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MOLECULAR STUDY OF ACTIN IN *NAEGLERIA FOWLERI*

By

Jonghee Ahn

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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ABSTRACT

MOLECULAR STUDY OF ACTIN IN NAEGLERIA FOWLERI

By

Jonghee Ahn

Naegleria fowleri is a free-living amoebo-flagellate, and the causative agent of primary amoebic meningoencephalitis (PAM). This organism like many other species has multiple actin genes. Actin genes are present in most organisms and conserved throughout evolution. Analysis of Northern blots showed the existence of actin genes and a possible role in virulence of the amoebae. The multiplicity of actin genes was demonstrated by Southern blots. Two cDNA actin genes were isolated, sequenced and compared with many other actin genes from various organisms. The results suggested that N. fowleri is more closely related to Acanthamoeba than to Trypanosoma, which is contradictory to speculations on data from small subunit ribosomal RNA sequencing which place N.fowleri closer to Trypanosoma than Acanthamoeba in the evolutionary tree. N. fowleri may be a more advanced organism than thought previously.

Dedicated to my family

ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. R. Neal Band, my major professor, for his guidance and encouragement during the course of this study.

I also wish to thank Dr. Will J. Kopachik, my advisor, and Dr. Donald B. Jump, my committee member for their helpful suggestions in preparing my thesis.

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INTRODUCTION

The free-living, pathogenic amoeba Naegleria fowleri, is widely distributed in soil and freshwater throughout the world. These organisms have the unusual ability to undergo transformation from an amoeboid trophozoite to a temporary, non-feeding, non-dividing biflagellate. The life cycle of Naegleria also includes a dormant cyst. Although Naegleria flagellates do not encyst, amoebae are able to encyst (Marciano-Cabral, 1988).

Primary amebic meningoencephalitis (PAM) is a rapidly fatal human disease of the central nervous system caused by this organism. Unlike a "true" parasite, this is an opportunistic pathogen whose virulence is affected by several factors. Although determinants of pathogenicity are largely unknown, it has been suggested that phagocytosis, phospholipolytic enzymes and catalase are responsible for its virulence (John, 1982).

N.fowleri, like all other eukaryotic cells, keep their shape and motility with actin filaments. Actin also participates in cytokinesis. Many organisms have multiple isoforms of actin that often exhibit developmentally regulated and cell-type-specific expression (Lees-Miller et al. 1992). At least N. gruberi, translatable actin mRNA

disppears rapidly during the differentiation of amoebae to flagellates (Sussman et al, 1984).

Here, I reported the existence of a multigene family of actin genes

in Naegleria fowleri. Amino acid and nucleotide sequence comparisons show an extremely biased codon usage.

MATERIALS AND METHODS

Northern blot

DNA-RNA hybridization was carried out for 2 days at 50°C in 50% formamide, 5x Denhardt's solution (10% Ficoll/10% polyvinylpyrrolidone/10% bovine serum albumin), 1% ultrapure sodium dodecyl sulfate (SDS), 10% dextran sulfate, 100µg/ml denatured salmon sperm DNA, 1M NaCl, 50mM Tris-HCl (pH 7.5) and $3.2x10^5$ cpm/ml of an end-labelled DNA probe. The probe was end-labelled to reduce the background.

The probe was prepared with the synthetic oligomer (5'-TAGAAGCATTTTCTGTGCAC-3'). This conserved sequence was determined by comparison of available actin gene sequences in various species. This oligonucleotide was synthesized by the Michigan State University Macromolecular Synthesis Facility. The 20mer oligonucleotide was radiolabelled with γ-^{32}P ATP by the polynucleotide kinase (PNK) reaction (Sambrook, et al., 1989).

The blot was washed at room temperature, 15 min twice with 2x sodium chloride/sodium citrate (SSC).

Screening of cDNA library

Poly (A) + mRNA purified from 200ug total RNA was used as a template for oligo dT-primed cDNA synthesis by using a cDNA synthesis kit (Amersham). Blunt ended double stranded

cDNA was ligated into pUC18 which had been cut with Sma I and dephosphorylated with calf intestinal phosphatase. After Escherichia coli DH5'a cells were transformed, 1000 mini-plasmid preparations of cDNA clones were made and dot blotted onto GeneScreen membranes (Dupont). The library was screened with the same probe used in the Northern blot, but the washing step was done at reduced stringency (6x SSC/1% SDS at 40°C for 20 min, 3x SSC/1% SDS at 40°C for 20 min).

Southern blot

Southern blot transfer was done on Naegleria DNA, digested with restriction enzymes (BamH I, Hind III, EcoR I, Pst I) for 5 hr and separated by electrophoresis in 0.7% agarose (IBI) gel. Denaturation of the gel is accomplished with 0.4N NaOH/0.6N NaCl, and then transferred to GeneScreen.

BamH I and EcoR I was from Stratagene, Hind III and Pst I was purchased from BRL. The buffer used for restriction enzyme digestion was Stratagene universal buffer at concentrations recommended by Stratagene.

The oligolabelled probe for the Southern blot was first prepared by cutting the insert from actin cDNA plasmid with EcoR I, followed by agarose gel electrophoresis and electroelution of DNA into a dialysis membrane. This insert went through a further purification step by running with low melting agarose (FMC) and a second electroelution. This

double purified probe was labelled with a-^{32}P dCTP by random primer method (Feinberg, et al., 1983).

Hybridization was done for 2 days at 65°C in 1M sodium chloride, 1% ultrapure SDS, 10% dextran sulfate, 5x Denhardt's solution, 50mM Tris-HCl (pH7.5), 100µg/ml denatured salmon sperm DNA with oligolabelled probe.

The Southern blot was washed with 2x SSC for 10 min at room temperature twice, 1xSSC for 20 min at 60°C twice, 0.5x SSC for 20 min at 60°C twice. The blot was exposed to Kodak X-ray film overnight.

Preparation of DNA for sequencing

Plasmid DNA from the cDNA clone was prepared for double strand sequencing. Bacteria grown overnight (100ml) were harvested, resuspended in lysis buffer (25mM Tris-HCl, 10mM EDTA, 5mg/ml lysozyme) and incubated in ice for 10 min. The cells were lysed in 0.2N NaOH/1% SDS. After neutralization with 3M potassium acetate (pH4.8), the plasmid DNA was recovered by standard procedures. Ethanol precipitation, washing, RNA digestion, phenol-chloroform extraction, second precipitation and washing were carried out as previously described (Sambrook, et al., 1989). The plasmid DNA was dissolved in nanopure water before further experiment.

Denaturation for double strand sequencing

DNA (10μg, measured by spectrophotometer) was denatured with 0.4M NaOH at 37°C for 30 min. An 0.1 vol. 3M sodium acetate (pH 4.5-5.5) was added to neutralize and 4 vol. 100% ethanol was added to precipitate the DNA at -20°C overnight.

DNA sequencing

Naegleria fowleri actin cDNA clones were sequenced by the dideoxynucleotide chain termination method according to the method provided by US Biochemical with USB Sequenase version 2.0. For labelling, ^{33}P dATP is used as well as ^{35}S dATP.

Two sequence primers (5'-CCAATTGAACACGGTAT-3', 5'-CACAACCTTAATCTTCA-3') were synthesized to get the full length actin cDNA.

RESULTS

Northern blot analysis

The blot of 10μg of total celluar RNA was made by Wang-nan Hu. The RNA was size separated on agarose gels with formaldehyde as the denaturant. After electrophoresis the RNA was electroblotted onto GeneScreen. The hybridization of end-labelled probe to Northern blot occurred in the 1.2kb region of the blot which was determined by running RNA size makers on the same gel. The level of hybridization increased after subsequent mouse brain passages (compare m1 RNA to m2, m3 and m4 in Figure 1) while the axenically grown RNA showed the lowest hybridization (lane Ax). An equal amount of intact RNA was present in the five samples because the rRNA had equal ethidium bromide staining, I conclude that there is a higher expression of actin gene in virulent amoebae than non-viruelnt amoebae.

Southern blot analysis

Southern blot analysis was performed to determine the number of copies of actin genes (Figure 2). Total nuclear DNA was digested individually with four different restriction enzymes (BamH I, EcoR I, Hind III and Pst I) and the digestion products size separated by agarose gel electrophoresis. First, the hybridization was done with the

end-labelled probe but the signals were too weak to be clearly visualized. Therefore a random primer oligo-labelled probe of cDNA N.fowleri actin gene was made to give stronger signal. The 4-7 bands shown on Southern blot suggested that actin genes are a multigene family.

Dot blot and nucleotide sequence analysis

A cDNA library of 10^4 clones was made from LEE strain with SmaI digestion and pUC 18 ligation by Wang-nan Hu. This library was screened with the end-labelled probe.

Three positive clones were found. The inserts were sequenced but one of them had the SmaI site within the coding region of actin gene and it couldn't represent the full length cDNA actin. The actual insert sizes were 1120 base pair and 1177 base pair, designated act1, act2 respectively.

In the act1 sequence, one long open reading frame was present extending 1116 bases from the ATG putative start codon to TAA stop codon. A possible poly A additional signal was located 25 bases after the stop codon. The other sequence, act2, 1128 bases were identified within the coding region, missing 12 bases from start codon. It contained 24 base pairs of the 5' upstream region, 68 base pairs of the 3' region after the stop codon including poly A tailm as well as coding sequence. Also act1 had a possible poly A additional signal at 36 bases after the stop codon.

Comparison of the nucleotide sequence to the GENEMBL data base sequence showed a homology with 241 actin gene sequences. The highest homology shown here was 78.1% for act1 with a Candida albicans actin gene (accession number X16377). Other organisms, Dictyostelium (73.8%, accession number X03281), Saccaromyces (77.3%, accession number L00026) and Entamoeba (76.7%, accession number M16396) actin genes showed relatively high homology with that of N.fowleri. Also the deduced amino acid sequence was compared with other actin peptide and showed the highest homology (83.4%) for act1 with Physarum polycephalum (accession number P02576). Dictyostelium discoideum (accession number P02577) human (accession number P02570) and Acanthamoeba (accession number P02578) showed more than 80% homology with N.fowleri actin amino acid sequence (Table 2).

Because the structure of actin protein is now available from x-ray crystallography, we can deduce the ATP and divalent metal ion binding site as well as actin-myosin and actin-actin interaction site. The amino acid residues which involves ATP, metal ion binding and actin-myosin interaction are largely conserved while actin-actin contacts are not as highly conserved as other interaction sites.

There were 10 nucleotide differences out of 1116 base pairs between act1 and act2 genes in coding region. Six amino acid changes were predicted from the nucleotide difference, 4 of them had different side chain polarity.

The A+T content inside the coding region was 58.3% while in the 5' and 3' region, it was 67.4% (act1 both 5'and 3' ends),and 78.7% (act2, 3'end only). For translation, actin gene showed the preference of purine base at third position (Table 1).

DISCUSSION

Actin is present in the form of filaments, small filament bundles and meshworks in a wide variety of organisms (Taylor et al. 1979). Thick and thin microfilaments, morphologically similar to actin and myosin, have been seen in the cytoplasm of Naegleria fowleri (Lastovica et al., 1976). The Northern blot showed that there is at least one actin transcript of moderately high abundance in this organism (Fig. 1). The probes were hybridized to gel blots of total RNA from axenically growing amoebae (Lane Ax) and from amoebae taken from brains of mice after one, two, three or four serial infections of mice (m1, m2, m3, m4). This results showed that there is a higher expression of actin gene. Because of the increase in hybridization, the increased mRNA of actin may be necessary for the virulence in N. fowleri. Wong et al. (1977) noted that after prolonged periods of maintenence in axenic medium, strains of N. fowleri lost their original pathogenicity in mice Virulence was restored after serial mouse brain passage. The highly virulent amoebae exhibit faster movement in vivo than do weakly virulent amoebae (Cline et al. 1986). The increase of actin mRNA is correlated with increased motility possibly for host cell invasion and phagocytic activity.

The actin gene generally belongs to a multigene family, but there are exceptions such as in Saccharomyces cerevisidae and Tetrahymena spp. (Amar et al. 1988). The size of the

multigene family may be small, as in Acanthamoeba spp. and Physarum spp. which have three or four actin genes, or large as in Dictyostelium spp., mice or human which have 20 to 30 actin genes (Amar et al.1988). Cloned actin cDNAs hybridize to at least four fragments in genomic DNA which were digested with restriction endonucleases (Fig. 2) indicating that actin may be present in many copies in Naegleria genome. In addition, faintly hybridizing bands were detected which might be due to cross-hybridization to similar sequence of actin or parts of actin genes which have restriction sites within their genomic sequences.

The construction and identification of cDNA clones containing sequences of actin provides an understanding of actin protein structure as well as gene evolution. Actins are widely assumed to be evolutionary conserved proteins. The sequences determined here are between 65-80% for both nucleic acid and amino acid homology to other organisms. This is lower than comparisons of Schizosaccharomyces pombe actin genes to other actin sequences in various organisms (Lees-Miller et al. 1992). I have sequenced two actin genes in N. fowleri, one of which is 12 base pairs short of the start codon (Fig. 3). There are 13 nucleotide differences which cause 3 silent and 5 amino acid changes in actin protein. This is a big difference (79.6% amino acid homology) compared with Entamoeba histolytica (Edman et al. 1987) which was previously thought to be closely related to N. fowleri,

that showed just 4 nucleic acid changes, all of which were silent changes when translated into amino acid sequence.

Even though 3 of the changed amino acids have the same side chain polarity, one of them is polar/non-polar change and the other is non-polar/polar change. Thus, the overall charge of these two proteins remains the same from 4 to 375 amino acid but the isoelectric point can be different because the N-terminus is a very variant region in actin protein. It is now established that various forms of actin, differing from one another by certain changes in primary strcture of protein, exist in different cells and tissues and within a given cell (Taylor et al. 1979) or in an amoebae (Nellen et al. 1982). The two different actin proteins deduced from the actin gene cloned here can coexist in a N.fowleri, excuting different roles structurally and functionally.

The comparison of both act1 and act2 3' untranslated regions did not show high homology (23%), but they have 11 A's in the polyA tail as well as the eukaryotic polyadenylation signal (Fig3, underlined). From GENEMBL data base sequences, a comparison of both 5' and 3' untranslated region was done with various species. Unlike the coding region, there were no detectable similarities between N. fowleri and other organisms that have high homology in coding sequences. The 3' untranslated regions including poly A tail in N. fowleri actin genes are very short (50 and 57 base pair long, shown in Fig.3) which is also true for a- and ß-tubulin genes of Naegleria (Clark. 1990). This short untranslated

portion of actin mRNA was also observed in Acanthamoeba, Dictyostelium, and yeast (Nellen et al. 1982). It, therefore, seems that the group of smaller actin mRNA is confined to the simpler eukaryotes.

Deduced amino acid sequence comparison is contradictory to that of nucleic acid sequences (Fig 4). Among 8 organisms, Dictyostelium and Saccharomyces exhibit the highest homology (77.3%) followed by Entamoeba (76.7%) in nucleic acid comparison. However, in amino acid alignment, Acanthamoeba, Dictyostelium, and human show higher homlogy than Entamoeba (Tabel 2). No matter how high the homology is, there are two parts of amino acid sequences in N. fowleri which are very distinct in terms of side chain polarity. The region of 192-194 and 216-220 amino acids are quite different from other actin sequences while all other eight genes shared those sequences or have few replacements. As N-terminal amino acids showed high degree of replacement in other comparison (Lees-Miller et al. 1992), it is not remarkable that there is a variety of amino acids in this comparison. In one species of Naegleria, N. gruberi, an antibody for actin did not recognize determinants in actin of Acanthamoeba, Dictyosteium and Physarum (Fulton et al. 1986) This antibody defines a region possibly species specific. Lack of N-methylhistidine in N. gruberi (Fulton et. al. 1986.) may also participate in the uniqueness of actin. The reason for specific antibody specificity may be clarified by sequence analysis.

Recently, the atomic structure of actin was determined by X-ray crystallography in rabbit skeletal muscle (Kabsch et al. 1992). Actin generally consists of two domains which can be further subdivided into two subdomains. It is suggested that a five-stranded a sheet consisting of ß-meander and a right handed ßaßunit appears in each domain. Because Naegleria actin is homologous to their actins, possibly it has the same atomic structure.

Naegleria actin, although different enough to have unique antigen determinants, is conserved in many properties like calcium-, nucleotide-, myosin-, actin-binding sites (Lees-Miller et al. 1992). As shown in Fig 4, calcium-binding sites are completely conserved while nucleotide-binding sites are relatively well conserved in N. fowleri. The most variable replacements are found in actin-actin binding sites, however, those replacements have the same side chain polarity except 40, 41, 43, 110, 196 amino acids. The myosin binding sites are highly conserved throughout the peptide exept the variant N-terminus region where no homology was found among 9 species. DNAseI contact sites, composed of 50, 53, 61, 68, 69 amino acid residues (Edman et al. 1987) was conserved in N. fowleri actin. Therefore the N.fowleri actin polypeptide possesses the common structure of actin which allows the functional properties of it.

The relative frequency of synonymous codons for any amino acids suggests that codon usage differs among species (Starner et al. 1989). The preference of particular codons

implies that a functional role of codon usage may differ in many organisms as a consequence. As shown in Table 1, the amino acid codon usage in N. fowleri actin is very biased. Every amino acid is encoded by a preferred codon with exceptions of threonine, asparagine, histidine and serine. N. fowleri shows a strong preference for substitution of an adenine or thymidine residue in the third position of a codon. The adenine or thymine occurrence at the third position presents at a frequency of 64.5% in contrast to 30% in human actin. Comparison of the codon usage of N. fowleri actin with E. histolytica, S. cerevisidae and A. castellani actin (Edman et al. 1987) revealed that N. fowleri actin codon usage is more smilar to S. cerevisidae than to E. histolytica and very different from A. castellani. Also, this bias will prove useful in producing probable DNA sequences for oligonucleotide based gene isolation and for confirming the proper reading frame when genes are being sequenced.

As amoebas have long been considered the most difficult protozoa to place satisfactorily in a taxonomical scheme, actin is extremely valuable for probing phylogenic relationship. Many regions of actin remain conserved over large periods of time, and the rate of molecular evolution in general is relatively constant with time (Baverstock et al. 1989). Most of the molecular work to determine evolutionary relationships are by comparison of small-subunit ribosomal RNA sequences. These sequences are considered highly

conserved in evolution and because of slow divergence (Clark et al. 1989). According to rRNA sequencing, Naegleria is more closely related to Tetrahymena or Trypanosoma than Acanthamoeba (Baverstock et al. 1989). This is contradictory to the conclusion based on actin nucleotide and amino acid sequence which place Naegleria more closely to D.discoideum and A.castellanii. Table 2 shows the similarity between Naegleria fowleri and other species. Amino acid sequence comparison instead of nucleotide was used because Loomis et al. (1990) had suggested that amino acid sequence might be more reliable than untranslated nucleic acid sequences for evolutionary comparisons. N.fowleri has a higher H-value (defined by Sogin et al. 1986) with A.castellanii and D.discoideum than with E.histolytica. This discrepancy might be due to the species used for the rRNA sequencing. N. gruberi is considered to be the most divergent species in the genus Naegleria (Clark et al. 1988). Restriction endonuclease analysis of mitochondria DNA also showed the most divergent nature of N. gruberi within the genus as well as the most distant relatedness to N. fowleri (Milligan et al. 1988). Furthermore, ribosomal DNA digestion demonstrated that N. gruberi is very variant within the species (Clark et al. 1989). Therefore the possibility remains that N. fowleri is a more advanced eukaryote than N. gruberi and is closely related to Acanthamoeba. Similar contradiction in phylogenic analysis have been reported in Dictyostelium discoideum (Loomis et al., 1990). Cladistic analysis of amino acid

sequence in actin from a variety of eukaryotes shows that D.discoideum and E.histolytica are closely related and are closer to metazoa than are yeast. But in distance matrix, Dictyostelium is grouped with the metazoa, and Entamoeba forms a separate line. Therefore although rRNA sequencing could be a useful method to determine the phylogenic relationships in evolution, the comparison of highly conserved protein sequences can come to a different conclusion. One remarkable characteristic of Acanthamoeba is to form a cyst in the absence of exogenous nutrients. Also, Dictyostelium aggregates and form spores when staring (MacLeod et al., 1980). Acanthamoeba, Dictyostelium and Naegleria transform their shape when starving and the actin is an organelle which is resposible for the shape of an organism. Therefore among these three organisms, the unexpected high homology of actin might be due to the ability of transforming their shape in starvation.

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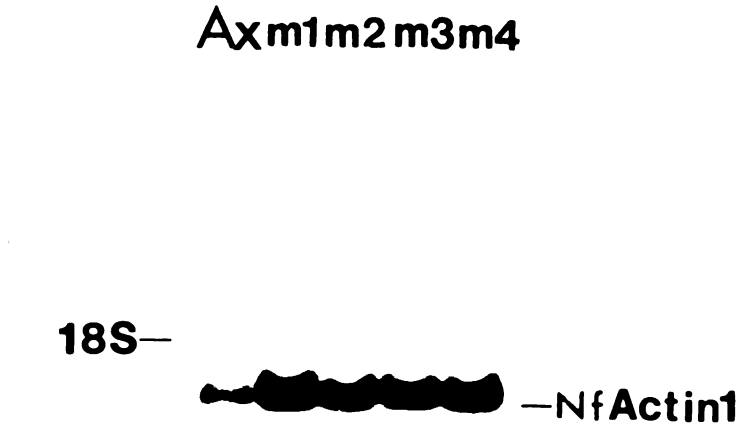


Figure 1. Autoradiograph of Northern blot of RNA from axenically grown amoebae and amoebae from mouse brain. Total RNA (10µg) was electrophoretically size separated on an agarose gel containing formaldehyde. Lane Ax (axenic amoebae); m1. m2, m3 and m4 amoebae after 1, 2, 3 and 4 passages of infection in mouse brain. The blot of the gel was probed with and ^{32}P-end labelled oligonucleotide homologous to an actin mRNA sequence.

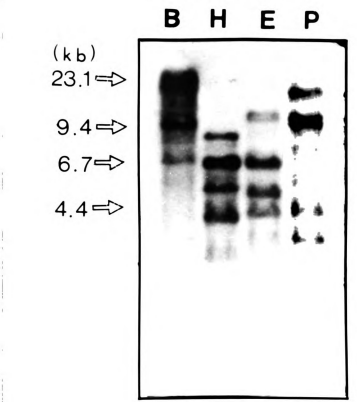


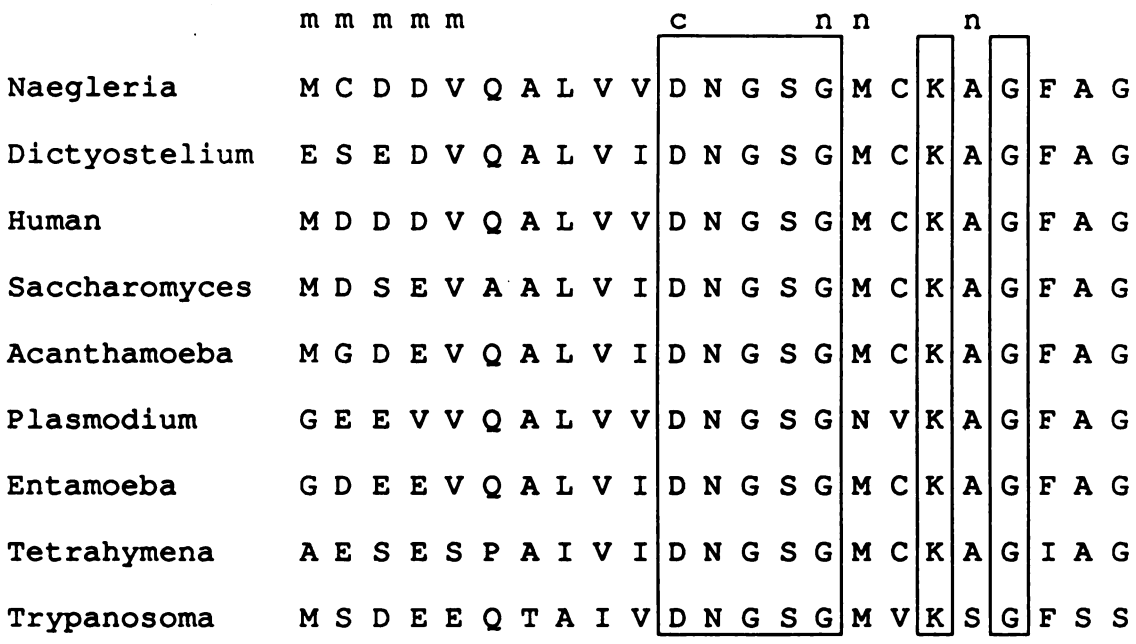
Figure 2. Autoradiograph of southern blot probed with the act2 cDNA. Genomic DNA (10µg) digested with BamH1(B), EcoR1(E), HindIII(H) and Pst(P) restriction enzymes was electrophoretically size separated, blotted onto GeneScreen and hybridized to ^{32}P-oligolabelled act2 cDNA insert. Size markers on the left were from lambda DNA digested with HindIII.

```
   -24
Act1  TTCCTCTCCAACAAGAACAACAAA

                                30                                  60
MetCysAspAspVal  GlnAlaLeuValVal  AspAsnGlySerGly  MetCysLysAlaGly
ATGTGTGACGACGTT  CAAGCACTCGTAGTT  GATAACGGATCTGGT  ATGTGTAAGGCTGGT
********Act2              a
                         Glu

                                90                                 120
PheAlaGlyAspAsp  AlaProArgAlaVal  PheProSerIleIle  GlyArgProLysGln
TTCGCTGGTGATGAT  GCACCAAGAGCTGTC  TTCCCTTCCATCATT  GGTAGACCAAAGCAA

                               150                                 180
LysSerIleMetVal  GlyMetGlyAsnLys  AspAlaTyrValGly  AspGluValGlnSer
AAGTCCATCATGGTT  GGTATGGGTAACAAG  GATGCCTATGTTGGT  GATGAAGTCCAATCC

                               210                                 240
LysArgGlyIleLeu  ThrLeuLysTyrPro  IleGluHisGlyIle  ValThrAsnTrpAsp
AAGAGAGGTATTTTG  ACTTTGAAGTATCCA  ATTGAACACGGTATT  GTCACCAATTGGGAT

                               270                                 300
AspMetGluLysIle  TrpHisHisThrPhe  TyrAsnGluLeuArg  ValAlaProGluGlu
GATATGGAAAAGATC  TGGCATCACACCTTC  TACAATGAATTGAGA  GTTGCTCCAGAGGAA

                               330                                 360
HisProValLeuLeu  ThrGluAlaProLeu  AsnProLysAlaAsn  ArgGluLysMetThr
CATCCAGTCTTGTTG  ACTGAAGCTCCATTG  AATCCAAAGGCTAAC  AGAGAAAAGATGACT

                               390                                 420
GlnIleMetPheGlu  ThrPheSerValPro  AlaMetTyrValAla  IleGlnAlaValLeu
CAAATCATGTTTGAA  ACCTTCTCTGTTCCA  GCCATGTATGTTGCC  ATTCAAGCTGTCTTG

                               450                                 480
SerLeuTyrAlaSer  GlyArgThrThrGly  IleValLeuAspSer  GlyAspGlyValSer
TCTTTGTATGCTTCT  GGTCGTACCACTGGT  ATTGTTTTGGACTCT  GGTGATGGTGTCTCT

                               510                                 540
HisThrValProIle  TyrGluGlyTyrAla  LeuProHisAlaIle  LeuArgLeuAspLeu
CACACTGTTCCAATT  TATGAAGGTTATGCT  TTGCCTCATGCTAGG  TTGAGATTGGATTTG
                                                c
                                               Ala

                               570                                 600
AlaGlyArgAspLeu  ThrAspTyrLeuIle  GluAspSerHisGly  ThrCysTyrSerPhe
GCTGGTAGAGATTTG  ACTGATTACTTGATC  GAAGATTCTCATGGA  ACGTGTTACTCATTC

                               630                                 660
AsnThrThrAlaGlu  ArgGluIleValArg  AspIleGluGlyLys  AlaLeuLeuTyrCys
AATACCACTGCTGAG  AGAGAAATTGTCAGA  GATATCGAAGGAAAA  GCTCTGTTATATTGC
                       tg caa             a
                       CysGly            Glu
```

```
                                690                                 720
PheAspPheGluGln  GluMetLysIleAla  AlaGluSerSerThr  ValGluLysSerTyr
TTTGACTTTGAACAA  GAAATGAAGATTGCT  GCTGAATCATCCACC  GTTGAAAAGTCTTAT
                                                t            ctc
                                                Ser          Leu

                                750                                 780
GluLeuProAspGly  AsnValIleThrVal  GlyAsnGluArgPhe  ArgCysProGluVal
GAATTGCCAGACGGT  AACGTGATTACTGTT  GGAAATGAAAGATTC  AGATGTCCAGAAGTT

                                810                                 840
LeuPheGlnProAsn  PheIleGlyMetGlu  AlaAlaGlyValHis  GluThrThrPheAsn
TTGTTCCAACCAAAC  TTCATTGGTATGGAA  GCTGCTGGTGTCCAT  GAAACTACTTTCAAC

                                870                                 900
SerIleGlyLysCys  AspIleAspIleArg  LysAspLeuTyrGly  AsnValValLeuSer
TCGATTGGAAAGTGT  GATATTGATATCAGA  AAGGATTTGTATGGT  AACGTTGTCTTGTCT

                                930                                 960
GlyGlyThrThrMet  PheGluGlyIleAla  GluArgMetThrLys  GluLeuThrAsnMet
GGTGGTACTACCATG  TTTGAAGGTATTGCT  GAGAGAATGACCAAG  GAATTGACCAACATG

                                990                                1020
AlaProAlaSerMet  LysIleLysValVal  AlaProProGluArg  LysTyrSerValTrp
GCTCCTGCTTCCATG  AAGATTAAGGTTGTG  GCTCCACCAGAAAGA  AAGTACTCGGTCTGG
                                                  a
                                                Lys

                               1050                                1080
IleGlyGlySerIle  LeuAlaSerLeuSer  ThrPheGlnGlnMet  TrpIleThrLysGlu
ATTGGAGGTTCCATC  TTGGCTTCATTGTCC  ACCTTCCAACAAATG  TGGATCACCAAGGAA

                               1110                                1128
GluTyrGluAspAla  GlyProGlyIleVal  HisArgLysSerPhe  stop
GAATATGAGGATGCC  GGTCCAGGTATTGTC  CACAGAAAGAGCTTC  TAA

ATTGACCTTGGATGCACATTATCAAATTCCAATGTAATAAAACATAAAAATCTATGT

AAAAAAAAAAA  1196

attggatgcacattatcaaattccaataaatccaataattgtaatacttcaaaaaaaaaaa 1189
```

Figure 3. Nucleotide and deduced amino acid sequence of N.fowleri is shown in capital letters in full length cDNA (Act1), while the partial cDNA (Act2) with small letters. Nucleotides are numbered relatively to the A of the ATG initiation codon in Act1, negative numbers indicate 5' flanking sequence. The deduced amino acid sequence of Act1 is shown above the nucleotide sequence. In Act2,Amino acid sequence is shown below the nucleotide sequence. The underline indicates a potential poly A additional site AATAAA.

23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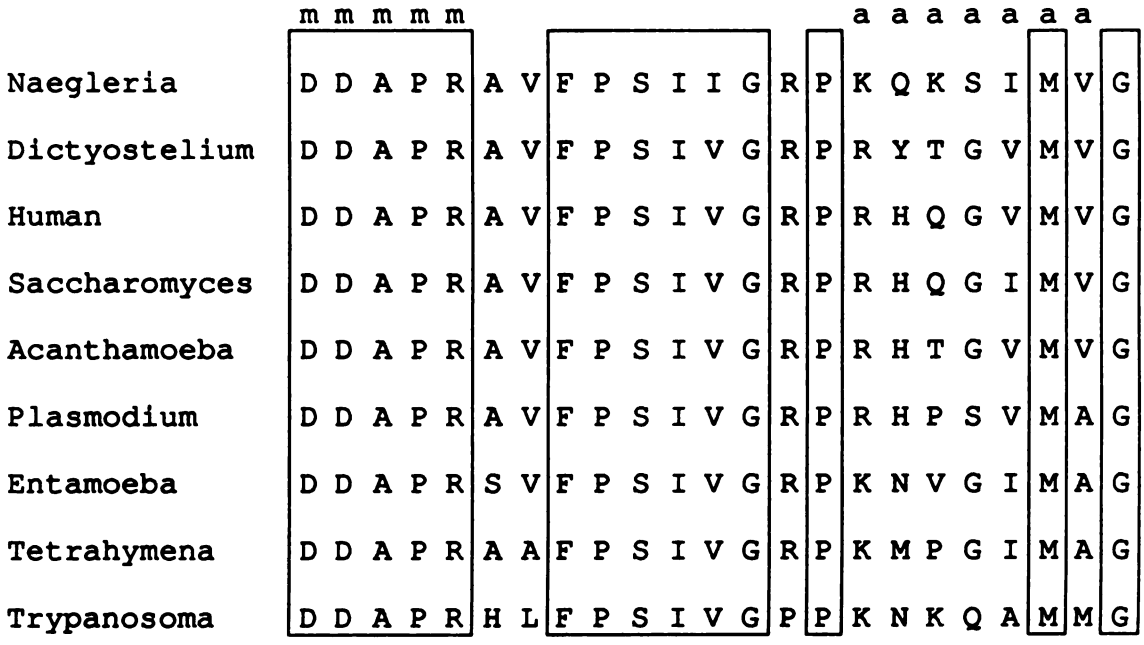
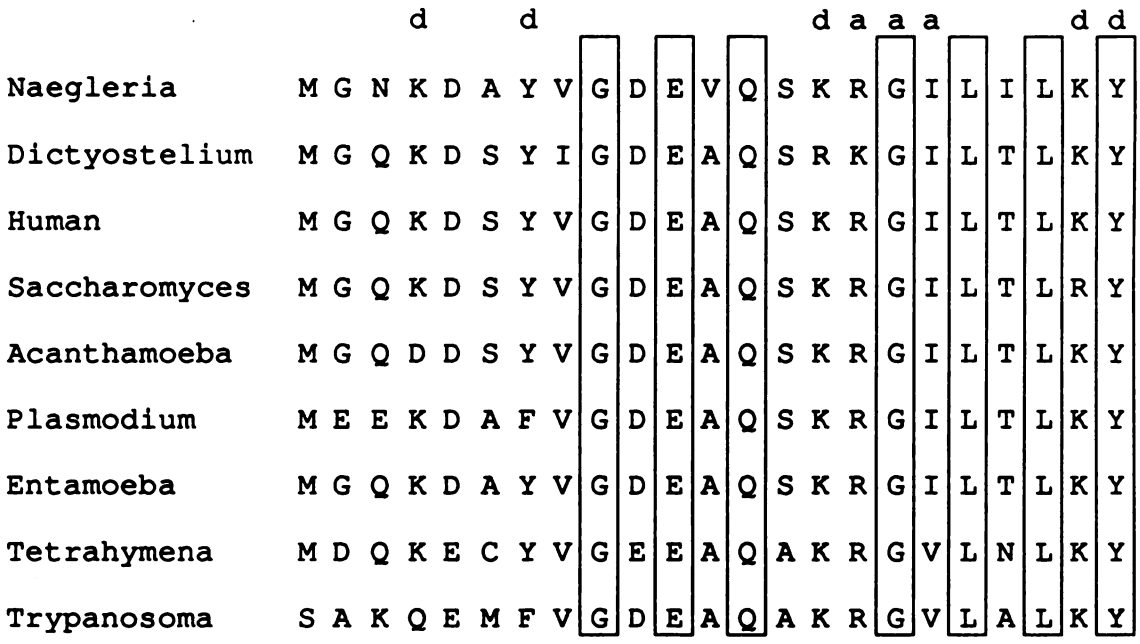
Dictyostelium E S E D V Q A L V I D N G S G M C K A G F A G
Human M D D D V Q A L V V D N G S G M C K A G F A G
Saccharomyces M D S E V A A L V I D N G S G M C K A G F A G
Acanthamoeba M G D E V Q A L V I D N G S G M C K A G F A G
Plasmodium G E E V V Q A L V V D N G S G N V K A G F A G
Entamoeba G D E E V Q A L V I D N G S G M C K A G F A G
Tetrahymena A E S E S P A I V I D N G S G M C K A G I A G
Trypanosoma M S D E E Q T A I V D N G S G M V K S G F S S

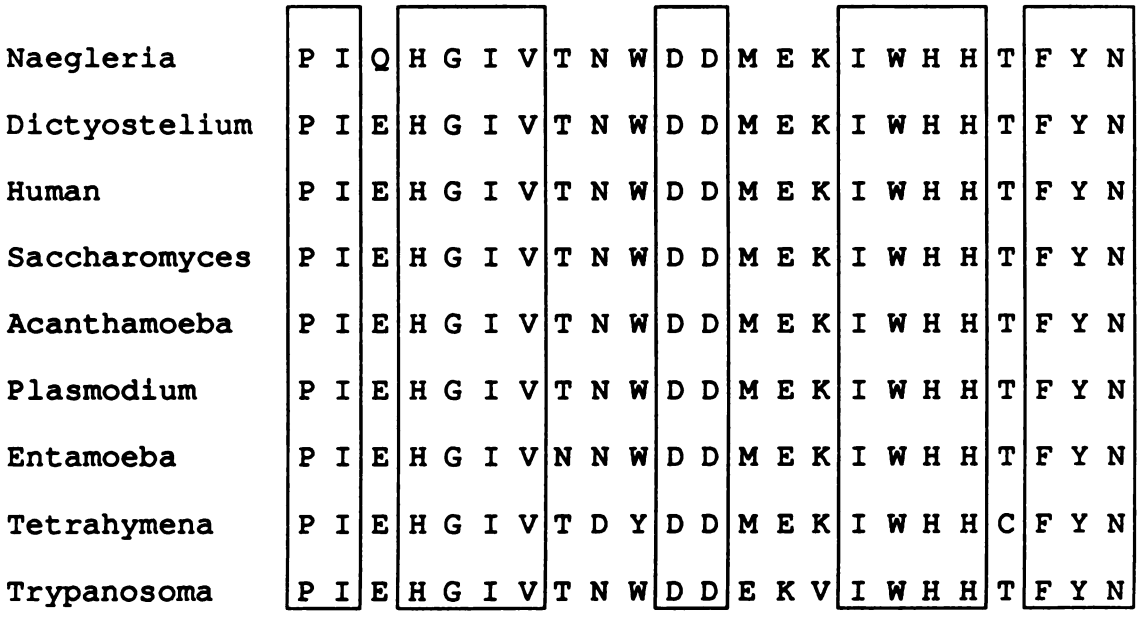
46


m m m m m a a a a a a a
Naegleria D D A P R A V F P S I I G R P K Q K S I M V G
Dictyostelium D D A P R A V F P S I V G R P R Y T G V M V G
Human D D A P R A V F P S I V G R P R H Q G V M V G
Saccharomyces D D A P R A V F P S I V G R P R H Q G I M V G
Acanthamoeba D D A P R A V F P S I V G R P R H T G V M V G
Plasmodium D D A P R A V F P S I V G R P R H P S V M A G
Entamoeba D D A P R S V F P S I V G R P K N V G I M A G
Tetrahymena D D A P R A A F P S I V G R P K M P G I M A G
Trypanosoma D D A P R H L F P S I V G P P K N K Q A M M G

69


d d d a a a d d
Naegleria M G N K D A Y V G D E V Q S K R G I L I L K Y
Dictyostelium M G Q K D S Y I G D E A Q S R K G I L T L K Y
Human M G Q K D S Y V G D E A Q S K R G I L T L K Y
Saccharomyces M G Q K D S Y V G D E A Q S K R G I L T L R Y
Acanthamoeba M G Q D D S Y V G D E A Q S K R G I L T L K Y
Plasmodium M E E K D A F V G D E A Q S K R G I L T L K Y
Entamoeba M G Q K D A Y V G D E A Q S K R G I L T L K Y
Tetrahymena M D Q K E C Y V G E E A Q A K R G V L N L K Y
Trypanosoma S A K Q E M F V G D E A Q A K R G V L A L K Y

92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Dictyostelium P I E H G I V T N W D D M E K I W H H T F Y N
Human P I E H G I V T N W D D M E K I W H H T F Y N
Saccharomyces P I E H G I V T N W D D M E K I W H H T F Y N
Acanthamoeba P I E H G I V T N W D D M E K I W H H T F Y N
Plasmodium P I E H G I V T N W D D M E K I W H H T F Y N
Entamoeba P I E H G I V N N W D D M E K I W H H T F Y N
Tetrahymena P I E H G I V T D Y D D M E K I W H H C F Y N
Trypanosoma P I E H G I V T N W D D E K V I W H H T F Y N

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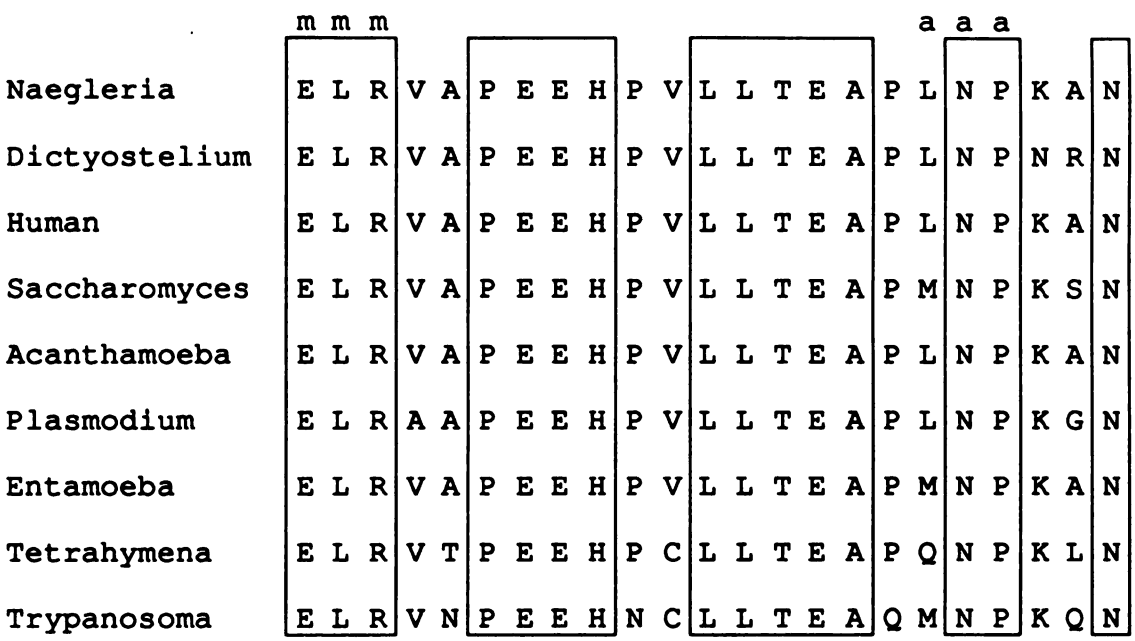
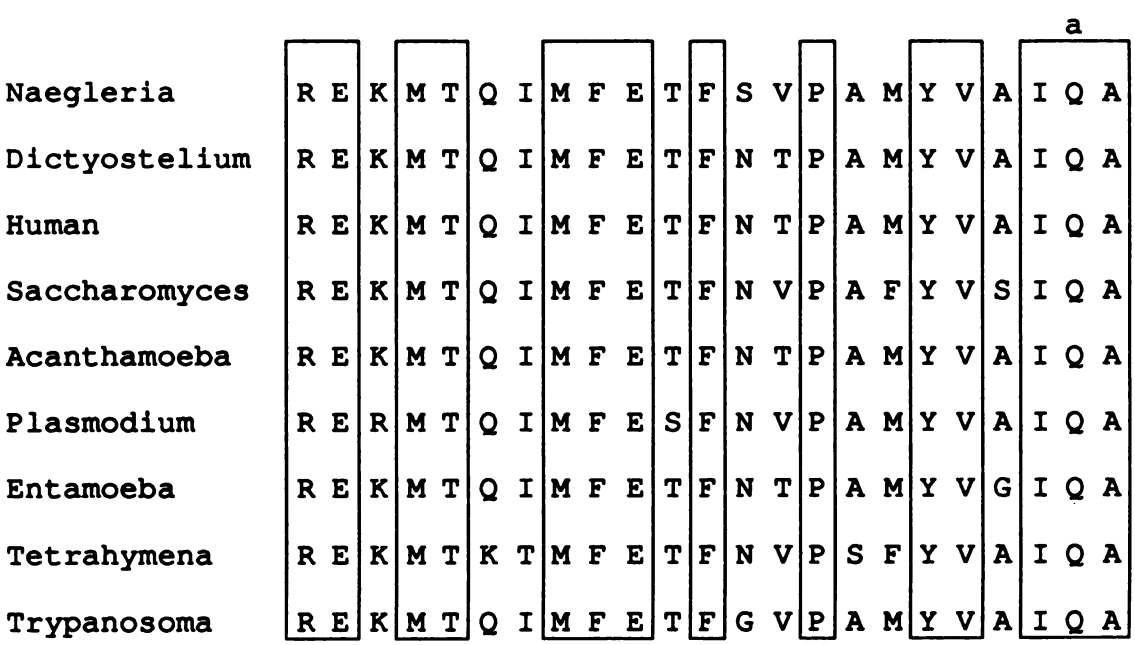
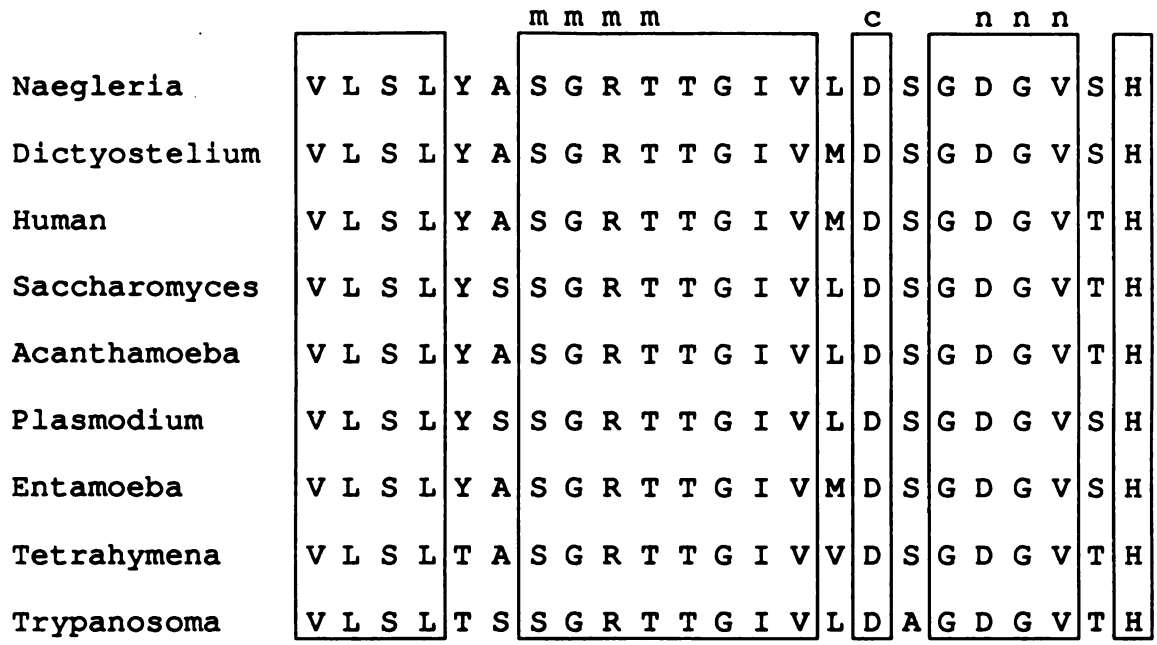

m m m
a a a
Naegleria E L R V A P E E H P V L L T E A P L N P K A N
Dictyostelium E L R V A P E E H P V L L T E A P L N P N R N
Human E L R V A P E E H P V L L T E A P L N P K A N
Saccharomyces E L R V A P E E H P V L L T E A P M N P K S N
Acanthamoeba E L R V A P E E H P V L L T E A P L N P K A N
Plasmodium E L R A A P E E H P V L L T E A P L N P K G N
Entamoeba E L R V A P E E H P V L L T E A P M N P K A N
Tetrahymena E L R V T P E E H P C L L T E A P Q N P K L N
Trypanosoma E L R V N P E E H N C L L T E A Q M N P K Q N

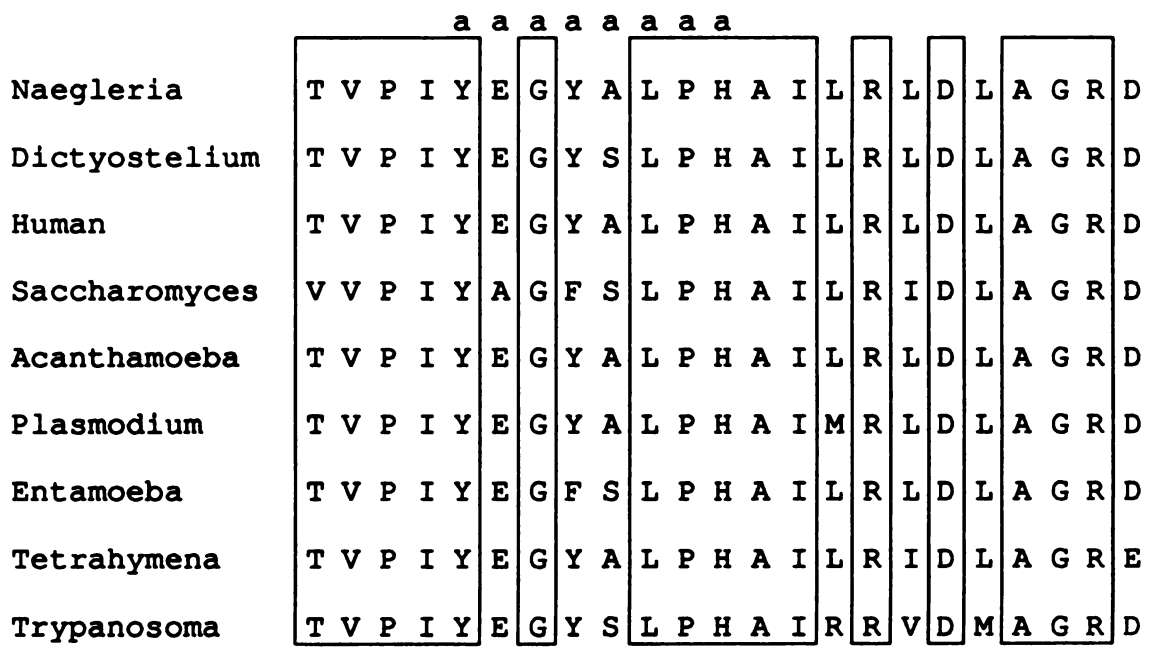
138


a
Naegleria R E K M T Q I M F E T F S V P A M Y V A I Q A
Dictyostelium R E K M T Q I M F E T F N T P A M Y V A I Q A
Human R E K M T Q I M F E T F N T P A M Y V A I Q A
Saccharomyces R E K M T Q I M F E T F N V P A F Y V S I Q A
Acanthamoeba R E K M T Q I M F E T F N T P A M Y V A I Q A
Plasmodium R E R M T Q I M F E S F N V P A M Y V A I Q A
Entamoeba R E K M T Q I M F E T F N T P A M Y V G I Q A
Tetrahymena R E K M T K T M F E T F N V P S F Y V A I Q A
Trypanosoma R E K M T Q I M F E T F G V P A M Y V A I Q A

161


m m m m
c
n n n
Naegleria V L S L Y A S G R T T G I V L D S G D G V S H
Dictyostelium V L S L Y A S G R T T G I V M D S G D G V S H
Human V L S L Y A S G R T T G I V M D S G D G V T H
Saccharomyces V L S L Y S S G R T T G I V L D S G D G V T H
Acanthamoeba V L S L Y A S G R T T G I V L D S G D G V T H
Plasmodium V L S L Y S S G R T T G I V L D S G D G V S H
Entamoeba V L S L Y A S G R T T G I V M D S G D G V S H
Tetrahymena V L S L T A S G R T T G I V V D S G D G V T H
Trypanosoma V L S L T S S G R T T G I V L D A G D G V T H

184


a a a a a a a a
Naegleria T V P I Y E G Y A L P H A I L R L D L A G R D
Dictyostelium T V P I Y E G Y S L P H A I L R L D L A G R D
Human T V P I Y E G Y A L P H A I L R L D L A G R D
Saccharomyces V V P I Y A G F S L P H A I L R I D L A G R D
Acanthamoeba T V P I Y E G Y A L P H A I L R L D L A G R D
Plasmodium T V P I Y E G Y A L P H A I M R L D L A G R D
Entamoeba T V P I Y E G F S L P H A I L R L D L A G R D
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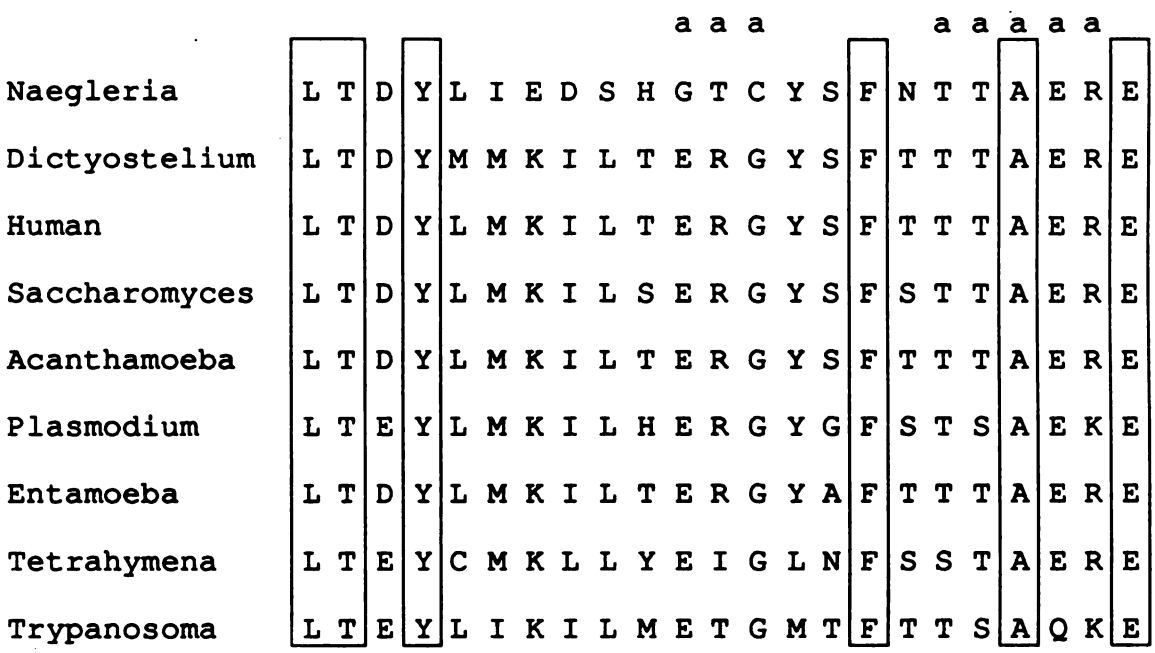
Trypanosoma T V P I Y E G Y S L P H A I R R V D M A G R D

207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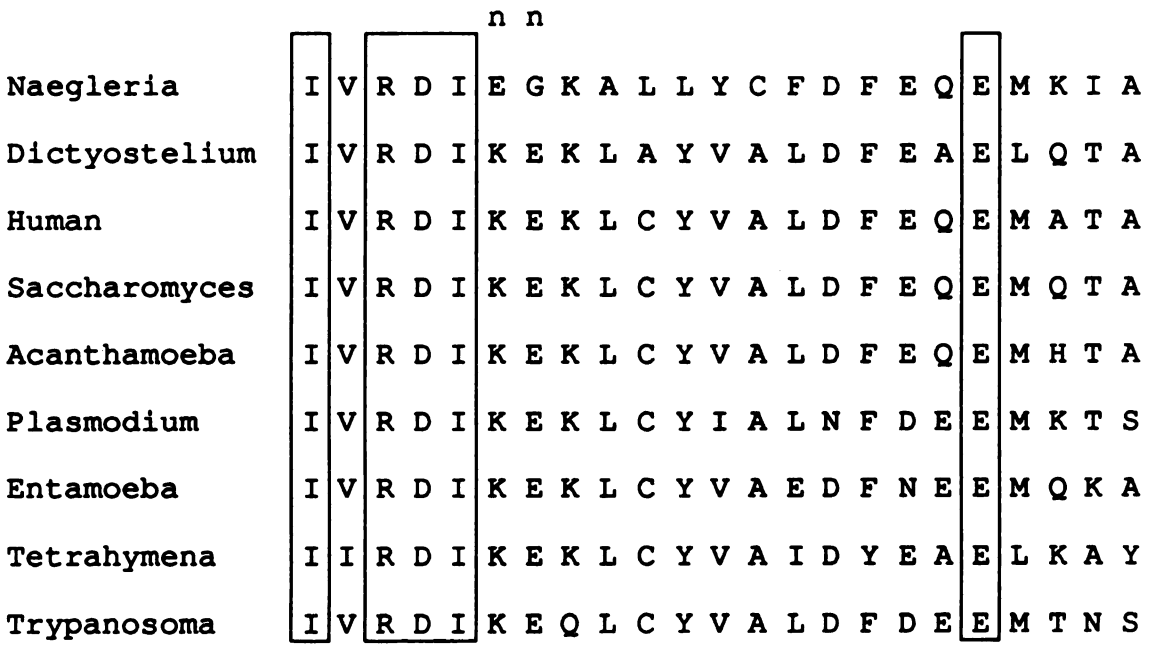
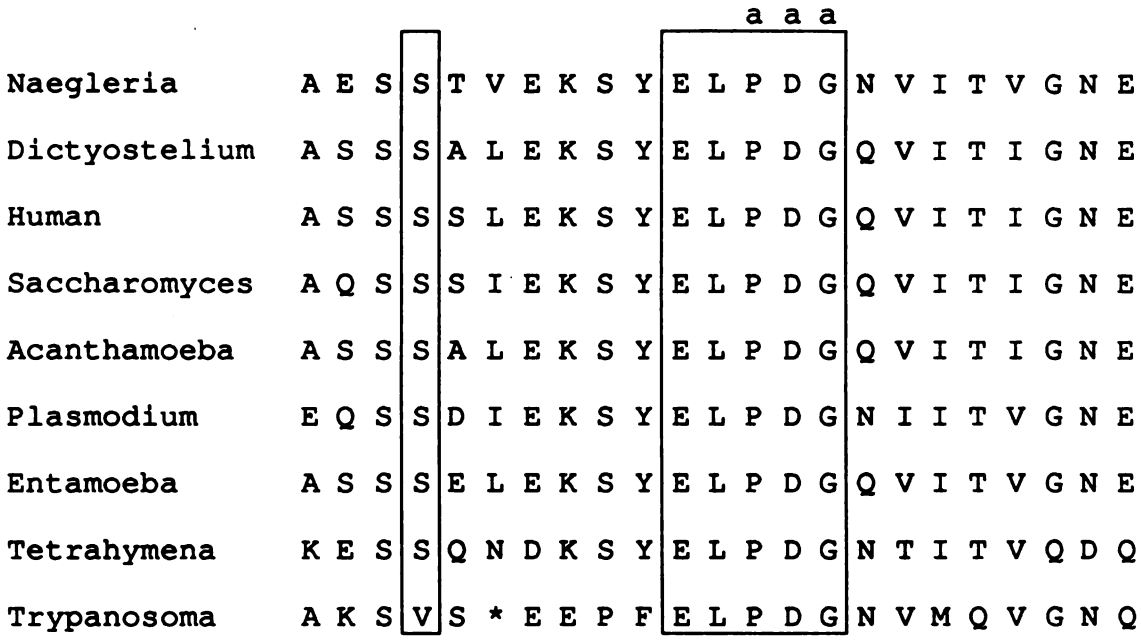
Naegleria L T D Y L I E D S H G T C Y S F N T T A E R E
Dictyostelium L T D Y M M K I L T E R G Y S F T T T A E R E
Human L T D Y L M K I L T E R G Y S F T T T A E R E
Saccharomyces L T D Y L M K I L S E R G Y S F S T T A E R E
Acanthamoeba L T D Y L M K I L T E R G Y S F T T T A E R E
Plasmodium L T E Y L M K I L H E R G Y G F S T S A E K E
Entamoeba L T D Y L M K I L T E R G Y A F T T T A E R E
Tetrahymena L T E Y C M K L L Y E I G L N F S S T A E R E
Trypanosoma L T E Y L I K I L M E T G M T F T T S A Q K E

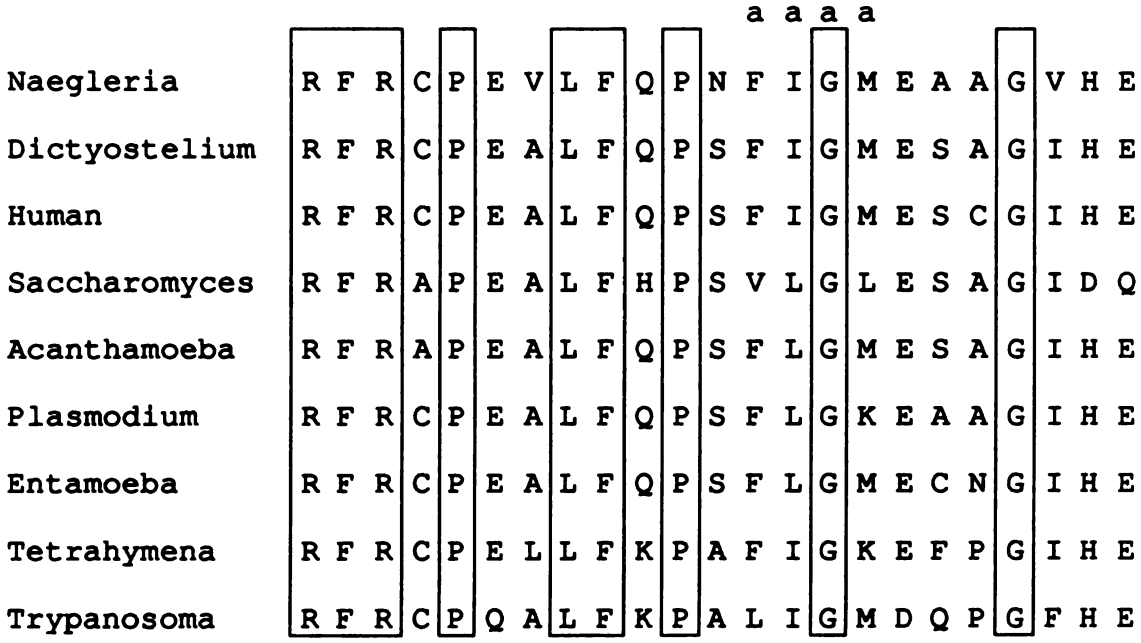
230


n n
Naegleria I V R D I E G K A L L Y C F D F E Q E M K I A
Dictyostelium I V R D I K E K L A Y V A L D F E A E L Q T A
Human I V R D I K E K L C Y V A L D F E Q E M A T A
Saccharomyces I V R D I K E K L C Y V A L D F E Q E M Q T A
Acanthamoeba I V R D I K E K L C Y V A L D F E Q E M H T A
Plasmodium I V R D I K E K L C Y I A L N F D E E M K T S
Entamoeba I V R D I K E K L C Y V A E D F N E E M Q K A
Tetrahymena I I R D I K E K L C Y V A I D Y E A E L K A Y
Trypanosoma I V R D I K E Q L C Y V A L D F D E E M T N S

253


a a a
Naegleria A E S S T V E K S Y E L P D G N V I T V G N E
Dictyostelium A S S S A L E K S Y E L P D G Q V I T I G N E
Human A S S S S L E K S Y E L P D G Q V I T I G N E
Saccharomyces A Q S S S I E K S Y E L P D G Q V I T I G N E
Acanthamoeba A S S S A L E K S Y E L P D G Q V I T I G N E
Plasmodium E Q S S D I E K S Y E L P D G N I I T V G N E
Entamoeba A S S S E L E K S Y E L P D G Q V I T V G N E
Tetrahymena K E S S Q N D K S Y E L P D G N T I T V Q D Q
Trypanosoma A K S V S * E E P F E L P D G N V M Q V G N Q

275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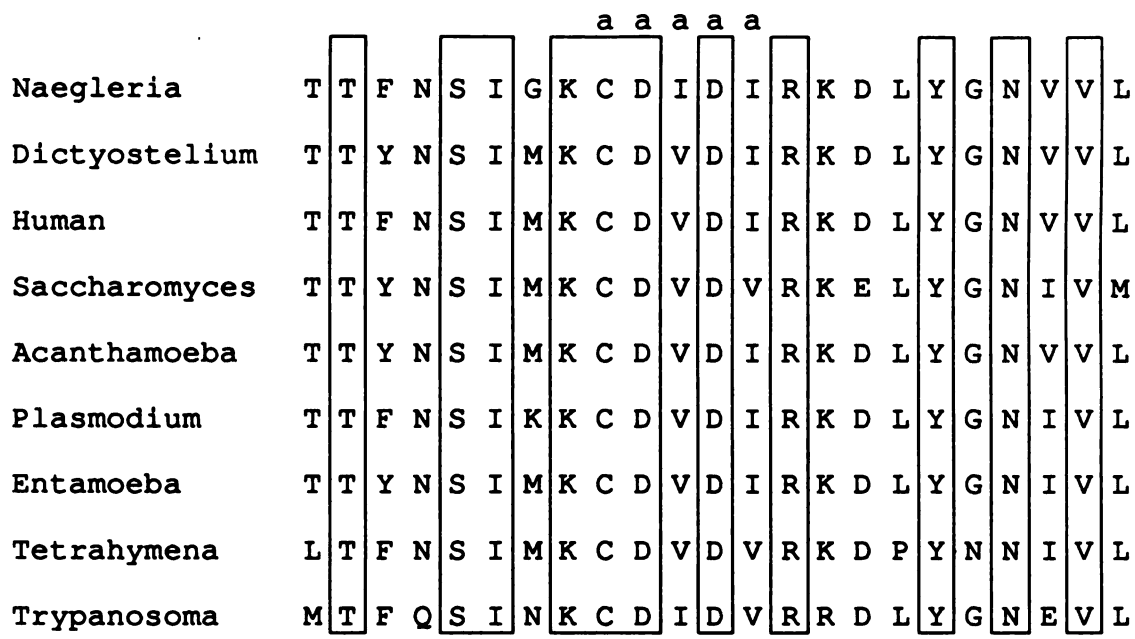
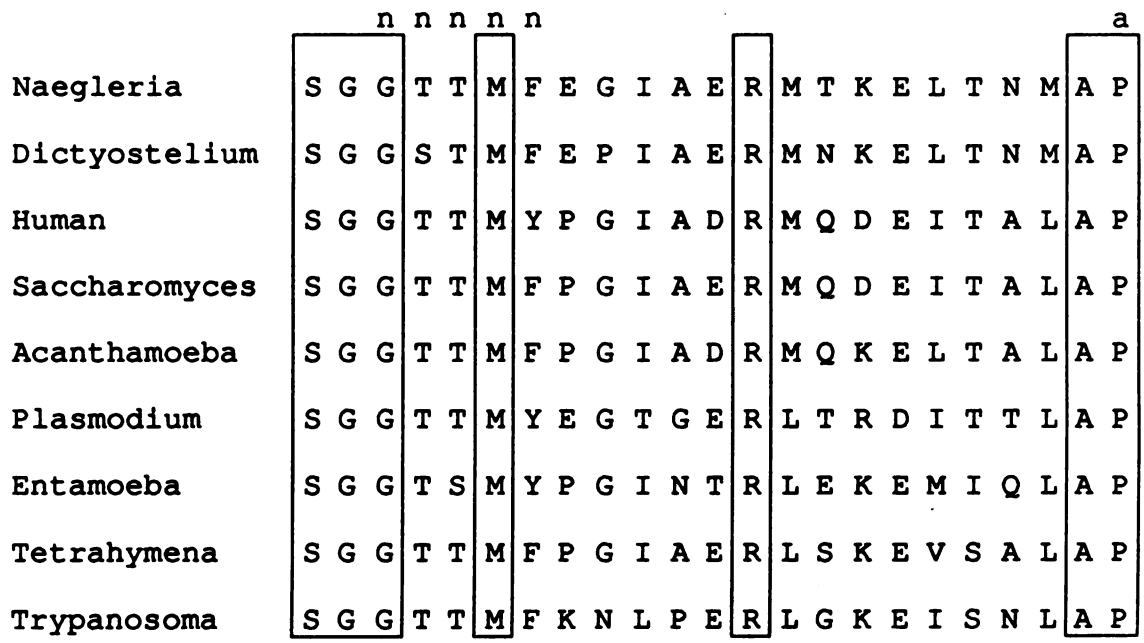
Naegleria R F R C P E V L F Q P N F I G M E A A G V H E
Dictyostelium R F R C P E A L F Q P S F I G M E S A G I H E
Human R F R C P E A L F Q P S F I G M E S C G I H E
Saccharomyces R F R A P E A L F H P S V L G L E S A G I D Q
Acanthamoeba R F R A P E A L F Q P S F L G M E S A G I H E
Plasmodium R F R C P E A L F Q P S F L G K E A A G I H E
Entamoeba R F R C P E A L F Q P S F L G M E C N G I H E
Tetrahymena R F R C P E L L F K P A F I G K E F P G I H E
Trypanosoma R F R C P Q A L F K P A L I G M D Q P G F H E

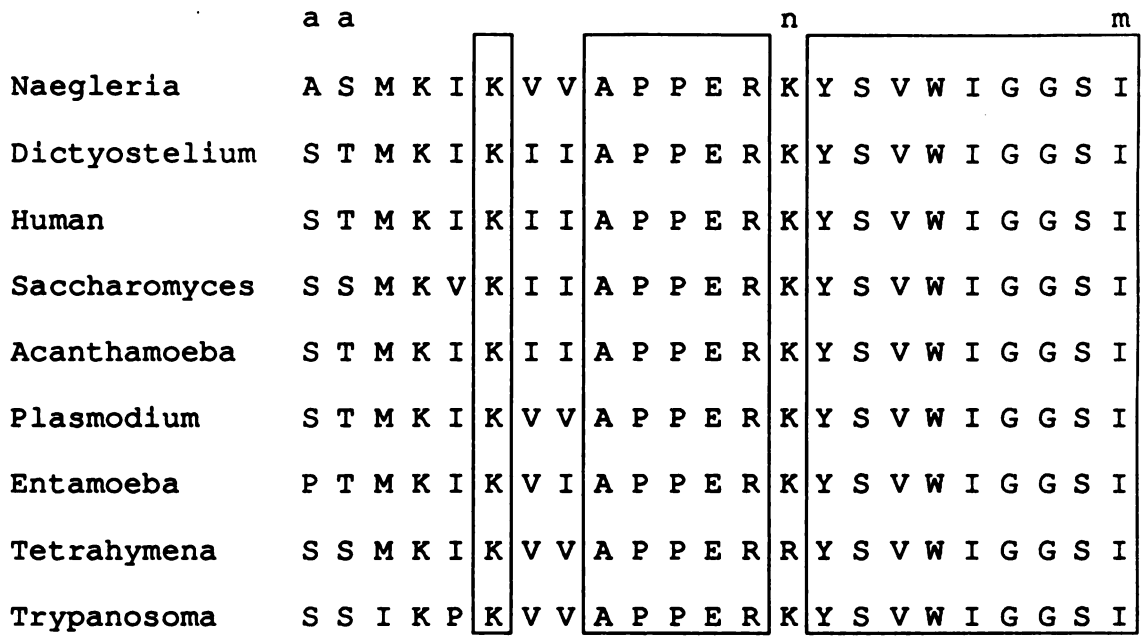
299


a a a a a
Naegleria T T F N S I G K C D I D I R K D L Y G N V V L
Dictyostelium T T Y N S I M K C D V D I R K D L Y G N V V L
Human T T F N S I M K C D V D I R K D L Y G N V V L
Saccharomyces T T Y N S I M K C D V D V R K E L Y G N I V M
Acanthamoeba T T Y N S I M K C D V D I R K D L Y G N V V L
Plasmodium T T F N S I K K C D V D I R K D L Y G N I V L
Entamoeba T T Y N S I M K C D V D I R K D L Y G N I V L
Tetrahymena L T F N S I M K C D V D V R K D P Y N N I V L
Trypanosoma M T F Q S I N K C D I D V R R D L Y G N E V L

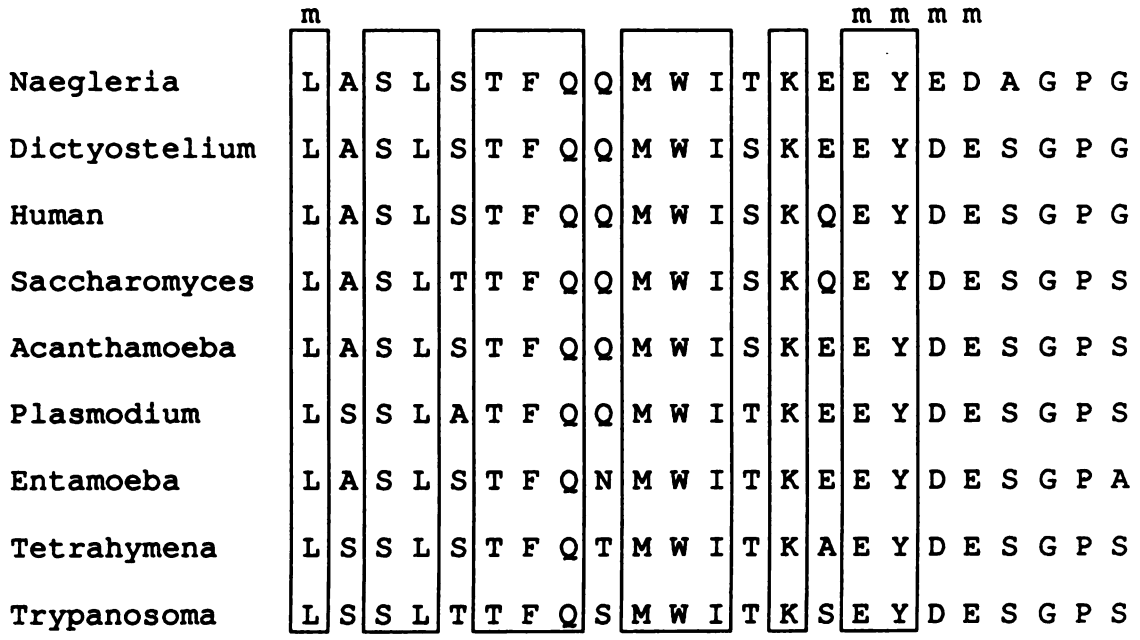
322


n n n n n a
Naegleria S G G T T M F E G I A E R M T K E L T N M A P
Dictyostelium S G G S T M F E P I A E R M N K E L T N M A P
Human S G G T T M Y P G I A D R M Q D E I T A L A P
Saccharomyces S G G T T M F P G I A E R M Q D E I T A L A P
Acanthamoeba S G G T T M F P G I A D R M Q K E L T A L A P
Plasmodium S G G T T M Y E G T G E R L T R D I T T L A P
Entamoeba S G G T S M Y P G I N T R L E K E M I Q L A P
Tetrahymena S G G T T M F P G I A E R L S K E V S A L A P
Trypanosoma S G G T T M F K N L P E R L G K E I S N L A P

345


a a
n
m
Naegleria A S M K I K V V A P P E R K Y S V W I G G S I
Dictyostelium S T M K I K I I A P P E R K Y S V W I G G S I
Human S T M K I K I I A P P E R K Y S V W I G G S I
Saccharomyces S S M K V K I I A P P E R K Y S V W I G G S I
Acanthamoeba S T M K I K I I A P P E R K Y S V W I G G S I
Plasmodium S T M K I K V V A P P E R K Y S V W I G G S I
Entamoeba P T M K I K V I A P P E R K Y S V W I G G S I
Tetrahymena S S M K I K V V A P P E R R Y S V W I G G S I
Trypanosoma S S I K P K V V A P P E R K Y S V W I G G S I

368


m
m m m m
Naegleria L A S L S T F Q Q M W I T K E E Y E D A G P G
Dictyostelium L A S L S T F Q Q M W I S K E E Y D E S G P G
Human L A S L S T F Q Q M W I S K Q E Y D E S G P G
Saccharomyces L A S L T T F Q Q M W I S K Q E Y D E S G P S
Acanthamoeba L A S L S T F Q Q M W I S K E E Y D E S G P S
Plasmodium L S S L A T F Q Q M W I T K E E Y D E S G P S
Entamoeba L A S L S T F Q N M W I T K E E Y D E S G P A
Tetrahymena L S S L S T F Q T M W I T K A E Y D E S G P S
Trypanosoma L S S L T T F Q S M W I T K S E Y D E S G P S

375

Naegleria	I	V	H	R	K	S	F	X
Dictyostelium	I	V	H	R	K	C	F	X
Human	I	V	H	R	K	C	F	X
Saccharomyces	I	V	H	H	K	C	F	X
Acanthamoeba	I	V	H	R	K	C	F	X
Plasmodium	I	V	H	R	K	C	F	X
Entamoeba	I	V	H	R	K	C	F	X
Tetrahymena	I	V	H	R	K	C	F	X
Trypanosoma	I	V	H	S	K	C	F	X

Figure 4. Amino acid sequence alignment and comparison of the actin sequences in N.fowleri (act1), Dictiostelium discoideum, human b-actin, Saccharomyces cerevisidae, Acanthamoeba castellanii, Plasmodium falciparum, Entamoeba histolytica, Tetrahymena thermophila, Trypanosoma brucei. In the species that have isoforms of actin, the most homologous amino acid sequence is chosen for the comparison. Amino acid identities are boxed only when shared by all nine members. The residues in actin that interact with the nucleotide (n), calcium (c), and myosin (m) and that involved in actin-actin interactions (a) are in small letters above the act1 sequemce.

TABLES

Table 1. Codon usage in N. fowleri actin gene. All 375 codons from the initiator ATG to the stop TAA are represented. The numbers are total number of occurrences of each codon.

ARG	CGT	1	LEU	TTA	1	SER	TCT	9
	CGC	0		TTG	22		TCC	7
	CGA	0		CTT	0		TCA	3
	CGG	0		CTC	1		TCG	2
	AGA	15		CTA	0		AGT	0
	AGG	0		CTG	1		AGC	1
ALA	GCT	22	GLY	GGT	25	PRO	CCT	3
	GCC	4		GGC	0		CCC	0
	GCA	2		GGA	6		CCA	15
	GCG	0		GGG	0		CCG	0
VAL	GTT	14	THR	ACT	11	ILE	ATT	18
	GTC	11		ACC	11		ATC	9
	GTA	1		ACA	0		ATA	0
	GTG	2		ACG	1			
ASN	AAT	5	ASP	GAT	17	CYS	TGT	5
	AAC	8		GAC	5		TGC	1
GLN	CAA	9	GLU	GAA	25	HIS	CAT	5
	CAG	0		GAG	5		CAC	4
LYS	AAA	1	PHE	TTT	4	TYR	TAT	10
	AAG	19		TTC	11		TAC	4
MET	ATG	15	TRP	TGG	4			

Table 2. Amino acid comparison of *Naegleria fowleri* with other species (Ac;*Acanthamoeba castellanii*, Dd;*Dictyostelium discoideum*, Hs;*Homo sapiens*, Eh;*Entamoeba histolytica*, Pf;*Plasmodium falciparum*, Sc;*Saccharomyces cerevisidae* Tt;*Tetrahymena thermophila*, Tb;*Trypanosoma brucei*). Matching column shows the number of identical amino acids between the two species. Similar replacement is the amino acid changes within the same side chain polarity while different replacement shows different polarity. H-value is calculated as H=m/(m+u+g/2), m is the number of sequence positions with matching amino acids in the two positions, u is with unmatching (replacements) positions, and g is the number of positions that have a gap in one sequence opposite an amino acid in the other. The greater H-value represents closer relationships between the two species.

comparison	matching	replacement		H-value
		similar	different	
N-Ac	318	46	11	0.848
N-Dd	316	47	12	0.845
N-Hs	312	50	13	0.832
N-Eh	307	56	12	0.817
N-Pf	302	61	12	0.805
N-Sc	296	68	11	0.789
N-Tt	276	72	27	0.736
N-Tb	272	82	21	0.725

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