms. However, A. castellanii exhibited a much higher G+C content (85%), similar to Drosophila melanogaster (G+C, 85%). This result suggests that those two species may have diverged early in

evolution. This codon usage information will be useful in designing oligonucleotides for probes and for the polymerase chain reaction.

The GenBank accession number for the cDNA clone of action gene transcript was #M90311.

Table 1. Codon usage by Naegleria fowleri and Acanthamoeba castellanii.*

Amino acid	Codon	Nf	Ac	Amino acid	Codon	Nf	Ac	Amino acid	Codon	Nf	Ac
ARG	CGT	30	19	LEU	TTA	16	0	SER	TCT	27	6
(R)	CGC	0	54	(L)	TTG	63	5	(S)	TCC	23	38
	CGA	11	3		CTT	9	4		TCA	12	0
	CGG	0	2		CTC	11	56		TCG	13	44
	AGA	59	4		CTA	0	0		AGT	23	0
	AGG	0	17		CTG	1	36		AGC	2	13
ALA	GCT	51	8	GLY	GGT	55	25	PRO	CCT	30	14
(A)	GCC	25	62	(G)	GGC	2	65	(P)	CCC	7	59
	GCA	21	1		GGA	42	8		CCA	63	0
	GCG	3	29		GGG	1	1		CCG	0	27
VAL	GTT	47	6	THR	ACT	50	5	ILE	ATT	78	8
(V)	GTC	27	51	(T)	ACC	29	69	(I)	ATC	20	92
	GTA	3	0		ACA	17	1		ATA	2	0
	GTG	23	43		ACG	4	24	CYS	TGT	79	0
ASN	AAT	72	3	ASP	GAT	80	7	(C)	TGC	21	10
(N)	AAC	28	97	(D)	GAC	20	93	HIS	CAT	60	9
GLN	CAA	92	10	GLU	GAA	89	5	(H)	CAC	40	91
(Q)	CAG	8	90	(E)	GAG	11	95	TYR	TAT	76	8
LYS	AAA	19	3	PHE	TTT	47	6	(Y)	TAC	24	92
(K)	AAG	81	97	(F)	TTC	53	94	END	TGA	0	50
MET	ATG	100	100	TRP	TGG	100	100		TAG	0	0
(M)				(W)					TAA	100	50

* % codon frequency given for N. fowleri (NF) actin I (GenBank accession number M90311) and serine carboxypeptidase (M88397); A. castellanii (AC) actin (V00002, J01016) and myosin 1B heavy chain (J02974, M13564, M13565). Codon frequency calculated with the Genetics Computer Group Sequence Analysis software package (Devereux et al., 1984).

Table 2. Third-Codon-Position Nucleotide Frequency for Actin Gene Coding Region*

	%A	%T	%G	%C	%G+C	Reference
Naegleria fowleri	20	40	20	20	40	this paper
(carboxypeptidase)	27	44	16	13	29	Hu, et al., 1992
Dictyostyelium discoideum	32	33	6	29	35	Warrick, 1987
Physarum polycephalum	27	17	35	21	56	Hamelin et al., 1988
Saccharomyces cerevisiae	21	36	14	29	43	Edman, et al., 1987
Tetrahymena thermophila	16	15	13	36	49	Martindale, 1989
Entamoeba histolytica	38	40	8	14	22	Edman, et al. 1987
Plasmodium falciparum	45	40	8	8	15	Wesseling, 1989
Acanthamoeba castellanii	5	10	29	56	85	Nellen & Gallwitz, 1982
Drosophila melanogaster	6	11	31	52	83	Starmer & Sullivan, 1989

*Carboxypeptidase from N. fowleri was included since it is the only other gene sequence published for this organism, except ribosomal nucleotide sequences (Baverstock et al., 1989).

Appendix 3

Note: The following methods (or protocols) used during my research, were developed by Dr. W. Kopachik, or they were commonly used procedures available in his laboratory.

MATERIALS AND METHODS

1. CULTURE OF *N. fowleri*

Low-virulent amoebae of the LEE strain of *N. fowleri* were obtained from Dr. David John, grown in 10 ml H4 liquid medium supplemented with 1 ml hemin in 250 ml culture flask (Band and Balamuth, 1969) at 37°C.

High virulent amoebae (LEEmp) were obtained by serial inoculation of the LEE strain amoebae into mouse brains for four consecutive passages. Ten 3 to 4-week-old male mice (*Mus musculus* BALB/c) were used for each passage. Amoebae were harvested and washed by centrifugation three times with LS saline (50 mM NaCl, 4.6 mM $MgSO_4$ and 0.36 mM $CaCl_2$). A series of 2 μl of LS saline containing *N. fowleri* (10^6 amoebae were used for the first passage and 10^4 for subsequent passages) were applied into the nares of each mouse, using a narrow bore 0-200 μl microcapillary pipet tip (Western Coast Scientific, Inc.). Within 4-6 hr after death pieces of brains were placed in 250 ml tissue culture flasks (Corning Science Products) containing 10 ml H4 medium with Penicillin-G (1000 units/ml), Streptomycin (100 units/ml)

and incubated at 37°C. Two hr later, mouse brain tissues were removed and fresh H4 medium without antibiotics was added to the residual amoebae. The amoebae were then cultured for 24 to 36 hr before protein analysis.

Bacterized (LEE.b) amoebae were grown in E. coli (strain K-12) (1 mg/ml LS saline) and incubated at 37°C. Before protein analysis, the amoebae were cultured in LS saline overnight without bacteria.

Amoebae were co-cultured with 3T3 fibroblast tissue culture cells, with dissociated mouse brain cells and with dissociated mouse liver cells in H4 medium without hemin at 37°C.

2. DNA ISOLATION

A. Plasmid DNA isolation (for high copy number plasmid, e.g. pUC18, pBluescript).

a. Large scale plasmid DNA preparation without CsCl.

The plasmid DNA isolated with this procedure is good for sequencing, restriction enzyme digestion, etc.

1. Grow 50 ml E. coli DH5α bacterial culture with 50 μg/ml ampicillin overnight.

2. Harvest bacteria with 50 ml tube.

3. Resuspend cells in 3 ml lysis buffer (50 mM Tris, 10mM EDTA, pH 8 and 20% Sucrose) with 20 mg lysozyme and keep on ice for 10 min.

4. Carefully dropwise add 6 ml freshly made 0.2 N NaOH/1% SDS buffer while swirling and pipeting them up and down with 10 ml pipette until mixed, then incubate on ice for about 5-10 min. (after cell lysis, the solution will be clear.)

5. Add 3.8 ml 3 M KAc (pH 4.8 or 5.2). Carefully mix by inversion and incubate on ice for about 15 min, or until all the white staff floating on the surface.

6. Centrifuge at 12,000 rpm for 10 min. at room temperature.

7. Filter the supernatant with No.1 Whatman filter into 30 ml Corex tube.

8. Add 2 vol. 100% cold ethanol, mix and keep the tube on ice for 30 min.

9. Spin 15 min at 4°C at 12,000 rpm using HB-4 rotor.

10. Discard the supernatant, rinse with 10 ml 70% ethanol and dry.

11. Dissolve the pellet in 3 ml TE buffer.

12. Add 150 μl RNase A (10 mg/ml) and incubate at 37°C for 1-2 hr.

13. Transfer the solution into a 15 ml orange cap tube; add 2 ml phenol/chloroform, vortex and pour into a 15 ml Corex tube.

14. Spin 10 min as in step 9.

15. Repeat step 13 and 14.

16. Extract DNA with equal volume of chloroform and spin 10 min. at 4°C at 12,000 rpm using HB-4 rotor.

17. Transfer DNA into a new 15 ml Corex tube and add 1/10 vol. 3 M NaAc (pH5.2), 2 vol. 100% ethanol. Keep on ice for 30 min.

18. repeat step 9 and 10.

19. Dissolve DNA in 1 ml TE and Rinse with 1 ml TE buffer. Combine them together and check O.D at 260/280.

b. Large scale plasmid DNA preparation with CsCl.

(Vertical rotor preparation of plasmid).

1. Grow 100 ml E. coli DH5α bacterial culture with 100 μg/ml ampicillin overnight.

2. Harvest bacteria using a 50 ml tube with HB-4 rotor at 7,000 rpm for 5 min. and resuspend in 3.8 ml sucrose buffer (50 mM Tris, 10mM EDTA, pH8 and 20% Sucrose) with 20

mg lysozyme. Keep on ice for 5 min, then add 100 μl RNAse (10 mg/ml) for anther 5 min.

3. Add slowly with a pipette while swirling 5 ml of 2% triton X-100/50 mM Tris/10 mM EDTA. Draw up and down the solution several times in the pipette to make sure all of the cells are exposed to the detergent. Leave on ice for 10 min (or until solution is clear).

4. Transfer the solution into a clear T865 tube. Spin in ultracentrifuge T865 rotor at 25 K for 20 min. Pellet should be slightly fluffy but doesn't have to be.

5. Transfer supernatant into a 15 ml orange cap tube and dilute to 10 ml with water containing 0.4 ml of 5 mg/ml ethidium bromide.

6. Dissolve 9.6 g CsCI in the solution by warming to 55°C for 15 min or longer. Spin in table top centrifuge at maximum speed for 10 min. Carefully take out the red protein floating on the top and load the solution into 15 ml polyallomer TV856 tubes. Balance to within 0.1 g and spin for at least 8 h at 50 K at 20°C.

7. Localize the lower band with the long wave-length UV lamp (366nm) in a dark room. Remove the lower band in about 1 ml volume. Extract with 5 M saturated isopropyl alcohol 4 times to remove ethidium bromide (the solution will be clear without any pink color). Dialyze against TE buffer in 2 liters with 2 changes in overnight.

8. Extract dialysate with phenol/chloroform, chloroform

alone. Precipitate with 1/10 vol. 3 M NaAc and 2 vol. ethanol.

9. Dissolve in 200 μl TE buffer. Take 5 μl for O.D. 260/280 nm reading to measure the concentration, and also check on gel.

c. Small scale plasmid DNA prep. (alkaline-SDS method)

The plasmid DNA isolated with this procedure is good for sequencing, restriction enzyme digestion, subcloning, etc.

1. Pick a single bacterial colony with the plasmid interest from a LB-agar plate containing ampicillin (50 μg/ml), inoculate in 5 ml of SOB medium with 100 μl ampicillin (10 mg/ml) and grow overnight at 37°C.

2. Spin down 1.5 ml culture in a Eppendorf tube.

3. Aspirate all the medium as much as possible.

4. Add 100 μl GTE buffer and vortex to mix.

5. Add 200 μl 0.2 N NaOH/1% SDS buffer and shake to mix well. Keep on ice for 2-3 min or solution clear.

6. Add 150 μl 3 M KAc buffer, mix and keep the tube on ice for 5 min.

7. Spin down at 12,000 rpm for 10 min. at room temperature.

8. Transfer supernatant to a new tube with a pipette.

9. Add 800 μl 100% cold ethanol, mix well and keep on ice for 30 min.

10. Spin down at 13,000 rpm at 4°C for 15 min.

11. Aspirate the supernatant and add 0.5 ml of 70% ethanol.

12. Spin down the DNA for 3 min in microcentrifuge and aspirate the ethanol and dry.

13. Dissolve DNA in 200 μl TE buffer and add 10-15 μl RNAse (10 mg/ml) at 37°c for 1-2 hr.

14. Add 250 μl TE buffer.

15. Add 300 μl phenol/chloroform and vortex 1 min.

16. Spin down in microcentrifuge for 5 min in high speed.

17. Transfer the supernatant into a new tube and repeat step 16.

18. Extract DNA with 300 μl chloroform.

19. Transfer the supernatant into a new tube and add 1/10 vol. of 3 M NaAc (pH5.2), 2 vol. 100% cold ethanol. Keep on ice for 30 min.

20. Repeat steps 10, 11 and 12.

21. dissolve DNA in 50 μl TE buffer.

22. Take 3 μl to check on gel and O.D. at 260/280 for concentration.

Solutions for plasmid preparation:

1.

SOB medium	LB medium	
20 g	10 g	bacto typone
5 g	5 g	yeast extract
2.5 ml	-	1 M KCI
2.5 ml	10 g	4 M NaCI
H_2O	to 1000 ml	

adjust pH to 7.6, autoclave, then add filter sterile 10 ml of 1 M $MgCI_2$ and 10 ml of 1 M $MgSO_4$ for SOB.

2. SOC medium: Add 10 ml of filter sterilized 2 M glucose per liter or 18.6 ml of 20% glucose to SOB solution.

3. LB-agar plate: Add 15 g bacto-agar/ 1 liter.

4. Alkaline-SDS lysis buffer (prepare fresh):

8.5 ml H2O, 1.0 ml SDS (10%) and

0.5 ml NaOH (4 M) -----Total 10 ml

5. 3 M KAc buffer: Dissolve 14.72 g KAc in 35 ml H_2O in 50 ml orange cap tube, add 5.75 ml Acetic acid, H_2O to 50ml.

B. N. fowleri Genomic DNA isolation (CsCI method) (Nellen, et al., 1987)

1. Grow 4 liters of N. fowleri cells (5-6 x 10^6/ml)
2. Harvest cells using GSA rotor at 5,000 rpm for 5 min.
3. Dissolve the cells in a 1:40 vol. ratio of ice cold HMN buffer 0.1% NP-40, and on ice for 5 to 10 min. (the solution should be clear)

4a. Check cell lysis under the microscope, if no lysis, add more NP-40.

4b. After cell lysis, spin at 8,000 rpm for 5 min to pellet nuclei.

5. Repeat the step 3.
6. Repeat the step 4b, and dissolve the pellet in 5 ml HMN buffer.
7. Drip into 0.1 M EDTA (pH8.2)/4% Sarcosyl

8. Put the tube in 65°C water bath for 5 min, solution should be clear.
9. Measure the amount of solution, add ethidium bromide to 0.4 mg/ml.
10. Add 0.95 g CsCI per ml (including ethidium bromide solution).
11. Put the tube into 55°C until all the solid CsCI dissolved.
12. Using table centrifuge to spin for 5 to 10 min at high speed to get rid of the upper protein film.
13. Transfer the solution into an ultracentrifuge tube.
14. Ultracentrifuge at 35 K at 15°C for 36 hr using the fixed angle rotor.
15. Take off the tube from the ultracentrifuge, look it under the long wave-length UV light. Sometime it will be seen 2 bands. The upper band may be the mtDNA etc. The lower one will be genomic DNA.
16. Carefully cut off the top of the tube with a sharp razor blade and take off the top red protein layer and other solution using a wide-bore pipet tip.
17. Then transfer the band from the top into a 50 ml orange cap tube and add 1.5 vol water to mix well.
18. Add 1 vol. phenol/chloroform and gently mix.
19. Spin down and transfer the upper aqueous phase into a dialysis tubing against TE buffer (4 L) for overnight and change once.

20. Transfer into 30 ml Corex tube and add 1/10 vol. 3M NaAc, 2 vol. ethanol to precipitate at -20°c for overnight.
21. Spin down using HB-4 rotor at 13,000 rpm for 15 min.
22. Discard the supernatant and rinse with 70% ethanol.
23. Dry with speed vacuum and add 1 ml TE.
24. Rock for a day at room temp. to dissolve the DNA.

3. SOUTHERN BLOT

1. Completely digest 10 μg of genomic DNA in a total reaction volume of 50 μl with 5 μl restriction enzyme at 37°C for 3 hr or overnight. Use only a cut off pipette tip with a wider bore so the DNA is not sheared excessively. Mix the reaction well by pipetting and flicking the tube because the high M.W. DNA is very viscous.

2. Prepare 0.7% agarose gel with 1X TBE buffer. Need 400 ml of gel in the 20 X 30 cm gel box and 800 ml running buffer. Use the large comb slots (0.9 cm X 1.5 cm) or else the DNA may smear during the run. Cover the gel with a thin (2 mm) layer of buffer.

3. Add 5 to 10 μl loading buffer to the sample and mix well. Then load the sample in the slots and use lambda DNA (1μg) cut with Eco RI or Hind III or both as a size marker at the ends of the gel. Run EC 500 at 80 V. for 10 min. to make all of the DNA stack in slot. Then lower voltage to 40 V. and run overnight for about 15 hr until the dye has run about 10 cm. Run all gels the same way so that blots done on different days could be easily compared later.

4. Shut off power and cut away the excess gel. Transfer the DNA in gel using a X-ray film as a support because the gel breaks easily. Cut off a small bit of one edge of gel as an orientation mark. Place in a large plastic box and add about 1 liter of buffer then about 2 to 3 drops of ethidium bromide until the solution is just pink. Shake for about 15

min. or until the DNA is stained. View on the transilluminator and take a picture with a ruler set along the side of the gel. At the same, turn on the Haake Cooler set at -10°C.

5. Replace the buffer with 1 liter of 0.2 N NaOH (8 g)/0.6 M (35 g) NaCI and shake for 30 min. to denature the DNA. Replace with 1 liter of 25 mM phosphate buffer pH 6.5. Change buffer twice within about 30 min.

6. Pour 5 Liters of phosphate buffer into the electroblot apparatus. Cut 4 sheets of 3 MM filter paper and 1 sheet of Gene Screen membrane to the size of the gel to be blotted. Soak the gene Screen for about 10 min. Assemble the blot sandwich: on the plastic grid in a box with 3 liter of phosphate buffer place a layer of foam, 2 layers of pre-wetted filter paper, the gel, the Gene Screen, 2 layers of pre-wetted filter paper, foam and then the other side of the plastic grid. Do all of this under the buffer to prevent air bubbles from lodging in the sandwich. Sandwich will not transfer in bubble regions. Clamp the sandwich with your hands and do not release your grip until the sandwich within the electroblotter slots and under the buffer.

7. Electroblot for ≈0.4 Amp for 1 hr. Check after 10 min. to make sure the Amp. is stable. Increase Amp. to ≈1 Amp. and continue for 6 hr. or longer. At the end remove the filter paper and dry it. View the blot with the short wave UV light and mark lightly with pencil any lambda size bands

or rDNA bands and the slot.

8. Fix the DNA on the blot by baking 80°C for 2 hr.

9. Seal the blot in a seal-a-meal bag with 1X 10 ml hybridization buffer (depend on the size of membrane). Prehybridize the blot at 65°C for at least 1 hr using the agitator set for slow movement of the buffer over the blot.

10. Cut the bag at a corner and pour off the prehybridization buffer. Then add a very hot inset probe at $\leq$ 10 ng/ml for at least 24 hr. Shake at 65°C.

11. For the post-hybridization, wash the blot as follows:

a. 2 X 2X SSC R.T. for 5 min each

b. 2 X 0.5X SSC/1% SDS at 65°C for 30 min.

Check background count (cpm) over region which should not have any counts; if less than 50 cpm, stop here.

If the counts still high:

c. 2 X 0.2X SSC/1% SDS at 65°C for 30 min.

Check counts

d. 2 X 0.1X SSC/1% SDS at 65°C for 30 min.

(There should be almost no detectable cpm even over the DNA which should hybridize.) Wash away excess SDS with a couple of rinses in 0.1X SSC.

12. Wrap the blot in plastic wrap and mount over 3 MM filter paper using tape. Mark all size markers, rDNA and anything other bands you saw on the blot with radioactive (^{35}S) ink. Put date, orientation marks as X on the filter.

Do not get outside wet or else the film in contact will be ruined. Set up blot against XAR-5 Kodak film and lay this over an intensifying screen in a cassette. Expose at -70°C for overnight or longer.

13. Keep blot for reuse as many as three times. For reuse, follow procedure for stripping old probe (0.1X SSC/ 1%SDS, boil for 30 min.). Store the dry blot at room temp. in plastic bag.

Solutions for Southern Blot:

1. Phosphate buffer for 1 M stock.

Mix 93.84 g NaH_2PO_4 and 45.44 g Na_2HPO_4 in 700 ml water, heat and stir then cool to room temp.; adjust pH to 6.5 and volume to 1 liter.

2. (pre-)Hybridization buffer:

NaCI	2.92 g
1 M tris pH 7.5	2.5 ml
50% dextran sulfate	10 ml
50 X Denhardts	5 ml
10% SDS (ultra-pure)	5 ml
10 mg/ml Salmon DNA	0.5 ml
water to total	50 ml

4. DOT BLOT

1. Dissolve about 1 μg DNA in 10 μl H_2O.
2. Add 2.5 μl of 2 N NaOH (0.4 N final).
3. Put the Eppendorf-tube in 80°C for 10-15 min
4. Add 10 μl of Neutralization buffer.
5. Spot 4 μl at a time on GeneScreen membrane, and allow to try before adding another 4 μl.
6. Bake the blot at 80°C for 2 hr. or use UV cross linking method.
7. Ready for pre-hybridization.

Neutralization buffer:

2.5 ml 1M Tris pH 7.5, 1.25 ml 2N HCl and 6.25 ml 20X SSC

5. RNA ISOLATION (Chirgwin, et al, 1979)

A. Isolation of total cellular RNA from N. fowleri

DAY 1

1. For each 10^8 cells (about 4 ml cell pellet from 200 ml shaking culture) dissolve in 5 to 10 ml of 4M guanidinium thiocyanate/0.1 M 2-mercaptoethanol/pH 5. Freeze sample in dry ice or place in -20 or -70°C freezer to make sure that all cells lyse. Samples can be stored indefinitely when frozen.

2. Thaw sample, then spin at 3000 rpm or more in a table top centrifuge to pellet insoluble material if any. Centrifuge for 15 to 20 minutes is usually enough.

3. Take an RNAse free AH-629 polyallomer tube and add 2.5 ml of 5.7M CsCl/0.1 M EDTA/pH 5 to it to form a CsCl cushion. Gently layer the sample over the cushion. Fill the tube up to within a couple of millimeters of the top. The sample can be up to 13 ml. Balance the tubes to within 0.1 g and load all of the buckets even if only 2 have samples. Centrifuge for 24 hrs at 25K at 15°C.
For the Beckman SW 41 use 2.2 ml of CsCl and spin at 33K and for the SW 28 add 5.5 ml of CsCl and spin at 27 K. Shorter spin times (18 hr) could be used without significant loss of RNA yield.

DAY 2

4. Remove all of the sample by aspiration down to the CsCl layer and then add 3 ml of dep'd water to the tube to

wash the walls. Aspirate the wash water and most of the CsCl so that about 1 ml is left. Invert the tube and keep inverted while cutting the bottom of the tube with a razor blade. Make a cup of the bottom so that you can get to the pellet easily.

5. Carefully add about 0.5 ml of dep'd water to wash the area around the pellet and drain this out. Do not disturb the small button-like translucent pellet of RNA which will be in the exact bottom of the tube. The small aggregates around the pellet are not RNA and should be avoided. They can be wiped out with a Kim-Wipe if necessary.

6. Take up 0.5 ml of dep'd water in a blue tip and jab the pellet to break it up then add the water and take up the pieces to transfer to a microfuge tube. Add another 0.5 ml to the cup to rinse and make sure that no RNA is left in the cup or in the blue tip. Now vortex the RNA continuously for about 2 min to dissolve the RNA then spin in the microfuge for 5 min to pellet the insoluble material, if any. Take up the supernatant and transfer to a baked Corex 13 ml tube and add 2 ml of dep'd water, 75 μl of 4M NaCl, and 7.5 ml of cold 100% ethanol. Cap with parafilm, invert to mix and store at -20°C for a few hours or overnight or indefinitely.

7. Spin the tube at 13,000 rpm in the HB-4 rotor for 20 min at 4°C to pellet the RNA. Drain or aspirate the supernatant and then gently wash the film of a pellet with

cold 80% ethanol using about 0.5 ml to remove the residual NaCl. Remove this wash ethanol by aspirating. Cap the tube with parafilm and poke holes in it and then place in a vacuum for 5 minutes or until the ethanol and water have evaporated. The pellet assumes the appearance of a dry cracked lake bed. Take up the pellet in 200 µl of dep'd water and vortex to dissolve the RNA. Transfer to a microfuge tube and then add another 200 µl of water to rinse the tube and transfer this to the microfuge tube also. RNA dissolves easily within 2 min of vortexing and if there is pellet material that does not dissolve in this time it is not RNA.

This RNA is good enough for use in Northern blots.

8. To further purify the RNA (for <u>in vitro</u> translation and construction of a cDNA library) add 0.4 ml of buffered phenol/chloroform and vortex to make a cloudy emulsion. Spin for 2 min. in the microfuge to separate the phases. Remove the upper water phase and leave any interface behind. Re-extract the water phase in another tube with chloroform/isoamyl alcohol 0.4 ml and spin as before. Remove the lower chloroform phase and re-extract with chloroform again. The small amount of residual RNA left in the phenol/chloroform phase could be recovered by adding 0.2 ml of dep'd water to it and vortexing and spinning as before.

If done add this to the ca. 0.3 ml first recovered from the chloroform. Add 4M NaCl to 0.1 M final concentration (12.5 µl to 0.5 ml) and 2 volumes of cold 100% ethanol (1.0 ml to the 0.5 ml). Vortex to mix and allow to precipitate at -20°C for at least 2 hours or overnight or indefinitely.

9. Spin in the microfuge for 10 min and wash the pellet with 50 µl of 80% ethanol. Dry in the Speedy Vacuum.

10. Add 1 ml of cold 2 M LiCl to the dry pellet and break up the pellet. Cap and rotate or rock for about 1 hour at 4°C. Spin again in the microfuge to recover the pellet and then wash the pellet with 50 µl of 2M LiCl. Remove the wash and add 0.5 ml of dep'd water to dissolve the pellet and then precipitate with NaCl and ethanol as before (step 8). Finally dissolve the RNA in about 100 to 200 µl of dep'd water and determine the OD at 260 and 280 nm. Add 5 µl of the RNA to 1 ml of water and read the OD. Get at least 0.1 OD units at 260nm, if necessary add more sample. Determine the OD and multiply by the dilution factor of 200 and multiply by 40 µg/ml to get the RNA concentration in µg/ml. The usual 260/280 ratio is about 2 to 2.3 but values as low as 1.6 are still good preparations.

NOTES

1. This procedure gives total RNA which can be translated in the reticulocyte assay because inhibitory material has been removed with the extra phenol/chloroform and LiCl washes. Do not use more than about 2 x 10^8 cells

in the 10 ml at step 1 because a large amount of gelatinous material will spin if the gradient is overloaded.

2. At step 10 the RNA will not dissolve easily occasionally if the RNA concentration is too high and especially after phenol/chloroform extraction. These RNA aggregates will need extra help in dissolving such as breaking up the aggregates with a blue tip and vortexing for a long time (over 10 min).

Solutions for RNA preparation:

1. All glassware should be baked to be RNAse free (at 350°F for at least 3 hours).

2. All plasticware is OK if not handled with bare hands which have RNAse. Wear gloves throughout the procedure. Have pipet tips and microfuge tubes and glassware that you will use only for RNA preparation.

3. Water. Use nanopure water that has been treated with diethypyrocarbonate at a final concentration of 0.2% as follows. Make a 10% solution of dep in 100% ethanol and dilute this 500 fold in the water. Leave overnight or at least 3 hours and then autoclave for 20 min to inactivate the residual dep. Store the water in small baked bottles. Use gloves when handling the dep because it is a strong denaturing agent.

4. 4 M NaCl. Treat with dep as above.

5. Polyallomer tubes. Treat with dep as above. Place the tubes in a large beaker with water and add the dep to

0.2% and mix up. Autoclave as before and store the tubes in a closed box.

6. Guanidinium thiocyanate/0.1 M 2-mercaptoethanol. Add 47.2 g of Fluka G/SCN or Kodak or BRL to about 70 ml of nanopure water and then in a hood add 7 ml of 2-mercaptoethanol. Cover and stir and gently warm to dissolve and make up to a final volume of 100 ml. Filter through a 0.45 μm Nalgene filter and store at -20°C in 50 ml plastic tubes. Check pH , it should be pH5 and will be if Fluka G/SCN is used. Adjust if necessary with acid.

7. 5.7 M CsCl/0.1 M EDTA. Add 48 g of baked CsCl technical grade to about 30 ml of nanopure water and then 5 ml of 0.5 M EDTA and stir and warm to dissolve at a final volume of 50 ml. Adjust the pH to 5 with acid. Filter through a Nalgene filter and store at -20°C or add dep to 0.2% and treat as for dep'd water.

8. Phenol/Chloroform. Shake phenol with 0.1 M Tris pH8 (usually 25 ml of phenol with 25 ml of Tris) to equilibrate. Spin in 50 ml plastic tube at 3000 rpm for 5 min to separate the phases. Repeat until the water phase is pH about 7-8. This may be several times. Add a pinch of 8-hydroxyquinoline to give the phenol some brown color for visibility and then add 2-mercaptoethanol to 0.1%. This makes the equilibrated phenol which can be stored at -20°C tightly capped. Add equal parts of this phenol to chloroform to make the 50:50 mixture.

B. Isolation of Poly(A)+ mRNA

(using Amersham's Hybond-mAP paper)

Materials required:

0.5 M NaCl	Clean, sterile (flame) forceps
70% ethanol	Clean, sterile (flame) scissors
Dep'd water	Disposable gloves
Eppendorf tubes	Filter paper, 3MM
Parafilm	Water bath, set at 70°C

Wear gloves at all times to prevent contamination of Hybond-mAP by ribonuclease.

1. Prepare 100 μg RNA in an Eppendorf tube. And cut 1 cm^2 Hybond-mAP paper.

2. Wet it in 0.5 M NaCl solution.

3. Place Hybond-mAP on 3MM filter paper, then transfer onto parafilm. Slowly spot RNA solution on Hybond-mAP, stay for 2-3 min.

4. Using a pipet tip to transfer RNA solution back into an Eppendorf tube (if any), then place Hybond-mAP onto 3MM filter paper, place the RNA solution onto Hybond-mAP paper, leave for 2 min.

5. Wash Hybond-mAP in 0.5 M NaCl for 5 min (rocking) in 13 ml orange cap tube. Repeat twice, using new solution, to remove free RNA.

6. Rinse Hybond-mAP in 70% ethanol for 2 min. Then put Hybond-mAP onto 3MM filter paper to remove excess ethanol.

7. Transfer Hybond-mAP into 200 μl dep'd water (cover

the paper) in an Eppendorf tube and incubate at 70°C water bath for 5 min.

8. Remove the mAP using sterile forceps. The poly(A)+ RNA remains in the water. Using Speed vacuum to move excess water until 10-20 μl left.

Ready for further processing.

6. NORTHERN BLOT

1. To 5.2 g of agarose in a 500 ml flask add 80 ml of 5X MOPS buffer pH 7 and 250 ml of nanopure water. Put over a low flame and allow to boil thoroughly to melt all of the agarose. Meanwhile tape the edges of the 20 x 30 cm gel box and put it in the hood and fix the 0.7 cm x 2 mm gel comb in place. When the agarose is melted cool to about 60° and add 69 ml of 37% formaldehyde while swirling to evenly mix the formaldehyde into the agarose. Pour the mixture in the gel box and smooth out any bubbles. It hardens in about 30 min.

The concentration of agarose is 1.3 % and the gel is about 0.7 cm thick. Up to about 60 µl of sample can be loaded in the slots.

2. While the gel is cooling denature the RNA samples. Normally about 10 µg of total RNA is run but 50 µg will successfully separate by this method.

Prepare the following in a microfuge tube:

5X MOPS buffer	4 µl
formaldehyde (37%)	3.5 µl
formamide	10 µl
RNA	2.5 to 4 µl

Mix and place at 55°C for 15 min. Add 2 µl of sterile loading buffer(50% glycerol/0.05% bromophenol blue). Make sure that the RNA is assayed correctly for concentration or else the lanes may have different amounts. It is best to determine the OD of all samples then make a dilution to 1 or

2 mg/ml for all samples. Reassay the OD of the dilution then calculate the amount to load. RNA transcripts for size markers: use 10 μl of SP6 and T7 transcripts.

3. Gently remove the comb so you don't rip the slots. Fill up the chambers in the gel box which require about 800 ml per side and add a covering layer of buffer to about 1mm to just cover the gel and fill up the slots. Do not overfill because the formaldehyde concentration must remain high. Add the samples and run the gel at about 40 volts overnight (ca. 11.5 hr with the EC 500; use 50 V. for the EC 600) so that the blue dye has run about 10 cm. Run all of the gels the same way so that in the future you can compare gels run on separate days.

Optional faster runs: 80 V 5½ hrs ≈ 10 cm EC 500

100 V 5½ hrs ≈ 10 cm EC 600

Save the running buffer for reuse: can be used at least 3 times.

4. OPTIONAL STEP. Cut out the gel area containing the lanes and put it in another box filled with 1 liter of 50mM NaOH/10mM NaCl. Shake gently for 30 min to denature and break up the larger RNA species. This is not usually done except if the RNA is very large (over 7 kb).

5. Meanwhile turn on the Haake circulator cooling bath set at -10°C.

FOR GENE SCREEN PLUS: Also prepare 6 liters of 12 mM Tris/6mM sodium acetate/0.3 mM EDTA/ph 7.5. A 20 X stock

solution of the electroblot buffer can be prepared: 29 g Tris base/40 ml 3 M sodium acetate pH 5/ 12 ml 0.5 M EDTA/≈ 10 ml concentration. HCl to pH 7.5 and brought up to 1 liter. If using GENE SCREEN, prepare 8 liters of 0.025 M sodium phosphate buffer(pH 6.5). A 1 M stock solution is prepared as follows: dissolve 93.84 g sodium phosphate monobasic and 45.44 g sodium phosphate dibasic in 700 ml distilled water, heat and then cool to room temperature, and bring up to 1 liter. Check and adjust the pH with about 9.0 g of NaOH pellets. Usually Gene Screen is used.

6. If used, pour out the NaOH/NaCl and add 1 liter of the Tris or phosphate buffer solution and shake for 15 min. and then pour that out and replace with one more liter of Tris or buffer. If the NaOH/NaCl is not used rinse the gel with 1 liter of phosphate buffer. Shake again for 15 min plus add 5 drops of 5mg/ml Ethidium bromide to stain the RNA. The rRNA on the gel is usually not visible in the stained gel but it will be stained on the blot.

7. Remove the gel and look at the RNA on the ultraviolet light box. The RNA will only be visible well if the Tris buffer had been used. The ribosomal RNA of 26S and 18S should be visible about 5 to 7 cm from the slot. Trim away any extra gel outside of the lanes and cut off the gel below the bromphenol blue area at the bottom of the gel. Good visualization at this stage is not really necessary. The bands will appear well on the membrane after blotting.

8. Cut out 4 pieces of Whatman 3MM paper and one piece of Gene Screen or Gene Screen Plus the same size as the gel and soak these in the Tris buffer. Assemble the electroblot sandwich in a large plastic box. First add about 2.5 liter of the appropriate buffer and soak one of the sponges. Then add 2 sheets of the paper making sure that no air bubbles are trapped between or under them. Add the gel on top of them while submerged to prevent air bubbles and then the Gene Screen or Gene Screen Plus. Smooth out the layers and then add the final two sheets of paper under the buffer as before. Add the other side of the sandwich and place in the electroblot apparatus which is filled with 4 liters of the buffer.

9. The Gene Screen or Gene Screen Plus side of the sandwich is on the positive side of the electroblotter. Start the electroblot at 0.4 amp for one hour and check the amperage in about 15 min to make sure that it is stable and still on. After one hour increase the amperage to ca. 0.9 amp for 3 hr. Remove the blot and air dry or briefly blot and then bake at 80°C for 2 hours. The air drying step is a good overnight stopping point. Look at the blot under the short wave length uv light and mark the slots, the rRNA bands the date and the samples in pencil on the blot to the side of the rRNA bands.

10. Prepare the prehybridization buffer in a 50 ml tube.

For Gene Screen Plus:

50% formamide	25 ml	Deionized and stored at -20°C
50X Denhardt's sol.	5 ml	Do not heat over 37° to thaw
20X SSPE	12.5 ml	
10% SDS	0.5 ml	Ultrapure
10 mg/ml salmon DNA	1.0 ml	Boiled and sheared
nanopure water	7.0 ml	

Check pH with paper Should be pH7.

For Gene Screen:

50% formamide	25 ml	Deionized and stored at -20°C
50X Denhardt's sol	5 ml	Do not heat over 37° to thaw
1 M Tris, pH 6.8	2.5 ml	
NaCl	0.92 g	Dissolve NaCl first in the aqueous
10% SDS	5 ml	ingredients not the formamide or SDS.
50% dextran sulfate	10 ml	
10 mg/ml salmon DNA	0.5 ml	

Check pH with paper. Should be 7 and pale green. If not and the pH is very green the formamide may need to be deionized and the buffer should not be used for hybridization.

50 % dextran sulfate = 100 g plus 155 ml of nanopure water; heat and stir to dissolve. May take a while.

11. Seal the blot in the seal a-meal-bag and add 10 ml of the prehybridization buffer. Place in a 42°C water bath

and allow the bubbles to float up to the top. Prehybridize at least one half hour but can be overnight or longer.

12. Add the probe to 10 ml of the prehybridization buffer so that the probe concentration is ≤ 10 ng/ml with a specific activity of at least 1 X 10^7 per μg. Usually add one half of the oligolabelling reaction of 100 ng DNA. Heat the hybridization buffer for about 10 min. at 80°C to denature the DNA then quick cool on ice briefly. Pour off the old prehybridization buffer and add the cooled hybridization buffer to the blot and seal again. Leave in the 42°C bath for two days before the posthybridization washes. Turn on the 65°C shaker bath before the posthybridization so that it heats up in time.

13. Remove the hybridization buffer and save in a plastic tube at room temperature (can be reused at least 3 times within 2 weeks). Wash the blot in 1 liter of 2 X SSC twice for 5 min with shaking at room temperature. Follow with a wash in 1 liter of 0.5X SSC/1% SDS (Sigma 95% SDS) at 65°C for 30 min. with shaking. Check counts of blot with geiger counter held over the top of blot for background which should be low and over the lower part below the rRNA band which should be noticeably higher. Usually necessary to wash again at 0.5X SSC/1% SDS at 65°C for another 30 min. Remove excess SDS by several quick washes over 30 min. with 0.1X SSC at room temperature. During the washes do not allow the blot to dry.

14. Wrap the blot in plastic wrap and then mount on 3MM paper. Do not get the outside wet or it will stick to the film during exposure. Mark the rRNA band location, the date and the orientation of the lanes and some other X marks on the paper with ink spiked with some ^{35}S. Place into light proof cassette against XAR-5 film with the film against an intensifying screen. Expose at -70°C for at least overnight.

7. RADIOACTIVE PROBE PREPARATION (Feinberg, et al, 1983)

A. Random primer method

OLIGO LABELLING

Dilute 60-100 ng of purified DNA fragment to 12 µl in dH_2O.

Boil for 5 min. to denature and then
Quick-cool in ice-water for 10 min.

Add following:

LS	18 µl	
BSA	1 µl	(16 mg/ml)
dNTPs	3 µl	(dGTP, dATP and dTTP)
(^{32}p)dCTP	5 µl	(50 µCi)
Klenow frag.	8 unit	(DNA polymerase I)
total	40 µl	

Incubate at room temp. from 5 hr to overnight.

Note: TM : 250 mM Tris.Cl pH 8.0 2.5 ml
25 mM $MgCl_2$ 0.25 ml
50 mM β-Mercaptoethanol 36 µl
water to total 10 ml

OL : 90 O.D. Units/ml Hexamers
1 mM Tris.Cl pH 8.0
1 mM EDTA pH 8.0

LS : 1 M Hepes pH 6.6 : TM : OL ,
25 : 25 : 7

B. End labelling

1. Mix the following:

H_2O	11 µl
oligonucleotide	1 µl (200 ng)
10 X PNK buffer	2 µl
{r-3^2P}dATP	5 µl
Polynucleotide Kinase	1 µl

2. incubate the mix for 45 min. at 37°C, then heat it to inactivation for 10 min. at 70°C.

3. add 70 µl STE (100 mM NaCI, 20 mM Tris pH 7.5 and 10 mM EDTA pH 8.0) buffer.

4. use Stratagene's "Nuctrap push columns" to separate unincorporated dNTPs from labelled oligonucleotides.

8. SEQUENCING (Sanger, et al)

A. Single-stranded sequencing using M13 phage

Preparation of single stranded M13 for sequencing

1. Grow 5 ml of DH5αF′ cells in SOB medium o/n, then transfer 100 μl to 5 ml new media to continue to grow to exponential phase (OD 550 at 0.2 to 0.7) and dilute 100 times (1 ml to 99 ml SOB).
2. Place 5 ml in 15 ml glass tube and then add a plaque from a plate by removing an agar with a cut off blue pipette tip. (using a plaque which are well isolated from others and have not been growing for more than overnight)
3. Shake at 37°C at 200 rpm for about 5 hours, then transfer to 3 microfuge tubes and spin 5 min. at SH-MT rotor 13,000 rpm.
4. Remove 1.3 ml supernatant (avoiding pellet) from each to 3 new tubes.
5. Add 200 μl of 20% PEG/2.5 M NaCl to each tube. Invert to mix several times then vortex gently. Leave at room temp. for 15 min. Spin again in SH-MT at room temp. at 13,000 rpm for 5 min. Remove supernatant with drawn-out pasteur pipette then spin 10 sec. in microcentrifuge (room temp.) to get rest of supernatant. Should see a small pellet in each tube and the pellet should be almost dry.
6. Add 150 μl of TE to each tube and vortex vigorously to

resuspend. (good point to stop)

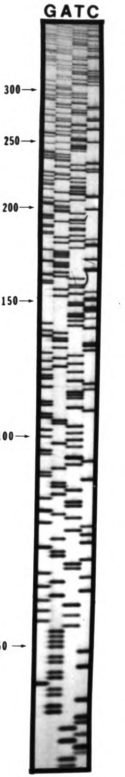
7. Extract with phenol once, phenol/chloroform a few times (maybe up to 5 times) and chloroform once. (vortex 1 min. and spin 7 min in microcentrifuge at room temp.)
8. After step 7, remove 70 µl upper aqueous from each tube to one new tube. Add 400 µl of a 25:1 ethanol:3M Na-acetate (pH 5) solution. Vortex to mix and store at room temp. for at least 15 min. or overnight.
9. Spin in SH-MT 15 min at 13,000 rpm to pellet. Remove most of supernatant. Add 200 µl of room temp. 70% ethanol to wash the pellet for 3 min, then spin 3 min in microfuge. Gently remove supernatant. Dry at room temp. or in Speedy Vacuum. Resuspend in 40 µl of dH_2O (not TE).
10. Run 5 µl of sample against M13 DNA control in 1% mini-gel. If any contaminants appear below the DNA (bands or fuzzy RNA) do not use for sequencing. Estimate 2 µg amount for sequencing and store rest at 4°C.
11. For sequencing, following the USB Sequenase protocol.

B. Double-stranded sequencing using plasmid DNA

Double stranded DNA sequencing is time and labor saving comparing to ss-DNA sequencing. By using the following protocol, we can easily read up to 300 bp.

I. PREPARATION OF DOUBLE-STRANDED DNA FOR SEQUENCING

see Plasmid mini-prep and large preparation



II. DENATURATION OF PLASMID FOR SEQUENCE ANALYSIS

1. Take 10 μg. plasmid in TE buffer in 0.5 ml micro-tube.
2. Add up to 0.4 M NaOH, then incubate at 37^0C for 15 min.
3. Add 0.1 vol 3 M NaAC (pH 4.5-5.5) to neutralize.
4. Add 2 to 4 vol. 100% ethanol (cold), mix well and leave the tube at -20^0C for 30 to 60 min or O/N.
5. Centrifuge for 15 min at 4^0C at 13,000 rpm.
6. Washing w/ 300 μl of 70% ethanol for 3 min., then spin 3-5 min. at micro-centrifuge.
7. Dry the pellet at room temp. for 30 min.
8. Keep the sample dry at 4°C until ready for sequencing.
9. Before sequencing, add 7 μl ddH_2O to dissolve the DNA.
10. Add 2 μl 5X sequence kit buffer, 1 μl Primer. (USB kit)
11. Put the tube in 65^0C water bath for 2 min., then cool at room temp. to $<30^0$C. (about 30 min.)
12. Continue follow the USB brief protocol.
13. We follow the Sequenase Version II protocol supplies by USB except following:

    1) use 1:15 dilution of labelling mix (instead 2:8).

    2) the temperature of labelling reaction must be <20°C.

    3) the termination reaction is done between 45-50°C (instead of 37°C) for 2-3 min.

    4) boil the sample for 2-3 min (instead of at 85°C).

C. Gel preparation

1. thoroughly mix the following components:

8% gel		6% :	
	50.4 g. Urea		50.4 g. Urea
	24 ml 40% acrylamide:bis		6.84 g/
	(38 g: 2 g to 100 ml)		0.36 g. bis
	12 ml 10 X TBE buffer		12 ml 10X TBE
	1.2 ml 10% ammonium persulfate		0.12 g. amp.
	44 ml dH_2O		68 ml dH_2O
	120 ml		120 ml

2. filter (Whatman No.1) the solution into a flask on ice.

3. place gel solution on room temp. until the temperature of the solution reaches 15°C.

4. add 60 µl TEMED and swirl gently to ensure thorough mixing, and pour the gel immediately.

5. insert the comb at the top of the gel in a horizontal position and clamp the plates at the top. Leave the gel flat on the bench horizontally for about 30 min.

6. buffer preparation: 1 X TBE 2000 ml.

7. after the gel is polymerized, tear off the tape of the bottom and affix plates to the sequencing apparatus. Pour running buffer (1 X TBE) into upper and lower reservoirs.

8. remove one comb and flush wells with 1 X buffer; insert one shark-tooth comb in the same position; then, do the same for the other comb.

9. pre-run the gel for 1 to 2 hours at 60 Watt (constant power). (The running power is 50-55 Watt.) The optimal plate temperature (measured with a thermometer outside the plate) for a good run is about 40°C.

10. (optional) check the leakage of the wells by loading 1 µl stop buffer.
(prepare samples while pre-run. see brief protocol supplies by manufacturer)

11. after gel-running, fix 30 min. using 15% methanol/ 5% acetic acid with gentle shaking every 5 min. and drying at 80°C for 40-50 min.

12. checking counts after drying the gel.

9. cDNA LIBRARY CONSTRUCTION

A. RNA and poly(A)+ mRNA preparation:

See RNA section for detail.

B. cDNA synthesis:

See Amersham's "cDNA synthesis system plus" kit for detail.

C. Vector preparation:

Digest 10 μg pUC18 plasmid with Sma I restriction enzyme for 3 hr at 37°C, then treated with CIP (10 units) for 30 min. at 37°C. Extract vector with phenol/chloroform once, chloroform once and ethanol precipitated. Ready to use.

D. Ligation and *E. coli* transformation:

Ligation: 0.5 μg of plasmid vector and poly(A)+ mRNA extracted from 200 μg total RNA were used with 20 units ligase (Boehringer Mannheim) for ligation in a 20 μl at 15°C for overnight. Add additional 1 μl ligase, plus 9 μl distilled H_2O to continue ligation at room temperature for 3 hr.

E. coli transformation: see next section for detail.

10. TRANSFORMATION OF BACTERIA E. coli

DAY 1.

1. Grow DH-5α cells directly from -70°C stock on LB plate overnight at 37°C.

DAY 2.

2. Pick about 10 colonies to grow in 5 ml SOB medium at 37°C for 1 to 1.5 hr.
3. Transfer about 0.5 to 1 ml overnight culture to 50 ml SOB in 500 ml flask. continuously to grow at 37°C to OD_{550} of 0.5 (about 1.5 to 2 hr, may be longer).
4. Cool on ice for 10 min, then transfer into a 50 ml pre-ice cold tube, then spin 5,000 rpm for 5 min to pellet cells. Resuspend in 10 ml Tfb I, and keep on ice 10 min Spin at 5,000 rpm for 5 min.

Note: Frozen cells for later use:

SEE next page for details.

if making fresh cells to use right away:

5. Resuspend pellet in 2 ml Tfb I. Add 70 μl freshly thawed DMSO, swirl and ice 5 min. Add 70 μl 2.25 M DTT/40 mM KAc pH 6.0, Swirl and ice 10 min. Add another 70 μl DMSO and ice for 5 min.
6. Transfer 200 μl of cells into Falcon 2057 tubes (keep on ice), and then add 10 to 20 μl of DNA (about 10 ng) to the tube, leave on ice for 1 hr. (the volume of DNA solution should not be more than 10% of total volume.)
7. Heat shock cells at 42°C for 1 min. (**critical step:**

sometimes has to test the optical time (i.e. 40", 50" etc.) to get the best result. It seems 40" to 1'is good.)

8. Cool on ice for 1 min.
9. Add 800 μl SOC medium (prewarmed to 37°C) into the tube and shaking for 2 hr at 37°C. (speed should not be more than 200 cycle/min.)
10. Spin down at table top centrifuge at max speed (2,100 rpm) for 10 min. Discard supernatant, add 200 μl SOC and mix well.
11. Put 50 μl X-gal (20 mg/ml) and 10 μl IPTG (100 mM) plus 40 μl SOC on plate containing 50 μg/ml ampicillin and spread evenly. Close lid for 10 min. and open it dry in the hood.
12. Plate out aliquot to LB plate under the hood. Spread evenly and leave the plate inversely at 37°C incubator for overnight. (no more than 20 hr)

Frozen cells for later use:

5'. Resuspend the pellet in 2 ml Tfb II. Add 70 μl freshly thawed DMSO, swirl and leave on ice for 5 min. Add another 70 μl DMSO and for additional 5 min.
6'. Transfer 210 μl aliquot to each Eppendorf tube on ice. Chill them very quickly in -70°C ethanol, then take them out and store in -70°C freezer until later use.
7'. When using frozen cells: take out from -70°C, warm it up in hand. Once thawed, put back into ice bath

immediately, then follow the step 6 of making fresh competent cell protocol.

Note: **A uncut vector should be used as a positive control to make sure that the transformation is OK.**

11. TWO DIMENSIONAL GEL ELECTROPHORESIS (O'Farrell, P. 1975, Garrels, J.I. 1979, Garrels, J.I. 1983, Kopachik, et al. 1985, Morrissey, J.H. 1981.)

1) 2-D gel sample preparation:

add 60-80 μCi ^{35}S-methionine to 1 x 10^7 cells and incubate at 37°C for about 8 hr

1. **Harvest cells**
2. **Add 18 μl 1% SDS/20 mM Tris pH 7.2 and 4 μl 55 mM DTT/20 mM Tris pH7.2.**
3. **Vertex and boil 2 min. in a tightly capped microcentrifuge tube**
4. **Spin down 10 sec. in microfuge and cool to rome temperature.**
5. **Add 2 μl DNAse/RNAse mixture and leave at room temperature for 1 min.**
6. **Freeze 2 min at -70°C, then spin 1 hr in speed vacuum to dry the sample.**
7. **Add 20 μl ddH_2O, 2 μl Ampholines (pH 3.5-7), 4.4 μl DTT, 4.4 μl NP-40 (44%), 30 mg Urea;**
8. **Dissolve all with vortexing and warming at 37°C.**

(Store the sample at -20°C or go to Protein determination)

2) TWO DIMENSIONAL GEL ELECTROPHORESIS

a) Isoelectric focusing gel electrophoresis:

1. Prepare gel. Measure out 0.582 g urea, add 0.35 ml 11.4% NP-40, 0.22 ml 11% acrylamide/0.55% bis and 50 µl ampholines (pH 3.5 to 10). Vortex and warm to 37°C to dissolve completely. Then spin 1 min to get rid of bubbles.

2. Degas for 5 min under vacuum from water aspirator. (optional: filter the solution by using 0.2 µm filter)

3. Add 10 µl of 10% APS freshly made. Vortex briefly to distribute but do not make aerated. Quickly load into acid washed capillary tubes (see notes below) up to a level of 11 cm. Place upright in poster putty. Allow to polymerize 1 hr.

4. Cut putty end so gel is 10.5 cm, total length is 15 cm; and overlay with 8 M urea (0.48 g/ml).

5. Prefocus the gel. Load gel carefully into gel box containing 1000 ml of H_3PO_4 (1.2 ml up to 1 L) in lower chamber adjust level of gel so that it just touches the acid. Submerge the upper end in 0.025 M NaOH (1 g to 1 L).

Start electrophoresis at 200 V. for 15 min., 500 V. for 15 min., 750 V. for 15 min. and note final current which should be ≤ 52 µAMP per gel.

6. Load sample: thaw sample and spin down for 5 min in microcentrifuge, then from the supernatant load about 150 µg of protein up to 10 µl volume. Can load up to 200 µg of protein for silver stained gels (more protein causes sticking in tube and stretching) in 20 µl. Keep all samples

to be compared later as close as possible in protein amounts and sample volume. For autoradiography of ^{35}S-methionine labelled samples try to get about 160,000 cpm or more in 10 µl. Remove NaOH until level is below upper gel end then load sample just above the gel level under the 8 M urea. Add urea to replace any displaced by the sample. Replace NaOH to cover upper end.

(Note: load equal amount count for ^{35}S gel and equal protein for silver stain gel)

7. Focus samples. Run gels 250 V. constant voltage for 15 hr, then 500 V. 1 hr, 750 V. 1 hr and 1000 V. 2 hr.

8. Remove gels and put in -20°C freezer (actually -16°C and gels may break if temperature is -20°C) for 2 min. Expel with lab air into equilibration buffer (5 ml per gel) and let stand for 5 min. Catch gel in a tea strainer and add to upper tank of SDS gel box ready to electrophorese samples.

Notes for IEF:

A. Equilibration buffer: 5 ml per gel. For 20 ml 0.169 g DTT, 0.8 g SDS, 2.0 ml glycerol, 1.24 ml 1 M Tris pH 6.8 and water to 20 ml.

B. Ampholines are from LKB and are pH 3.5 to 10 mixtures. Other brands will give different spot patterns.

b) SDS gel electrophoresis.

1. Pour separating gel of 11% acrylamide using 1.5 mm spacers. Assemble plates with spacers and clamps and seal edges thoroughly with molten 1% agarose. Each gel requires 25 ml of gel solution (see notes below for composition). Degas 5 min then add APS and TEMED. Swirl but do not make aerated. Add to plates with 25 ml pipette and then overlay with about a 3 mm layer of water. The gel is 10.5 cm long with about 2 cm from the top of the slot to the water overlay. Polymerize for 1 hr.

2. Assemble gel in a gel box and add electrophoresis buffer (see notes below) to upper and lower tanks. Add 50 μl of bromphenol blue dye to upper tank for a tracking dye.

3. Manipulate the acidic end (the swollen end) of the gel to the far left of the SDS gel with spacers or spatulas and anchor that end. Then brush the rest into the slot with a wafting motion using the water pressure generated to push the gel into the slot.
(This step takes practice).

4. Electrophorese at 125 V. per gel until the dye reaches 10 cm.

Note for SDS gel electrophoresis:

A. Separating gel: 25 ml per gel of 10.5 cm depth and 1.5 mm thickness. For 11% acrylamide:
Make up of stock of 100 ml of 30% acrylamide 0.8% bis acrylamide and store at 4°C in darkened bottle.

Make up at stock of 1.5 M Tris pH 8.8 with 0.4% SDS.

For 30 ml of gel solution mix 10.9 ml of acrylamide stock, 11.2 ml of Tris-SDS buffer and 7.8 ml of water. After degassing add 150 µl of freshly made 10% ammonium persulfate and 14 µl of TEMED. Pour quickly within 5 min.

B. Electrode buffer. Need 1500 ml for 2 gels boxes. For 1500 ml add 4.5 g Tris, 21.6 g of glycine, and 1.5 g of SDS to 1.5 liter of water. The pH is about 8.3 and does not need to be adjusted.

Fixation for autoradiography.

1. Place gel plates in plastic box and pry away the plates at any place except the rabbit ears (they break easily). Let gel fall into 100 ml of methanol/ acetic acid. Wash 3 times with gently rocking for 40 min each. For gels from in vitro translations use 300 ml of fixative to wash away the greater amount of background counts.

2. Add 60 ml of ENHANCE in hood for 1 hr using rocking motion. Pour off into container for disposal.

3. Add lots of cold water to precipitate fluors in the gel and rock for 1 hr. Pick up gel by supporting it from below with a glass plate or film .

4. Place wet 3MM paper on top and smooth out bubbles. Dry with vacuum and heat for 1 hr at 60°C. Do not leave to long (cracks appear) or too short (the undried gel with shatter if the vacuum is release to soon).

5. Place against XAR 5 film and expose for 1.6 x 10^6 cpm times days. (1 day for a gel with that amount of counts loaded)

Silver Staining of gels:

Use filtered water (0.2 μm) and gel solutions throughout the 2 D gel procedure because the dust in unfiltered solutions will stain and give vertical streaks in the gel. Keep the bromphenol blue tracking dye at minimal levels for the SDS gel because it will stain . Do not press

hard on the gel at any time while changing solutions because this distortion will cause spots on the gel after development. Use gloves when handling the gels. Use gentle rocking throughout the staining procedure.

1. 50% methanol/10% acetic acid (100 ml) for 30 min. Pour off.

2. 5% methanol/7% acetic acid (100 ml) for 30 min. Pour off.

3. 10% glutaraldehyde for 1 hr (100 ml) Pour off.

4. Wash extensively with water. Several quick (5 min) changes using a lot of water, then a few changes over a 2 hr period and proceed. Or leave overnight or overweekend. Pour off.

5. 5 μg/ml DTT for 30 min (100 ml). Pour off.

6. 0.1 % silver nitrate for 30 min (100 ml). Pour off.

7. Quick rinse with about 100 ml water and another 100 ml water for about 1 min. Follow with 2 small rinses with developer. Developer is freshly made 3% Na_2CO_3 with 50 μl of formaldehyde added per 100 ml.

8. Add 100 ml of developer and develop on light box until spots appear in about 2 to 5 min. Stop development with 5 ml of 2.3 M citric acid and rock for about 15 min. Pour off.

9. Wash with lots of water and then store in sealable plastic bag.

12. PROTEIN IN VITRO TRANSLATION USING RABBIT RETICULOCYTE (AMERSHAM'S KIT)

1. 25 µl lysate (Rabbit reticulocyte)
   4 µl ^{35}S-methionine
   3 µl poly(A)+ RNA (from total 100 µg RNA)
   1) gently mix with pipet, don't vortex.
   2) lysate should be thawed on ice.
2. Incubate for 1 hour in 30°C.
3. Add 2 µl DNAse/RNAse at room temperature for 2 min.
4. Freeze (thoroughly) in -70°C for 3 min to indefinitely.
5. Dry down in Speed Vacuum.
6. Add 50 µl of translation buffer.
7. TCA cpm count on 2x2 µl.

Stock for in vitro translation buffer:

9.95 M urea	597 mg urea	
4% NP-40	40 µl	
2% LKB pH 5-7	20 µl	
100 mM DTT	100 µl	1M DTT
0.3% SDS	30 µl	10% SDS

water to 1 ml work.

13. SMALL AMOUNT DNA PRECIPITATION WITH ETHANOL

If the DNA concentration is very low (e.g. below 1,000-10,000ng/ml in the final volume which including ethanol), this method should be used if possible.

1. Add 0.1 vol. of 3 M NaAc, 2 vol. of 100% cold ethanol into 1.5 ml microcentrifuge tube, mix well and stored at -20°C for overnight to 24 hr. ($MgCl_2$ may be added to 0.01 M final solution of DNA before add salt).

2. Fill the whole microfuge tube with 80% ethanol.

3. Microfuge tubes are then put into ultracentrifuge tubes which have been filled with ice-cold water containing 20% ethanol to prevent freezing. The ultracentrifuge rotor should be pre-cold in the refrigerator for overnight.

4. Balance the tubes with no more than 0.1 gram difference.

5. Ultracentrifuge at 25,000-27,000 rpm (SW27 rotor) for 1 hr. at 4°C.

6. Supernatant should be carefully removed with micropipet until 10-30 μl is left, then use 80% ethanol to wash once, dry the sample in speed vacuum and dissolve in appropriate buffer.

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