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의학 박사학위 논문

***In vivo* transfection system of the  
*nfa1* gene cloned from pathogenic  
*Naegleria fowleri***

아주대학교 대학원

의학과

정석률

***In vivo* transfection system of the  
*nfa1* gene cloned from pathogenic  
*Naegleria fowleri***

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이 논문을 의학 박사학위 논문으로 제출함.

2004년 2월

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2003년 12월 19일

***In vivo* transfection system of the *nfal* gene  
cloned from pathogenic *Naegleria fowleri***

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**A Dissertation Submitted to The Graduate School of Ajou University  
in Partial Fulfillment of the Requirements for the Degree of**

**DOCTOR OF PHILOSOPHY**

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**December 19, 2003**

-ABSTRACT-

***In vivo* transfection system of the *nfa1* gene  
cloned from pathogenic *Naegleria fowleri***

The *nfa1* gene cloned from pathogenic amoeba, *Naegleria fowleri* consists of 360 bp and encodes the Nfa1 protein (13.1 kDa) which is located in pseudopodia of the amoeba by immunochemistry as shown in the previous paper. On the contrary, in nonpathogenic *N. gruberi* having an *nfa1* gene, the Nfa1 protein was not detected by western blotting using an anti-Nfa1 antibody. It was identified that other free-living amoebae, *Acanthamoeba* spp. except for nonpathogenic *A. royreba* had an *nfa1* gene by RT-PCR, which suggested the possible involvement of *nfa1* gene in *in vitro* cytotoxicity and *in vivo* pathogenicity of *N. fowleri*.

In this study, DNA of the *nfa1* gene was transfected into nonpathogenic *N. gruberi* to address whether the *nfa1* gene cloned from *N. fowleri* is related with *in vitro* cytotoxicity and *in vivo* pathogenicity. Eukaryotic transfection vector, pEGFP-C2 containing a CMV promoter and GFP gene, was used. Experimental vector was pEGFP-C2/*nfa1*UTR (*nfa1*UTR containing 5' UTR, *nfa1* ORF, and 3' UTR). After transfection using SuperFect<sup>TM</sup> reagent, the green fluorescence expressed from *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector was observed in the cytoplasm of *N. gruberi* and the transfected *N. gruberi* was preserved over 9 months by G418 selection. The transfected *nfa1* gene was observed by PCR using *nfa1*- and vector-specific primers from the genomic DNA of *N. gruberi* transfected with pEGFP-

C2/*nfa1*UTR vector. The *nfa1* and GFP gene were also identified by RT-PCR in the transgenic *N. gruberi*. The Nfa1 protein from the transgenic *N. gruberi* was expressed and identified as a 13.1 kDa of band by western blotting using an anti-Nfa1 antibody. Finally, *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector could induce the *in vitro* cytotoxicity but not *in vivo* pathogenicity. In conclusion, I have established an efficient transfection system for *N. gruberi* for the first time. Stable transfection into *N. gruberi* was established by using my system and the expressed Nfa1 protein enhanced *in vitro* cytotoxicity of *N. gruberi* but not the *in vivo* pathogenicity, suggesting the possible involvement of another factor(s) for *in vivo* pathogenicity of amoeba.

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Key Words: *Naegleria fowleri*, *Naegleria gruberi*, *nfa1* gene, *in vivo* transfection, *in vitro* cytotoxicity

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## **ABBREVIATIONS**

AP	Alkaline phosphatase
BCIP	Bromo-chloro-indolyl-phosphate
CAT	Chloramphenicol acetyltransferase
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
DEAE	Diethylaminoethyl
DEPC-DW	Diethylpyrocarbonate-treated distilled water
EGFP	Enhanced GFP
EMEM	Earle' s minimal essential medium
FBS	Fetal bovine serum
GAE	Granulomatous amebic encephalitis
gDNA	Genomic DNA
GFP	Green fluorescent protein
IFA	Indirect immunofluorescence
LDH	Lactate dehydrogenase
MCS	Multicloning site
NBT	Nitro blue tetrazolium

<i>neo</i>	Neomycin phosphotransferase
<i>nfa1</i>	<i>Naegleria fowleri</i> antigen 1
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAME	Primary amoebic meningoencephalitis
PBS	Phosphate buffered saline
PBST	PBS containing Tween 20
RSV-LTR	Rous sarcoma virus-long terminal repeat
SDS	Sodium dodecyl sulfate
UTR	Untranslated regions

# I. INTRODUCTION

## A. Free-living amoebae

Free-living amoebae belonging to the genera *Naegleria*, *Acanthamoeba*, and *Balamuthia* are important causes of disease in humans and animals. Human disease caused by free-living amoebae was first reported in 1965 by Fowler and Carter, who studied four patients with Primary amoebic meningoencephalitis (PAME) in South Australia.<sup>1</sup> *Naegleria* spp. and *Acanthamoeba* spp. have been commonly found in lakes, swimming pools, tap water, and heating and air conditioning units. There are six species of *Naegleria*: *N. fowleri*, *N. gruberi*, *N. australiensis*, *N. lovaniensis*, *N. jadinii*, and *N. thornstoni*.<sup>2,3</sup> *Naegleria fowleri*, sometimes called *N. aerobia*<sup>4-6</sup> produces an acute, and usually lethal, central nervous system (CNS) disease of PAME.<sup>7-9</sup> The infection results from introduction of water containing amoebae into the nasal cavity and subsequent passage of the organisms to the CNS via the olfactory apparatus.<sup>10-12</sup> *N. gruberi*, a nonpathogenic species, was the first member of the genus to be described and has been used extensively to define the molecular biology of cellular differentiation.<sup>13</sup> *Acanthamoeba* spp. and *Balamuthia mandrillaris* are opportunistic free-living amoebae capable of causing granulomatous amoebic encephalitis (GAE) in individuals with compromised immune systems.

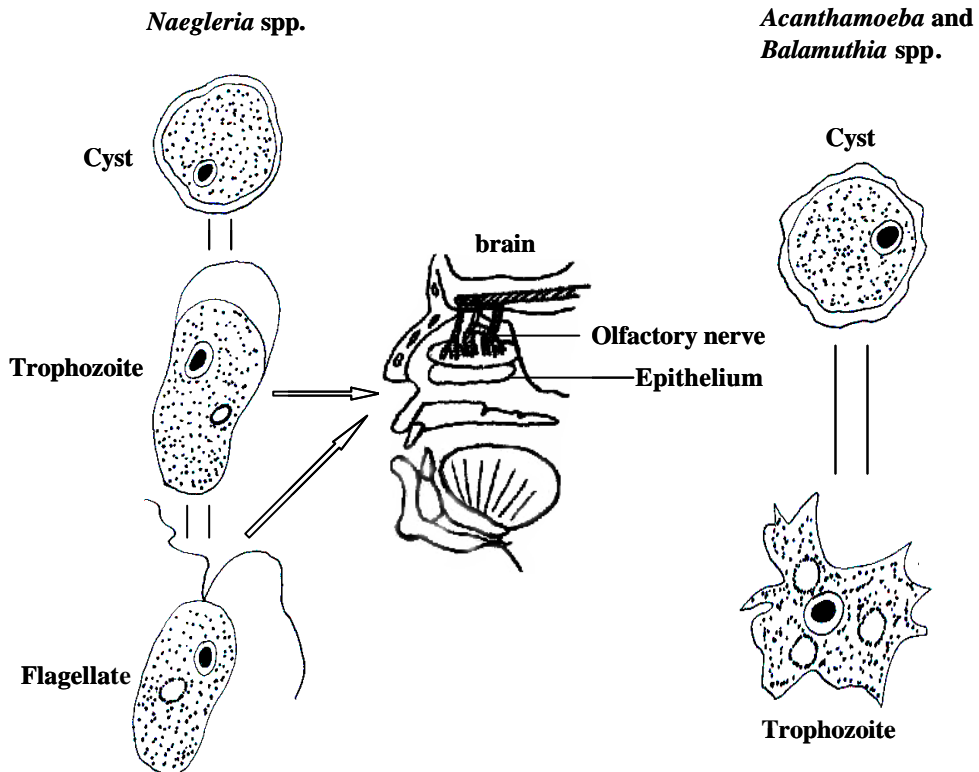


*N. fowleri* has three stages, cysts, trophozoites, and flagellate forms, in its life cycle (Fig. 1). Alternatively, the trophozoites can occur in two forms, amoeboid and flagellate. The amoeboid form (the only form recognized in humans) is elongated with a broad anterior end and tapered posterior end. The size ranges from 7 to 20  $\mu$ m. There is a large, central karyosome and no peripheral nuclear chromatin. The cytoplasm is somewhat granular and contains vacuoles. The amoeboid form replicates by promitosis (nuclear membrane remains intact). Amoeboid form can turn into temporary flagellate forms which usually revert back to the amoeboid stage. The flagellate form is pear-shaped, with two flagella at the broad end. These flagellate forms do not divide, but when the flagella are lost, the amoeboid forms resume reproduction. *N. fowleri* trophozoites are found in cerebrospinal fluid (CSF) and tissue, while flagellate forms are found in CSF. Cysts from nature and from agar cultures look the same and have a single nucleus almost identical to that seen in the trophozoite. They are generally round, measuring from 7 to 10  $\mu$ m, and there is a thick double wall. Unlike *N. fowleri*, *Acanthamoeba* and *Balamuthia* have only two stages, cysts and trophozoites, in their life cycle (Fig. 1). No flagellate stage exists as part of the life cycle.

## **B. Primary Amoebic Meningoencephalitis (PAME)**

PAME is a rapidly fatal disease which occurs generally in previously healthy children and young adults with a history of swimming in freshwater lakes or ponds.

Presumably, infection results from introduction of water containing amoebae into the nasal cavity and subsequent passage of the organisms to the CNS via the olfactory



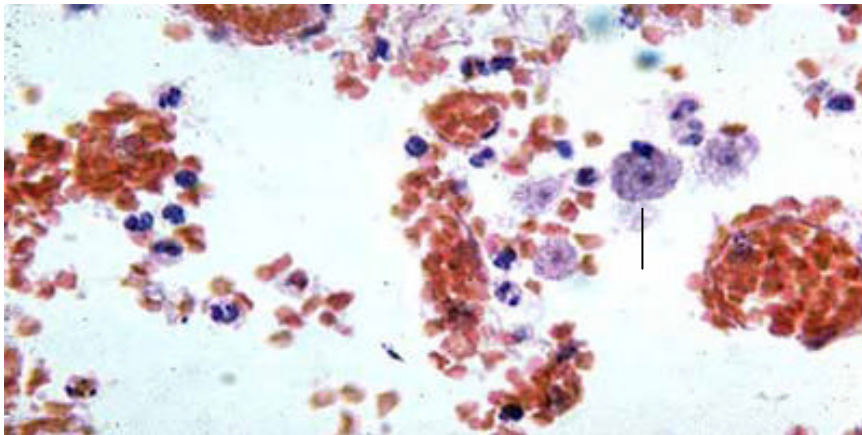
**Fig. 1. The life cycles of free-living amoebae.** Blank arrows show the route of infection in *Naegleria* spp.. *Naegleria* spp. unlike other free-living amoebae have three stage life cycles. Black arrows represent the morphological changes of free-living amoebae.

apparatus.<sup>7-9</sup> Acute hemorrhagic necrotizing meningo-encephalitis follows invasion of the CNS. Only amoebic trophozoites are found in the lesions of patients with PAME (Fig. 2).

PAME is a disease with an abrupt onset and a fulminant course. It is characterized by the sudden onset of bifrontal or bitemporal headache, fever (from 38.2 to >40 °C), nausea, vomiting (usually projectile), signs of meningeal irritation, and encephalitis. There is often a rapid progression from fever and early signs of leptomeningitis, encephalitis, or meningoencephalitis to coma and seizures. Nausea, vomiting, photophobia, and other symptoms related to increase intracranial pressure may also be prominent. The final diagnosis of PAME is based on the isolation and culture of free-living amoebae from CSF or the demonstration of amoebic trophozoites in biopsied brain tissue. Clinical diagnosis by experienced practitioners is based on the characteristic stromal infiltrate. Antibodies may be detected in serum; however, serologic tests usually are of no value in the diagnosis of infections with free-living amoebae.<sup>14</sup> Amphotericin B reportedly cured one case of PAME.<sup>15</sup> High morbidity and mortality may be reduced with rapid diagnosis and earlier treatment.

### **C. Pathogenicity**

The studies of mechanisms of pathogenicity by free living amoebae have been reported. In same free living amoeba, *Acanthamoeba*, with *Naegleria*, cytopathogenic effects of *Acanthamoeba* on host cells require (i) adhesion of the amoeba



**Fig. 2. PAME in mouse brain tissues due to the infection of *N. fowleri*.** Arrow indicates *N. fowleri* trophozoite surrounded by inflammatory cells ( $\times 400$ ). The trophozoites are mainly observed in PAME.

to host cell,<sup>16</sup> (ii) secretion of proteases,<sup>17</sup> and (iii) phagocytosis. It has been shown that adhesion is one of the crucial steps for the pathogenicity of amoeba<sup>16</sup> as non-pathogenic amoeba exhibit significant decreased binding to host cells. Yang et al.<sup>16</sup> reported that the first step in the pathogenesis of *Acanthamoeba* infection involve attachment of the parasite to the mannose residues of the plasma membrane glycoproteins of host cells. This binding, in turn, may promote phosphorylation of selective mannose residues by the amoeba enzyme, and it may be the presence of the mannose-6-phosphate residues on the surface of host cells that is required for infection to occur. According to Khan,<sup>18</sup> the binding of *Acanthamoeba* to corneal epithelial cells was acanthopodia-dependent as no binding of non-pathogenic amoeba (which exhibit significant acanthopodia) with epithelial cells was observed. Also, he reported that pathogenicity would be a complex process which involves both contact-dependent and contact-independent pathways in order to kill host cells quickly and to reduce the degree to which defense can be induced.

#### **D. Gene transfer to eukaryotic cells**

Gene transfer to animal cells has been practiced now for over 40 years. Techniques are available for the introduction of DNA into many different cell types

in culture, either to study gene function and regulation or to produce large amounts of recombinant protein. Three techniques fall into three categories: transfection by biochemical methods, transfection by physical methods, and virus-mediated transduction. Some methods of these are as below. The first is DNA-calcium phosphate coprecipitate method. The ability of mammalian cells to take up exogenously supplied DNA from their culture medium was first reported by Szybalska and Szybalski.<sup>19</sup> The formation of a fine DNA/calcium phosphate coprecipitate first settles on to the cells and is then internalized. It is thought that small granules of calcium phosphate associated with DNA are taken up by endocytosis and transported to the nucleus, where some DNA escapes and can be expressed.<sup>20</sup> However, since the precipitate must coat the cells, this method is suitable only for cells grown in monolayers, not those growing in suspension or as clumps. Second, other chemical transfection methods are available. By the calcium phosphate method, some cell lines are adversely affected by the coprecipitate due to its toxicity and are hence difficult to transfect. Alternative chemical transfection method has been developed to address this problem. One such method utilized diethylaminoethyl dextran (DEAE-dextran), a soluble polycationic carbohydrate that promotes interactions between DNA and the endocytotic machinery of the cell. It has been reported that although efficient for the transient transfection of many cell types, DEAE-dextran cannot be used to generate stably transformed cell lines. The third is the method using phospholipid as gene delivery vehicle. It interacts with the target cell membrane and facilitates DNA uptake. The procedures are very efficient in

terms of the number of transformants obtained, but they are also labor-intensive and so have not been widely adopted as a general transfection method. However, an important advantage is that they are gentle, allowing the transfer of large DNA fragments without shearing. More widespread use has been made of artificial phospholipids vesicles, which are called liposomes.<sup>21</sup> The low-toxicity transfection method, commonly known as lipofection, which is applicable to many cell types that are difficult to transfect by other means, including cells growing in suspension.<sup>22</sup> Fourth, the electroporation method involves the generation of transient, nanometer-sized pores in the cell membrane, by exposing cells to a brief pulse of electricity. DNA enters the cell through these pores and is transported to the nucleus. The most critical parameters are the intensity and duration of the electric pulse, and these must be determined empirically for different cell types. Finally, there are direct transfer methods. One such procedure is microinjection, a technique that is guaranteed to generate successful hits on target cells but that can only be applied to a few cells in any one experiment. In animals, this technique is most often used to transfect multiple cells in tissue slices rather than cultured cells.<sup>23,24</sup>

## **E. Consideration of the transfection**

Although the different expression systems vary in their total potential yield, in term of vector design and experimental methodology, the following consideration should apply when high-level expression is required.

## **1. Use of strong and constitutive promoter**

Very active promoters provide the highest levels of transgene expression. In viral vectors, the strongest endogenous promoters are used, for examples, human cytomegalovirus (CMV) immediate early promoter, baculovirus polyhedron promoter, adenoviral E1 promoter, and vaccinia virus p7.5 promoter, etc. Although these function widely, they are not necessarily active in all mammalian cells, e.g., the rous sarcoma virus long terminal repeat (RSV-LTR) promoter functions more poorly in the *Acanthamoeba* than ubiquitin promoter from *Acanthamoeba*.<sup>25</sup> According to Kong et al,<sup>26</sup> myosin gene was transcribed efficiently in *Acanthamoeba* under ubiquitin promoter.

## **2. Reporter genes used commonly as screenable marker genes**

Reporter genes are also known as screenable marker genes. Importantly, the assays used to detect reporter gene activity are quantitative, so they can also be used to measure transformation efficiency. The use of reporters is advantageous, because it circumvents the necessity to derive different assays for individual genes and also allows the activities of transgenes and homologous endogenous genes to be distinguished in the same cell. Details of the comparison of commonly used reporter



genes are illustrated in Table 1. Especially, because green fluorescent protein (GFP) exhibits the autofluorescence in transfected cells, it makes the efficiency of transfection measure easily.

**Table 1. Details of the comparison of commonly used reporter genes**

<b>Reporter genes</b>	<b>Advantages</b>	<b>Disadvantages</b>
Chloramphenicol acetyltransferase (CAT)	No endogenous activity	Narrow linear range; use of radioisotopes
Betagalactosidase (bacterial)	Well-characterized and stable; simple colorimetric readouts; sensitive bio- or chemiluminescent assays available	Endogenous activity (mammalian cells)
Luciferase (firefly)	High specific activity; no endogenous activity, broad dynamic range	Requires substrate (luciferin) and presence of O <sub>2</sub> and ATP
Alkaline phosphatase (human placental)	Secreted protein; inexpensive and highly sensitive luminescent assay	Endogenous activity in some cells; interference with compounds being screened
GFP (jellyfish)	Autofluorescent (no substrate needed); no endogenous activity	Requires posttranslational modification; low sensitivity

### **3. Selection of transfected cells with antibiotics for stable transfectants**

If animal cells are exposed to toxic concentrations of certain drugs, rare individual cells can survive because they have spontaneously undergone a mutation that confers resistance to that drug. For example, *neo* gene (Neomycin phosphotransferase from *Escherichia coli*) confers resistance to aminoglycoside antibiotics. The concentration of antibiotics is determined with lethal dose which makes the untransfected cells die after about one week. In addition to *neo* gene, there has been used puromycin N-acetyltransferase from *Streptomyces alboniger* conferring resistance to puromycin, or hygromycin phosphotransferase from *E. coli* conferring resistance to hygromycin B, etc.

### **4. Choice of pEGFP-C2 vector**

pEGFP-C2 vector encodes a red-shifted variant of wild-type GFP<sup>27-29</sup> which has been optimized for brighter fluorescence and higher expression in mammalian cells (Excitation maximum = 488 nm; emission maximum = 507 nm). pEGFP-C2 vector encodes the GFPmut1 variant<sup>30</sup> which contains the double-amino-acid substitution of

Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the enhanced GFP (EGFP) gene contains more than 190 silent base changes which correspond to human codon-usage preferences.<sup>31</sup> Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site<sup>32</sup> to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-C2 vector is between the EGFP coding sequences and the SV40 poly A. Genes cloned into the MCS will be expressed as fusions to the C-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T-antigen. A neomycin-resistance cassette (*neo<sup>r</sup>*), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex thymidine kinase gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette ( $P_{amp}$ ) expresses kanamycin resistance in *E. coli*. Fusions to the C-terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo*. The target gene should be cloned into pEGFP-C2 vector so that it is in frame with the EGFP coding sequences, with no intervening in-frame stop codons. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418.<sup>33</sup> pEGFP-C2 vector can also be used simply to express EGFP in a cell line of interest (e.g., as a transfection marker).

## **F. Introduction of *N. fowleri* antigen 1 (*nfa1*) gene and theme of this study**

Transfection system enables the study of gene function and regulation and provides the technology to define the mechanisms regulating stage differentiation.<sup>34</sup> Especially, it also will be able to contribute to the study of mechanisms of pathogenicity. Transfection systems have been available for several important species of protozoa, including *Acanthamoeba* sp., *Entamoeba histolytica*, members of the kinetoplastida and the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp..<sup>35-39</sup> However, chromosome number and ploidy, as well as the occurrence of sexual reproduction, are unknown in *Naegleria* spp., therefore, classical mapping and genetic analysis is limited.<sup>35</sup> The basic but important technique for DNA transfection into *Naegleria* sp. has not been yet established.

At a molecular level, we previously reported the *nfa1* gene encoding a 13.1 kDa antigenic protein cloned from *N. fowleri*.<sup>40</sup> An *nfa1* gene consists of 360 bp, and the Nfa1 protein is located in pseudopodia of trophozoite, which was identified by immunocytochemistry with a transmission electron microscopic observation. The Nfa1 protein was not detected in nonpathogenic *N. gruberi* by western blotting using an anti-Nfa1 polyclonal antibody derived from BALB/c mouse, but the *nfa1* gene was detected in it by RT-PCR. It supports the fact that the *nfa1* gene may be related with the pathogenicity of *Naegleria* spp..<sup>41</sup>

In this study, we transfected the *nfa1* gene into nonpathogenic *N. gruberi* in order

to develop a stable transfection system, which would be helpful for this study elucidating the mechanism of pathogenicity in *Naegleria* infection. For the transfection of the *nfa1* gene into *N. gruberi*, we have constructed some vectors based on the pEGFP-C2 eukaryotic transfection vector, pEGFP-C2/*nfa1*, pEGFP-C2/*nfa1*UTR, and other vectors containing an ubiquitin promoter. After each vector was transfected with SuperFect™ reagent, expressed GFP was observed under a fluorescent microscopy, and transfected *nfa1* gene was detected by PCR. Also, the Nfa1 protein expressed from transgenic *N. gruberi* was identified by western blotting using an anti-Nfa1 antibody which was obtained from mice immunized with a recombinant Nfa1 protein expressed from the *N. fowleri nfa1* gene. Finally, the experiments of *in vitro* cytotoxicity and *in vivo* pathogenicity were performed to identify the pathogenicity of transgenic *N. gruberi*.

## II. MATERIALS AND METHODS

### A. Materials

#### 1. Animals

7-week-old female BALB/c mice used in this experiment were purchased from DaeHan Biolink (KIST, Daejeon, Korea) for *in vivo* pathogenicity.

#### 2. Reagents

*N. fowleri* (ATCC No. 30215) and *N. gruberi* (ATCC No. 30960) were purchased from American Type Culture Collection (ATCC, Manassas, VA) for the transfection, *in vitro* cytotoxicity, and *in vivo* pathogenicity.

pEGFP-C2 vector was purchased in Clontech Laboratories (Palo Alto, California) for transfection and modified according to some purpose. pCR<sup>®</sup>T7/NT-TOPO<sup>®</sup> vector was purchased in Invitrogen (San Diego, CA) to clone an *nfal* gene. pGL3 vector and ubiquitin promoter were kindly provided by Dr. Kong at Kyung-

pook University in Korea. The *nfa1*UTR sequence was kindly presented by Prof. Park at Yonsei University in Korea. All primers were constructed in Bioneer Inc. (Daejeon, Korea)

SuperFect<sup>TM</sup> reagent was purchased in Qiagen (GmbH, Germany) for transfection.

All used restriction enzymes were purchased in New England Biolab Inc. (Beverly, MA). FBS was purchased in Hyclone Laboratories (Logan, UT). The penicillin, streptomycin, and G418 antibiotics was purchased in Gibco BRL (Gaithersburg, MD).

25 cm<sup>2</sup>-culture flask and 24 well cell culture plate were purchased in Nunc A/S (Roskilde, Denmark). The 0.22 μm syringe filter was purchased in Nalgene Europe Ltd. (Neerijse, Belgium).

Total RNA was prepared using an isolation kit RNazol<sup>TM</sup> B (TEL-TEST, Fiendwood, TX, USA). For reverse transcription, we used the Superscript First Strand Synthesis System kit (Invitrogen, San Diego, CA). Taq DNA polymerase was purchased in Promega (Madison, WI).

For western blotting and indirect fluorescent antibody (IFA) test, the secondary antibody of a goat anti-mouse IgG conjugated with alkaline phosphatase (AP), bromo-chloro-indolyl-phosphate (BCIP), and nitro blue tetrazolium (NBT) were purchased in Sigma Chemical Co (St. Louis, MO) and the secondary antibody of an AffiniPure rabbit anti-mouse IgG conjugated with rhodamine TRITC was obtained in Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

The Earle's minimal essential medium (EMEM) was purchased in Gibco BRL (Gaithersburg, MD) to culture chinese hamster ovary (CHO) cells.

CytoTox96<sup>®</sup> Non-radioactive Cytotoxicity Assay Kit for *in vitro* cytotoxicity was purchased in Promega (Madison, WI).

## **B. Methods**

### **1. Culture of *Naegleria* spp.**

Trophozoites of *N. fowleri* (NF69 strain; ATCC No. 30215) were axenically cultured in Nelson's medium<sup>42</sup> at 37 °C. Trophozoites of *N. gruberi* (ATCC No. 30960) were axenically cultured in 1034 modified PYNFH medium<sup>43</sup> at 33 °C. They were subcultured every week with about 80% confluence and *N. gruberi* was used as the host for the transfection. Especially, optimal temperature of *N. gruberi* commented in ATCC is 25 °C but it has been risen to 33 °C for the transfection, *in vitro* cytotoxicity, and *in vivo* pathogenicity.

### **2. Construction of eukaryotic expression vectors**

The eukaryotic expression vectors transfected to nonpathogenic *N. gruberi* were modified from the pEGFP-C2 vector (Clontech Laboratories, Palo Alto, California). The pEGFP-C2 vector containing CMV promoter encodes a red-shifted variant of



wild-type EGFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. It was used as a control construct.

An *nfa1* gene (GeneBank Accession No. AF230370) was cloned into the downstream of a gene encoding EGFP in a pEGFP-C2 vector. The various primers for cloning are illustrated in Table 2.

The *nfa1* gene was amplified from previously an *nfa1* gene-cloned vector, pCR<sup>®</sup>T7/NT-TOPO<sup>®</sup> (Invitrogen, San Diego, CA), by polymerase chain reaction (PCR) using an *nfa1* F1 primer containing *Hind* III site and an *nfa1* R1 primer containing *Eco* RI site, which produced 609 bp fragment. This fragment was cloned into pEGFP-C2 vector using *Hind* III and *Eco* RI restriction sites to construct a pEGFP-C2/*nfa1* vector.

The *nfa1*UTR gene (kindly presented by Prof. Park at Yonsei University) was cloned from a genomic DNA (gDNA) of *N. fowleri*. The *nfa1*UTR gene containing 5' upstream regions, open reading frame (ORF), and 3' downstream regions was amplified by PCR using an *nfa1*UTR F1 primer containing *Bgl* II site and an *nfa1*UTR R1 primer containing *Eco* RI site, which produced 615 bp fragment. This fragment was cloned into pEGFP-C2 vector using *Bgl* II and *Eco* RI sites to construct a pEGFP-C2/*nfa1*UTR vector.

An ubiquitin promoter of 1894 bp from *Acanthamoeba* (kindly presented by Dr. Kong at Kyung-pook University) was cloned into pEGFP-C2/*nfa1* vector between EGFP and *nfa1* because it could act strongly more than CMV promoter in same free-living amoeba, *Acanthamoeba*.<sup>25</sup> The ubiquitin promoter was amplified from

previously cloned pGL3 (Promega, Madison, WI) vector by PCR using an Ubi F1 primer containing *Sac* I site and an Ubi R1 primer containing *Hind* III site, which produced a 1901 bp fragment. This fragment was cloned into pEGFP-C2/*nfa1* vector using *Sac* I and *Hind* III restriction sites to construct a pEGFP-C2/Ubi/*nfa1* vector.

**Table 2. Primers used to clone the vectors for the transfection**

<b>Primer</b>	<b>Sequences</b>	<b>Cloned vector</b>	<b>Enzymes site</b>
<i>nfa1</i> F1	5'-GAGCTCAAGCTTTATACATATGCGG-3'	pEGFP-C2/ <i>nfa1</i>	pEGFP-C2/ <i>nfa1</i>
<i>nfa1</i> R1	5'-TAGTTATTGCTCAGCGGTGG-3'	pEGFP-C2/ <i>nfa1</i>	pEGFP-C2/ <i>nfa1</i>
<i>nfa1</i> UTR F1	5'-ACTCAGATCTCCGAATAGTAGCACCACC-3'	pEGFP-C2/ <i>nfa1</i> UTR	pEGFP-C2/ <i>nfa1</i> UTR
<i>nfa1</i> UTR R1	5'-CCCGGAATTCCGATCATAACAATCATC-3'	pEGFP-C2/ <i>nfa1</i> UTR	pEGFP-C2/ <i>nfa1</i> UTR
Ubi F1	5'-CAAACAAACTAGCAAATAGGCTG-3'	pEGFP-C2/Ubi/ <i>nfa1</i>	pEGFP-C2/Ubi/ <i>nfa1</i>
Ubi R1	5'-TCGATAAGCTTCCATGGAATTCTGT-3'	pEGFP-C2/Ubi/ <i>nfa1</i>	pEGFP-C2/Ubi/ <i>nfa1</i>
<i>nfa1</i> UTR F2	5'-GGGGAAGCTTCCGAATAGTAGCACCACC-3'	Ubi/pEGFP-C2/ <i>nfa1</i> UTR	Ubi/pEGFP-C2/ <i>nfa1</i> UTR
<i>nfa1</i> UTR R2	5'-TTTGATCCCGATCATAACAATCATC-3'	Ubi/pEGFP-C2/ <i>nfa1</i> UTR	Ubi/pEGFP-C2/ <i>nfa1</i> UTR

Primers were used to add restriction enzyme sites to *nfa1* or *nfa1*UTR or ubiquitin gene. Each gene amplified with primers above was cloned into a pEGFP-C2 vector through the sticky end ligation.

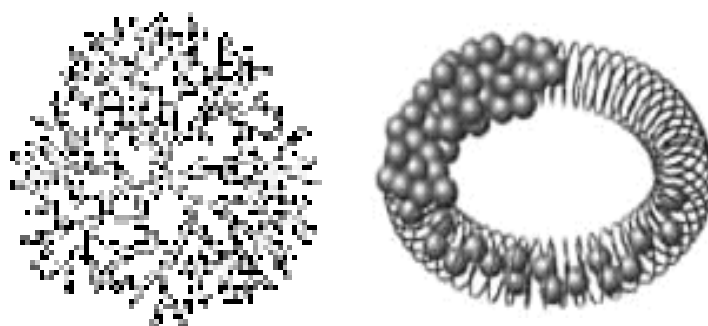
Alternatively, the ubiquitin promoter was inserted into the site, where CMV promoter was deleted, for the purpose of acting on the EGFP and *nfa1* gene. It was thought that ubiquitin promoter might be able to act on an *nfa1* gene and express it more efficiently than CMV promoter. The amplified ubiquitin promoter was cloned into CMV promoter-deleted pEGFP-C2 vector using *Ase* I and *Nhe* I restriction sites to construct an Ubi/pEGFP-C2 vector by blunt-end ligation, and then *nfa1* gene digested from pEGFP-C2/*nfa1* vector containing *Bsr* GI and *Hind* III site is cloned to an Ubi/pEGFP-C2/*nfa1* vector.

An *nfa1*UTR gene was amplified by PCR using an *nfa1*UTR F2 primer containing *Hind* III site and an *nfa1*UTR R2 primer containing *Bam* HI site, which produced 649 bp fragment. This sequence was cloned into an Ubi/pEGFP-C2 vector using *Hind* III and *Bam* HI sites to construct an Ubi/pEGFP-C2/*nfa1*UTR vector. All cloned vectors were sequenced with ABI Perkin Elmer 373A automated DNA sequencer (Applied Biosystems, Foster city, CA).

### **3. Transfection and G418 selection**

The vectors were transfected into *N. gruberi* trophozoites using SuperFect™

reagent (Fig. 3; Qiagen GmbH, Germany). It consists of activated-dendrimer molecules with a defined spherical architecture.<sup>44</sup> Branches radiate from a central core and terminate at charged amino groups which can then interact with negatively charged phosphate groups of nucleic acids. It assembles DNA into compact



**Fig. 3. Schematic representation of an activated dendrimer (left) and model of the SuperFect™-DNA complex (right).** There is the highly branched structure in dendrimer. SuperFect™ Reagent (black balls) interacts with DNA (black) to form a ring-like (toroid-like) structure.

structures that bind to the cell surface and are taken into the cell by nonspecific endocytosis. The reagent buffers the pH of the endosome, leading to pH inhibition of endosomal nucleases, which ensures stability of SuperFect™-DNA complexes. Due to highly controlled chemical synthesis the activated-dendrimer molecules in SuperFect™ reagent (Qiagen GmbH, Germany) have a precise size and a defined shape. This ensures consistent transfection-complex formation and reproducible transfection results. *N. gruberi* ( $5 \times 10^5$  trophozoites per 25 cm<sup>2</sup>-culture flask (Nunc A/S, Roskilde, Denmark)) was cultured for 24 hr at 33 °C in 5 ml of 1034 modified PYNFH medium containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and antibiotics containing penicillin and streptomycin (Gibco BRL, Gaithersburg, MD) to concentration of 10,000 unit ml<sup>-1</sup>, respectively. On the day of transfection, a total of 5 µg of supercoiled plasmid DNA dissolved in Tris-EDTA (TE) buffer (pH 8.0) with PYNFH medium without serum, proteins and antibiotics was added in 1.5 ml eppendorf tube to the final volume of 150 µl. After SuperFect™ reagent (30 µl, Germany) have a precise size and a defined shape. This preincubated at RT was added to the tube, and the mixture was incubated at room temperature for 10 min to allow the transfection-complex formation. While the complex formation takes place, *N. gruberi* trophozoites were washed once with 4 ml of 1× phosphate

buffered saline (PBS), and resuspended in 1 ml of PYNFH complete medium. The resuspended *N. gruberi* was added to reaction tube above, and immediately transferred into a 25 cm<sup>2</sup>-culture flask (Nunc A/S, Roskilde, Denmark). *N. gruberi* was incubated for 5 hr 30 min at 33 °C, and then 5 ml of PYNFH complete medium was added to the flask. After *N. gruberi* was incubated for 24 hr at 33 °C, the medium was removed, and fresh complete PYNFH medium was added. G418 (1 mg ml<sup>-1</sup>) (Geneticin; Gibco BRL, Gaithersburg, MD) was added to *N. gruberi*-cultivating flask for 48 hr after transfection.

The lethal dose of untransfected *N. gruberi* to G418 (Gibco BRL, Gaithersburg, MD) was determined that it was died after 1 week. It was subcultured every week with 80% confluence.

#### **4. Observation of EGFP expression and measurement of transfection efficiency in *N. gruberi***

The expression of EGFP in *N. gruberi* transfected with six vectors, respectively, was observed under a fluorescent microscopy (model BX60; Olympus Optical Co., Japan) using standard FITC excitation/emission filters (488 nm/507 nm). For FACS analysis, *N. gruberi* was washed twice with PBS and resuspended in 1 ml of PBS. The EGFP was measured with a CellQuest 3.2 FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA). The *N. gruberi* expressing the EGFP was sorted aseptically and incubated at 33 °C.

## 5. PCR of a gDNA in *N. gruberi*

For a gDNA preparation, trophozoites of *N. gruberi* were harvested and collected in an 1.5 ml eppendorf tube. Pellets incubated with the digestion solution (125  $\mu$ l of 10% sodium dodecyl sulfate (SDS), 6.25  $\mu$ l of proteinase K (20 mg ml<sup>-1</sup>), 500  $\mu$ l of TE buffer (pH 8.0)) for 30 min at 37 °C. After centrifuged at 10,000 $\times$ g for 5 min at 4 °C, the supernatant was transferred to a 1.5 ml eppendorf tube, mixed with 630  $\mu$ l of phenol (pH 7.4), and centrifuged 10,000 $\times$ g for 10 min at 4 °C. It was reacted with a solution containing chloroform and isoamylalcohol (24:1) by the same methods mentioned above. The 0.1 volume of 3 M sodium acetate (pH 7.5) and 2 volume of absolute ethanol were added to supernatant. After centrifugation, the pellet was dried at room temperature. The primers used to identify the EGFP and *nfaI* gene in transgenic *N. gruberi* or the *nfaI* gene in control *N. gruberi* were illustrated in Table 3. The program for PCR consists of 35 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min 30 s.

## 6. Reverse transcription (RT) PCR

Total RNA was prepared using an isolation kit RNAzol<sup>TM</sup> B (TEL-TEST, Friendswood, TX, USA) solution. Briefly presented, after 500  $\mu$ l of RNAzol<sup>TM</sup> B solution was mixed with pellet of  $1 \times 10^5$  trophozoites by pipetting, 10  $\mu$ l of

chloroform was added to the mixture. It was incubated on ice for 10 min and centrifuged at 10,000×g for 15 min at 4 °C. The supernatant was transferred to a new eppendorf tube and reacted with 250 µl of isoamylalcohol. Incubated for 15 min at 4 °C, it was centrifuged at 10,000×g for 15 min at 4 °C. The pellet was washed with 1

**Table 3. Primers used for PCR of gDNA or cDNA**

Primer	Sequences	Positions	Amplified gene	DNA
GFP F1	5'-ACAACATCGAGGACGGCAGCGTGCAGCTCG-3'	1121-1150 bp in pEGFP-C2	fragment between GFP and <i>nfa1</i>	cDNA
<i>nfa1</i> R2	5'-TTAAAGCACTCCCTTGTACTTCAT-3'	337-360 bp in <i>nfa1</i>	fragment between GFP and <i>nfa1</i>	cDNA
<i>nfa1</i> F2	5'-ATGCCCACTACTATCCATCACCA-3'	1-24 bp in <i>nfa1</i>	fragment between GFP and <i>nfa1</i>	gDNA
GFP F2	5'-CATGGTCCTGCTGGAGTTCGTG-3'	1166-1287 bp in pEGFP-C2	<i>nfa1</i>	gDNA
<i>nfa1</i> R3	5'-AACTCTTCACGAGCAAATGCCAAACGCTTTAAAGCAC-3'	361-397 bp in <i>nfa1</i> UTR	<i>nfa1</i> UTR	gDNA
VS1	5'-GTTTGGACAAACCACAACCTAGAATGCAGTG-3'	1599-1628 in pEGFP-C2	<i>nfa1</i> UTR <i>nfa1</i>	cDNA

Each primer was used to identify the transcription of the *nfa1* gene or integration of it into chromosome of *N. gruberi*.



ml of 70% ice-cold ethanol once and dried at room temperature. The total RNA was suspended with 10  $\mu$ l of diethylpyrocarbonate (DEPC)-treated distilled water (DW) and stored at -70  $^{\circ}$ C.

For reverse transcription, we used the Superscript First Strand Synthesis System kit (Invitrogen, San Diego, CA) to generate cDNA with 5  $\mu$ g of total RNA from *N. gruberi*. RT reaction was performed according to the manufacturer's recommendation for first-strand synthesis using gene specific primers. PCR fragments were amplified from cDNA using Taq polymerase (Promega, Madison, WI). The program used consists of 35 cycles at 94  $^{\circ}$ C for 1 min, 50  $^{\circ}$ C for 1 min, and 72  $^{\circ}$ C for 1 min 30 s. GFP-F1 and *nfa1* R2 primer were used to amplify the 717 bp fragment between the GFP and *nfa1* gene in pEGFP-C2/*nfa1*UTR vector.

## 7. Western blotting

Lysates of amoeba trophozoites were prepared by freezing-thawing as previously described.<sup>45</sup> The lysates were filtered through 0.22  $\mu$ m filters (Nalgene Europe Ltd., Neerijse, Belgium) to obtain soluble proteins, and the protein concentration (adjusted to 10 mg ml<sup>-1</sup>) was determined by Bradford assay.<sup>46</sup> Lysates containing 80  $\mu$ g of protein were electrophoresed on a 15% SDS-polyacrylamide gel electrophoresis (PAGE) and were blotted on a nitrocellulose membrane. The membrane was blocked

with 3% bovine serum albumin (BSA) overnight and reacted with an anti-Nfa1 polyclonal antibody (1:200 dilution with 3% BSA) which was obtained from mouse immunized with a recombinant Nfa1 protein expressed from the *N. fowleri nfa1* gene. The membrane was washed with PBS containing Tween 20 (PBST) three times for 5 min and reacted with secondary antibody of a goat anti-mouse IgG conjugated with alkaline phosphatase (AP; Sigma Chemical Co., St. Louis, MO) (1:10,000 dilution with 3% BSA), and developed with a solution with 33  $\mu$ l of bromo-chloro-indolyl-phosphate (BCIP; Sigma) and 66  $\mu$ l of nitro blue tetrazolium (NBT; Sigma) in 10 ml of AP buffer.

## **8. Indirect immunofluorescence (IFA) test**

Trophozoites were cultured on 24 well cell culture plate (Nunc A/S, Roskilde, Denmark) overnight. After the culture medium was discarded, the trophozoites were washed with 0.85% saline three times and were done with cold 0.85% saline at third time. 200  $\mu$ l of 10% formalin in 0.85% saline was added and the plate was incubated at room temperature for 30 min. The trophozoites was washed with 0.85% saline three times, added 200  $\mu$ l of 1%  $\text{NH}_4\text{OH}$  to render them permeable, and then incubated at room temperature for 5 min. The following washing steps were same above. After blocking with 3% BSA in PBS, the cells were incubated with anti-Nfa1 polyclonal antibody (1:200 dilution with 3% BSA) at RT overnight. After several washing with PBST, the amoebae were reacted with secondary antibody of an

AffiniPure rabbit anti-mouse IgG conjugated with rhodamine TRITC (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) (1:2,000 dilution with 3% BSA) at room temperature for 2 hr and washed with PBST. The trophozoites were analyzed under a fluorescent microscopy using standard FITC excitation/emission filters (488 nm/507 nm).

### **9. *In vitro* cytotoxicity**

As Chinese Hamster Ovary (CHO) cells are useful in observing *in vitro* cytotoxicity of amoeba.<sup>47</sup> CHO cells were cultured as monolayer in Earle's minimal essential medium (EMEM; Gibco BRL, Gaithersburg, MD) at 37 °C. The details of this experiment using 96 well cell culture plate (Nunc A/S, Roskilde, Denmark) were as follows:  $3 \times 10^4$  CHO cells only,  $3 \times 10^4$  CHO cells cultured with  $3 \times 10^4$  trophozoites of *N. fowleri*,  $3 \times 10^4$  CHO cells cultured with  $3 \times 10^4$  trophozoites of *N. gruberi*, and  $3 \times 10^4$  CHO cells cultured with  $3 \times 10^4$  trophozoites of transfected *N. gruberi*. The total volume per well was 200  $\mu$ l with EMEM. CHO cells and trophozoites were observed using an inverted microscope at cultured intervals.

Lactate dehydrogenase (LDH) release assay was used to measure *in vitro* cytotoxicity because the LDH could be released from lysed cells. For LDH assay, 50  $\mu$ l of reacted supernatant in each well was transferred on 96 well assay plate (Nunc A/S, Roskilde, Denmark). After 50  $\mu$ l of the reconstituted assay buffer in CytoTox96<sup>®</sup> Non-radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) for

LDH assay was added, the plate was incubated 30 min at room temperature and then 50  $\mu$ l of stop solution was added. The reactants were read at 490 nm with ELISA reader. The formula of *in vitro* cytotoxicity was as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Absorbance of experimental group} - \text{Absorbance of control group}}{\text{Absorbance of control group}} \times 100$$

### **10. *In vivo* pathogenicity**

$1 \times 10^4$  trophozoites were inoculated intranasally into 7-week-old female BALB/c mice (purchased from KIST, Daejeon, Korea) in order to induce of *in vivo* pathogenicity, namely, PAME. After 0.05 mg of secobarbital as an anesthetic per mouse body weight in grams was injected intraperitoneally,  $1 \times 10^4$  trophozoites were inoculated into the nasal cavity of the mice with a micropipette. When infected mice died or death was apparent, an autopsy or a biopsy was performed. In the brain, PAME was observed grossly.

### III. RESULTS

#### A. Description of *nfa1*UTR, ubiquitin gene, and the construction of transfection vectors

The *nfa1*UTR gene cloned from a gDNA of *N. fowleri* consists of 614 bp containing 135 bp of 5' untranslated regions (UTR), 360 bp of ORF of an *nfa1* gene, and 119 bp of 3' UTR (Fig. 4) and the ubiquitin promoter from *Acanthamoeba* sp. consists of 1894 bp (Fig. 5). Six vectors were constructed (Fig. 6, 7, 8) and overall the transfection efficiency of *N. gruberi* using these vectors was about 20%-30% measured at 48 hr after transfection by FACS analysis (Fig. 9). *N. gruberi* expressing EGFP was increased maximum about 60% when *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector was selected with 1 mg ml<sup>-1</sup> of G418 for 15 days after transfection (data not shown). When 0.5 M beta-mercaptoethanol was added in the transfection system, the transfection efficiency of *N. gruberi* was improved (data not shown).

#### B. Transfection and expression of EGFP in *N. gruberi*

Trophozoites were transfected using SuperFect<sup>TM</sup> reagent (Qiagen GmbH,

Germany) and subsequently allowed to be assayed during the following 48 hr. Expression of EGFP in *N. gruberi* transfected with six vectors was examined. No

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-135
gatctccgaatagtagcaccaccctttctgaacactataaaaaacgccaaactgtcatgacttctatgagaacaacaaaaca
-56
caacaaaaaaaaacacttgacaacaatcacaacaaccacaaccacaagaacaacaaca(5' UTR)
-1

1
M A T T I P S P F N W D S S F C V G N N
atg gcc act act att cca tca cca ttc aac tgg gac tct tct ttc tgc gtt ggt aac aat

21
E L N E Q H K K L F A L I N A L D A N R
gaa ttg aat gag caa cac aag aag ctc ttt gct ctc atc aat gct ttg gat gcc aac aga

41
S S A S A L K E L L D F V V M H F K A E
tcc agt gct tca gca ttg aag gaa ttg ctt gat ttc gtc gtt atg cat ttc aag gct gag

61
E D L F A K V N F S D S T S H K E T H D
gag gac ttg ttc gca aag gtg aat ttc tct gat tct act tct cac aaa gag act cat gat

81
K F V Q D A L G L K T V G D A E I Q F I
aag ttt gtt caa gat gct ttg ggt ttg aag act gtt gga gat gct gaa att caa ttc atc

101
K Q W L V N H I K G S D M K Y K G V L *
aag caa tgg ttg gtg aat cac att aag gga tct gat atg aag tac aag gga gtg ctt taa

(3' UTR)
agcgtttggcatttgctcgtgaagagtttaataaatatcaagattgtattactgatcaagattattgtagtattattgat
119
caatacaaaaaaagcattgtttgatgattgtgtatgatcgg

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**Fig. 4. The sequences of *nfa1* and *nfa1*UTR.** *nfa1*, Deduced amino acid sequences of *nfa1*, 5' UTR, and 3' UTR are shown. The asterisk is a stop codon. The expected size of Nfa1 protein from *nfa1* gene is about 13.1 kDa.

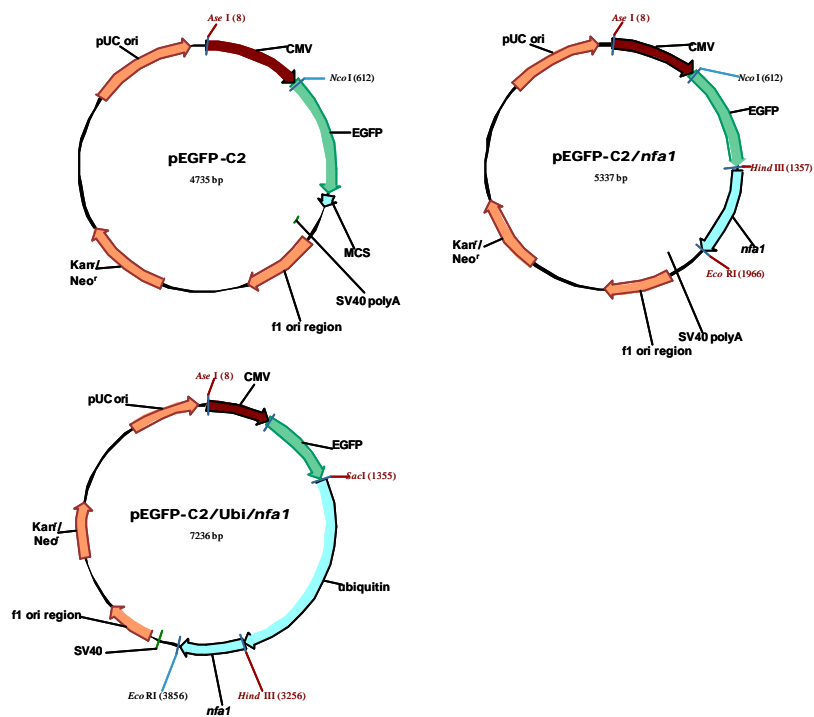
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1894

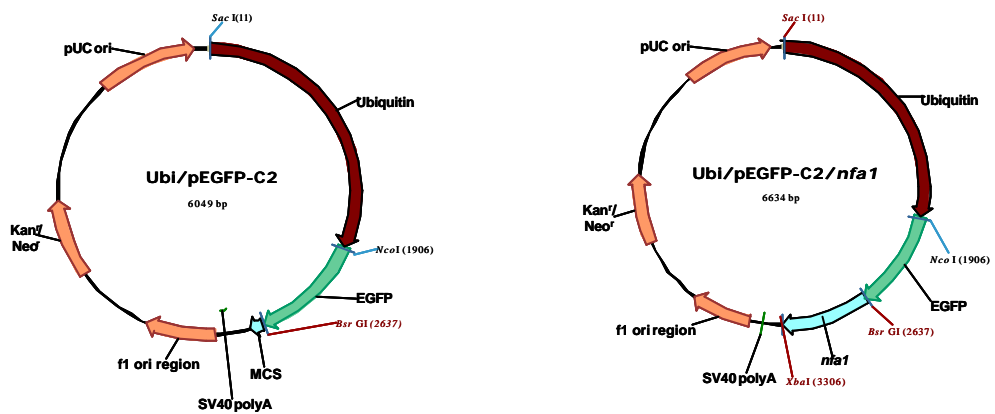
**Fig. 5. Nucleotide sequences of the ubiquitin promoter in *Acanthamoeba* spp..**

Ubiquitin promoter of 1894 bp in pGL3 vector was subcloned into an eukaryotic expression vector for transfection. It was used instead of CMV promoter.

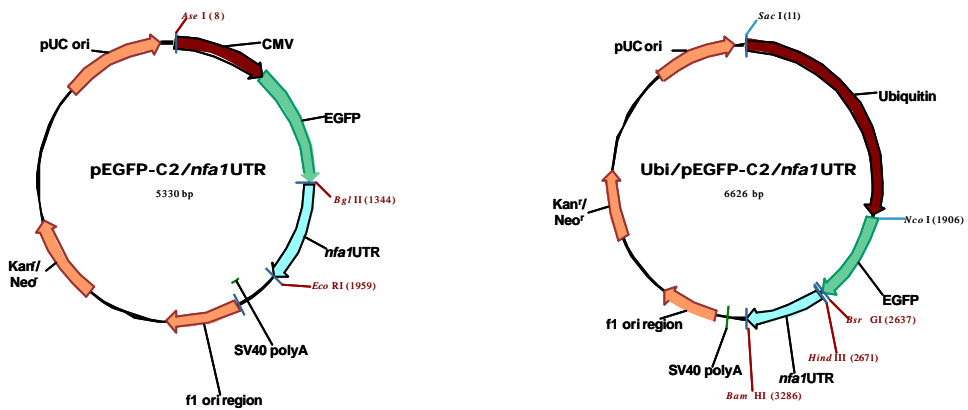


**Fig. 6. Construction of eukaryotic transfection vectors.** pEGFP-C2 vector was used as a backbone for other vectors. In pEGFP-C2/*nfa1* vector, an *nfa1* gene was inserted into MCS. In pEGFP-C2/Ubi/*nfa1* vector, ubiquitin promoter was used instead of CMV promoter.

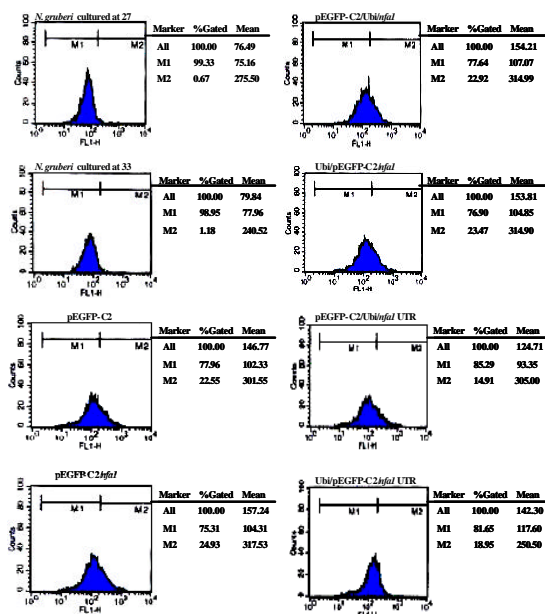




**Fig. 7. Construction of eukaryotic transfection vectors.** In Ubi/pEGFP-C2 vector, CMV promoter was replaced with ubiquitin promoter. In Ubi/pEGFP-C2/*nfa1* vector, ubiquitin promoter was replaced with CMV, and an *nfa1* gene was inserted. The ubiquitin might act as a promoter to transcript the GFP or *nfa1* gene in these vectors.



**Fig. 8. Construction of eukaryotic transfection vectors.** In pEGFP-C2/*nfa1*UTR vector, an *nfa1*UTR gene containing 5' UTR, ORF, and 3' UTR was inserted. 5' UTR might act as a promoter to transcribe the *nfa1* gene and 3' UTR be related with mRNA stability. In Ubi/pEGFP-C2/*nfa1*UTR vector, CMV promoter was replaced with ubiquitin promoter, and an *nfa1*UTR gene was inserted.



Samples	GFP (%)
<i>N. gruberi</i> cultured at 33	1.18
<i>N. gruberi</i> cultured at 27	0.67
<i>N. gruberi</i> transfected with pEGFP-C2	22.55
<i>N. gruberi</i> transfected with pEGFP-C2/Ubi/hfa1	22.92
<i>N. gruberi</i> transfected with pEGFP-C2/Ubi/hfa1	22.92
<i>N. gruberi</i> transfected with Ubi/pEGFP-C2/hfa1	23.47
<i>N. gruberi</i> transfected with pEGFP-C2/hfa1 UTR	14.91
<i>N. gruberi</i> transfected with Ubi/pEGFP-C2/hfa1 UTR	18.15

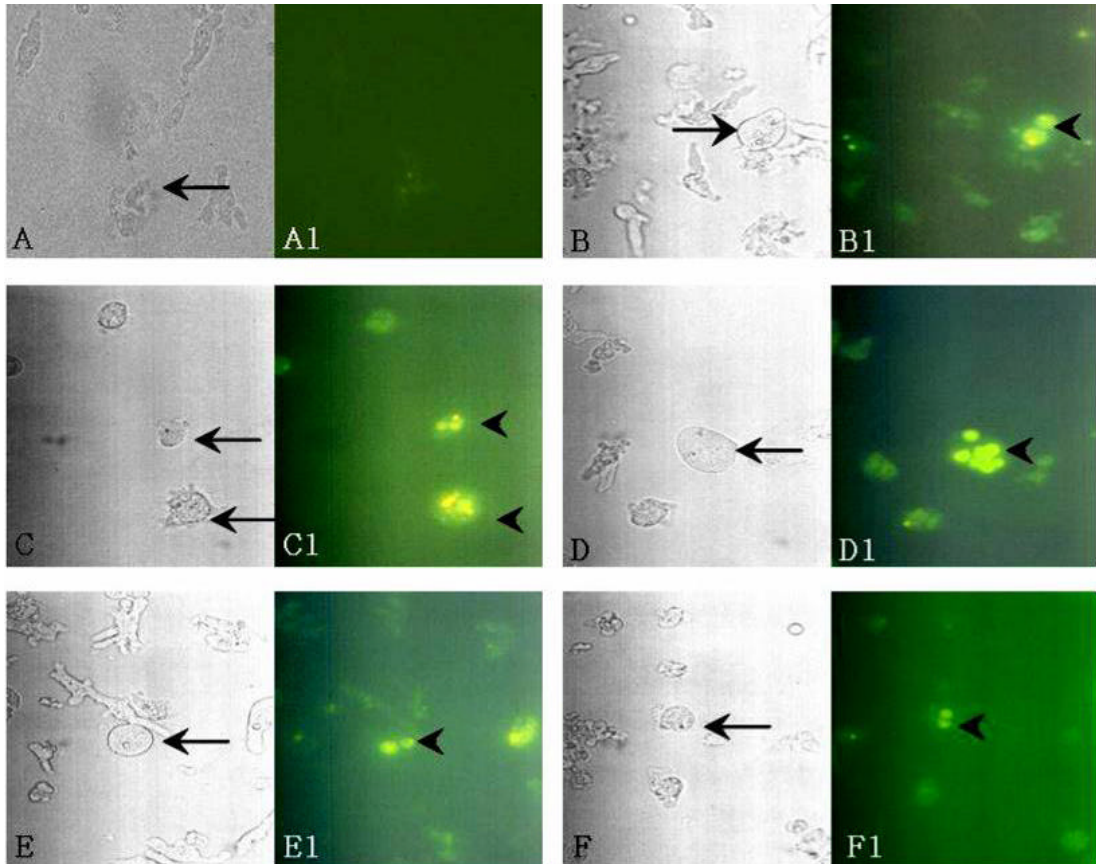
**Fig. 9. Transfection efficiency by FACS analysis.** The histograms (left) showed the transfection efficiency measured at 48 hr after transfection with the mean value by FACS analysis. GFP (%) represented the transfected cells expressing the GFP (right). The transfection efficiency was about 20-30% overall.

fluorescence was observed in trophozoites of *N. gruberi* used as a control group (Fig. 10). At 24 hr post transfection, GFP expression was observed in the cytoplasm of trophozoites of *N. gruberi* transfected with the six different EGFP expressing vectors, respectively but only *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector has been survived over 9 months since G418 antibiotics was treated 48 hr after transfection. Nfa1 protein expressed from *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector could be identified using western blotting 48 hr after transfection (Fig. 11).

### **C. *nfa1* gene in free-living amoebae**

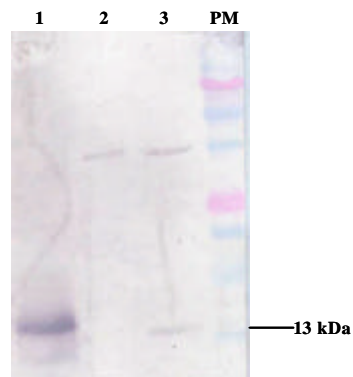
To identify whether *Naegleria* spp. have the *nfa1* gene, PCR was done with gDNA using two *nfa1*-specific primers (*nfa1* F2 and *nfa1* R2). It was shown that the *nfa1* gene of 360 bp exist in nonpathogenic *N. gruberi* and *N. lovaniensis* as well as pathogenic *N. fowleri* (Fig. 12). However, it was previously reported that the expression of Nfa1 protein was not detected in nonpathogenic *N. gruberi* by immunoblotting.<sup>34</sup> In addition, to investigate the transcription of *nfa1* gene in *Acanthamoeba* spp., RT was done using the *nfa1* gene-specific primer (*nfa1* R2), and then PCR was done using two *nfa1*-specific primers (*nfa1* F2 and *nfa1* R2) (Fig. 13). Except for nonpathogenic *A. royreba*, *A. culbertsoni*, *A. healyi*, *A. hatchetti*,

*Acanthamoeba* sp. YM4, *A. castellanii*, and *A. polyphaga* have the cDNA of *nfa1* gene.

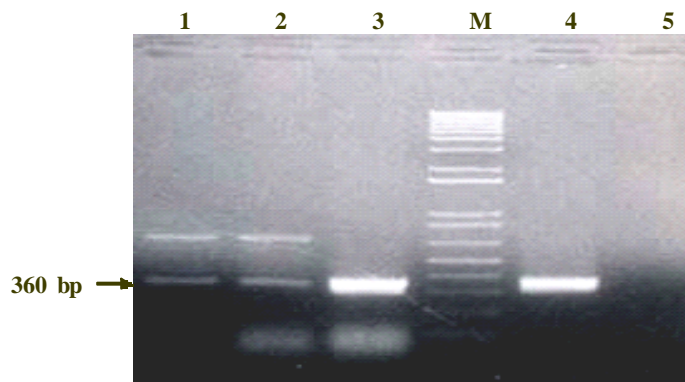


**Fig. 10. Photographs of transfected *N. gruberi* trophozoites.** The fluorescence of EGFP was observed 48 hr after transfection. A and A1; untransfected *N. gruberi*. B and B1; pEGFP-C2-transfected *N. gruberi*. C and C1; pEGFP-C2/*nfa1*-transfected *N. gruberi*. D and D1; pEGFP-C2/Ubi/*nfa1*-transfected *N. gruberi*. E and E1; Ubi/pEGFP-C2/*nfa1*-transfected *N. gruberi*. F and F1; pEGFP-C2/*nfa1*UTR-transfected *N. gruberi*. Arrows in panel A, B, C, D, E, and F indicate *N. gruberi*

observed under light microscopy. Arrow heads in panel A1, B1, C1, D1, E1, and F1 indicate *N. gruberi* observed under fluorescent microscope.  $\times 400$ .



**Fig. 11. Western blotting for identifying the Nfa1 protein.** Transient transfection of *N. gruberi* with pEGFP-C2/*nfa1*UTR vector was performed. Whole cell lysates were prepared and analyzed by western blotting. Lane 1; *N. fowleri* NF69. Lane 2; *N. gruberi*. Lane 3; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector.



**Fig. 12. Identification of an *nfa1* gene from gDNA of *Naegleira* spp. by PCR.**

PCR was performed with *nfa1*-specific primers (*nfa1* F2 and *nfa1* R2). Lane 1; *N. gruberi* cultured at 27 °C. Lane 2; *N. gruberi* cultured at 33 °C. Lane 3; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. M; 1kb+ DNA ladder. Lane 4; *N. fowleri* NF69. Lane 5; negative control (DW).



**Fig. 13. Identification of cDNA of an *nfa1* gene in *Naegleria* spp. and *Acanthamoeba* spp. by RT-PCR.** RT reaction was performed with the gene-specific primer (*nfa1* R2) and PCR was done with the *nfa1* F2 and *nfa1* R2 primer. Lane 1; *N. gruberi* cultured at 33 °C. Lane 2; *A. culbertsoni*. Lane 3; *A. healyi*. Lane 4; *A. hatchetti*. Lane 5; *Acanthamoeba* sp. YM4. Lane 6; *A. castellanii*. Lane 7; *A. royreba*. Lane 8; *A. polyphaga*. Lane 9; *nfa1* gene of *N. fowleri* NF69.



#### **D. Integration of the pEGFP-C2/*nfa1*UTR vector into chromosomal DNA of *N. gruberi***

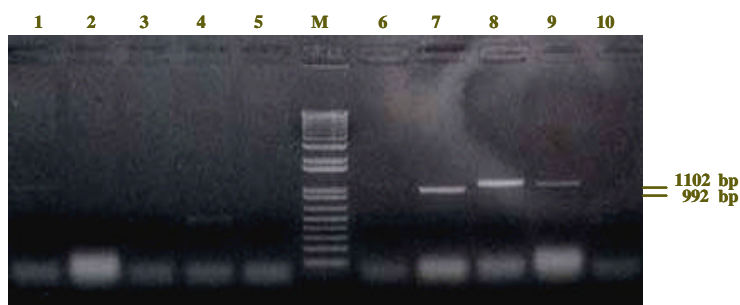
After the treatment of G418, the integration of vector into chromosomal DNA was examined. The possible integration of pEGFP-C2/*nfa1*UTR vector into chromosomal DNA of *N. gruberi* was examined by PCR from a gDNA of transgenic *N. gruberi* as illustrated in Fig. 14, Fig. 15, and Fig. 16. The DNA fragments amplified by PCR using the vector-specific primers (GFP F1 and VS1) were shown in lane 7, 8, and 9 (Fig. 14).

The fragment of 992 bp, which contained 3' regions of GFP, *nfa1* ORF, and 3' regions to MCS in Ubi/pEGFP-C2/*nfa1* vector, was amplified in *N. gruberi* transfected with Ubi/pEGFP-C2/*nfa1* vector (lane 7 of Fig. 14). No fragment (lane 7 of Fig. 15) was amplified using GFP F2 primer and *nfa1* R3 primer in Ubi/pEGFP-C2/*nfa1* vector compared with lane 7 of Fig. 14. The reason why it did was unclear, but no fragment was observed although the other primers were used and the conditions of PCR were changed.

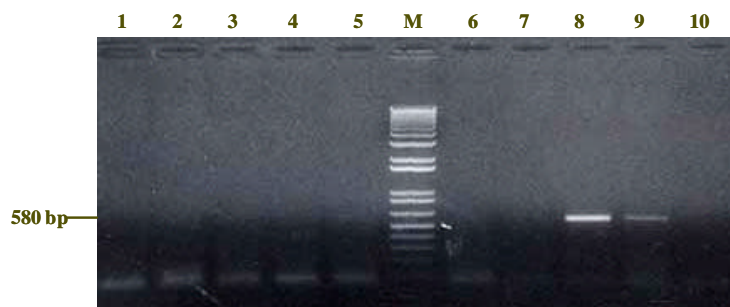
The amplified fragment of 1102 bp (lane 8 of Fig. 14), which contained 3' regions of GFP, *nfa1*UTR, and 3' regions to MCS in pEGFP-C2/*nfa1*UTRvector, was exact in size. On using other vector-specific primer (GFP-F2) and an *nfa1*-specific primer (*nfa1* R3), 580 bp fragment of expected size was amplified (lane 8 of

Fig. 15).

The fragment of about 1102 bp (lane 9 of Fig. 14), which contained 3' regions of



**Fig. 14. Identification of transfection from gDNA by PCR.** PCR was performed with the vector-specific primers (GFP F1 and VS1). Lane 1; *N. gruberi* cultured at 27 °C. Lane 2; *N. gruberi* cultured at 33 °C. Lane 3; *N. fowleri*. Lane 4; *N. gruberi* transfected with pEGFP-C2 vector. Lane 5; *N. gruberi* transfected with pEGFP-C2/*nfa1* vector. Lane 6; *N. gruberi* transfected with pEGFP-C2/Ubi/*nfa1* vector. Lane 7; *N. gruberi* transfected with Ubi/pEGFP-C2/*nfa1* vector. Lane 8; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. Lane 9; *N. gruberi* transfected with Ubi/pEGFP-C2/*nfa1*UTR vector. Lane 10; negative control (DW).



**Fig. 15. Identification of transfection from gDNA by PCR.** PCR was performed with the vector-specific (GFP F2) and *nfa1*-specific primer (*nfa1* R3). Lane 1; *N. gruberi* cultured at 27 °C. Lane 2; *N. gruberi* cultured at 33 °C. Lane 3; *N. fowleri*. Lane 4; *N. gruberi* transfected with pEGFP-C2 vector. Lane 5; *N. gruberi* transfected with pEGFP-C2/*nfa1* vector. Lane 6; *N. gruberi* transfected with pEGFP-C2/Ubi/*nfa1* vector. Lane 7; *N. gruberi* transfected with Ubi/pEGFP-C2/*nfa1* vector. Lane 8; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. Lane 9; *N. gruberi* transfected with Ubi/pEGFP-C2/*nfa1*UTR vector. Lane 10; negative control (DW).

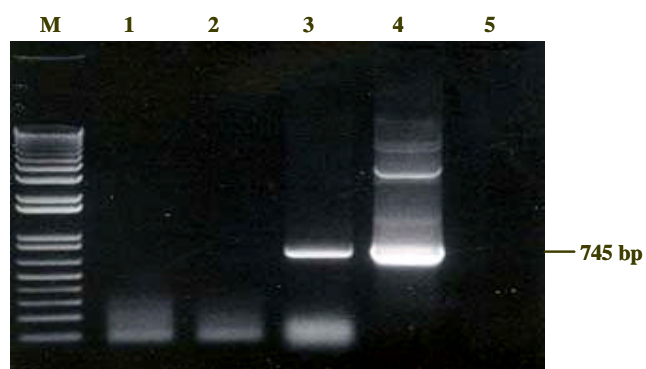
GFP, *nfa1*UTR, and 3' regions to MCS in Ubi/pEGFP-C2/*nfa1*UTR vector, was different from the expected size of 1302 bp in *N. gruberi* transfected with Ubi/pEGFP-C2/*nfa1*UTR vector. It seemed that about 200 bp fragment was deleted in the part between GFP and *nfa1*UTR gene. The lane 9 of Fig. 15 was shown as similar with the lane 9 of Fig. 14. The 580 bp fragment (lane 9 of Fig. 15) with the deletion of about 200 bp was amplified. Therefore, we could not measure the ability of the ubiquitin and CMV promoter at the front of GFP and *nfa1* gene because it was integrated with deletion between GFP and *nfa1* gene.

As shown in Fig. 16, vector-specific primer (GFP F1) and *nfa1*UTR-specific primer (*nfa1* R3) were used for PCR of gDNA. The size was expected to be 745 bp, which contained 3' regions of GFP, *nfa1*UTR, and 3' regions to MCS in pEGFP-C2/*nfa1*UTR vector, was amplified. Therefore, pEGFP-C2/*nfa1*UTR vector was integrated into chromosomal DNA of *N. gruberi* without deletion.

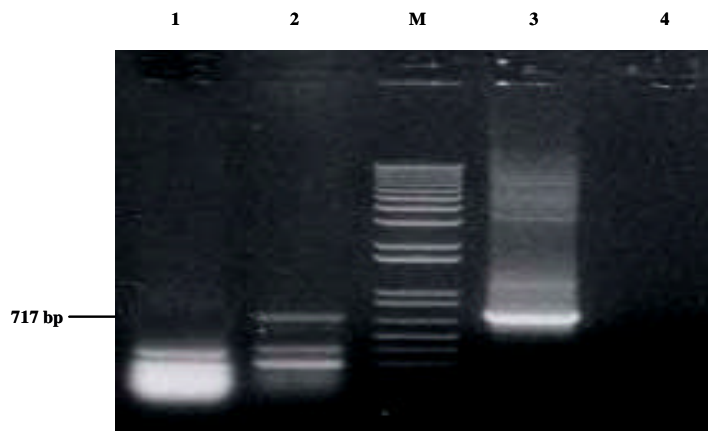
### **E. Transcription and expression of the *nfa1* gene in *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector**

*N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector was chosen for RT-PCR to observe the transcription of *nfa1*UTR gene containing 5' UTR, ORF, and 3' UTR. RT was performed using vector-specific primer (VS1), and PCR was done using a

vector-specific primer (GFP F1) and an *nfa1*UTR-specific primer (*nfa1* R2) (Fig. 17). The fragment of predicted 717 bp was transcribed in *N. gruberi* transfected with



**Fig. 16. Identification of transfection from gDNA by PCR.** PCR was performed with the vector-specific (GFP F1) and *nfa1*-specific primer (*nfa1* R3). Lane 1; *N. gruberi* cultured at 27 °C. Lane 2; *N. gruberi* cultured at 33 °C. Lane 3; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. Lane 4; positive control (plasmid DNA from *E. coli* DH5 $\alpha$  transformed with pEGFP-C2/*nfa1*UTR vector). Lane 5; negative control (DW).



**Fig. 17. RT-PCR of cDNA from *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector.** RT reaction was performed with a vector-specific primer (VS1) and PCR was done with the GFP F1 and *nfa1* R2 primer. Lane 1; 33 *N. gruberi*. Lane 2; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. M; 1kb+ DNA ladder. Lane 3; positive control (plasmid DNA from *E. coli* DH5 $\alpha$  transformed with pEGFP-C2/*nfa1*UTR vector). Lane 4; negative control (DW).

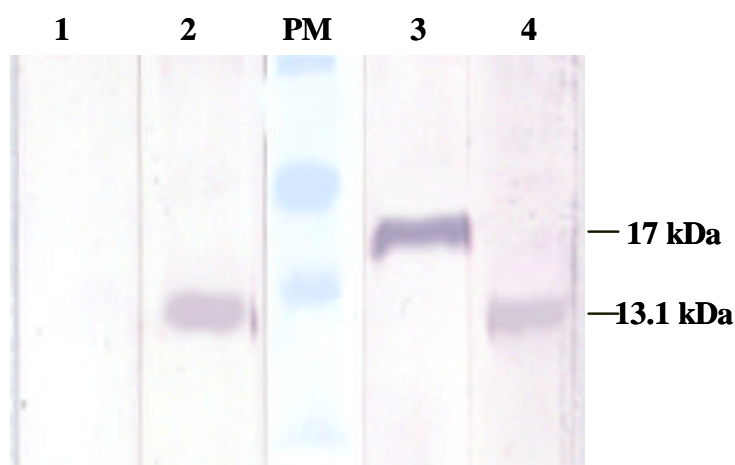
pEGFP-C2/*nfa1*UTR vector (Fig. 17).

To investigate the expression of Nfa1 protein in transgenic *N. gruberi*, the amoeba lysate was immunoblotted with an anti-Nfa1 polyclonal antibody (Fig. 18). As the control groups, the lysate *N. fowleri* (13.1 kDa of Nfa1 protein) and a recombinant Nfa1 protein (with His-tag of about 17 kDa) were used (lane 2, 3 in Fig. 18). *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector could express a 13.1 kDa of Nfa1 protein identified in lane 4. Therefore, *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector could transcribe the *nfa1* gene and express the Nfa1 protein.

## **F. Cellular localization of Nfa1 protein**

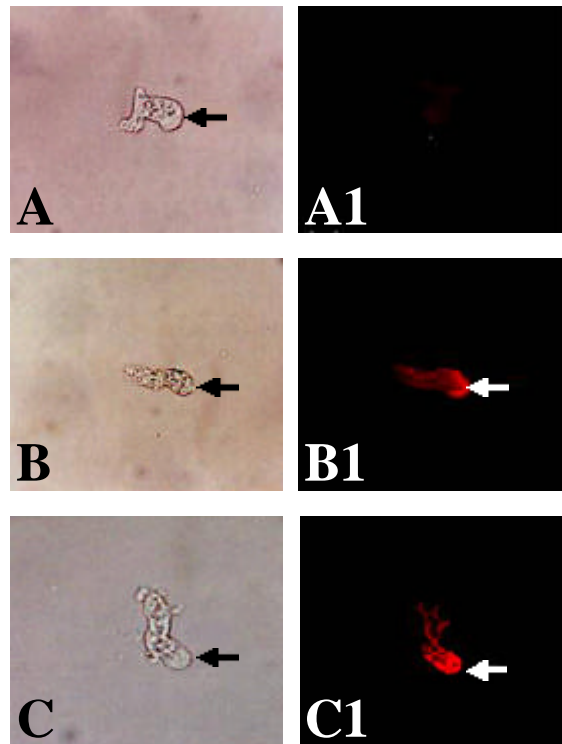
Previously, polyclonal antibodies against the Nfa1 protein were raised in mouse and tested for their specificity by immunoblotting with Nfa1 protein. As judged by indirect immunofluorescence, Nfa1 protein had a distinctly focal distribution in the pseudopodia of the cells (Fig. 19). The IFA test was performed 9 months after the transfection. The Nfa1 protein was localized in the pseudopodia of the *N. gruberi* transfected pEGFP-C2/*nfa1*UTR vector. The intensity of fluorescence was observed more strongly in *N. fowleri* than *N. gruberi*.

**G. *In vitro* cytotoxicity of transgenic *N. gruberi* against CHO cells**



**Fig. 18. Identification of Nfa1 protein from lysate by Western blotting (after 15% SDS-PAGE) with anti-Nfa1 polyclonal antibody.** The lysate of *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector was prepared three months after the treatment of G418. Lane 1; 33 *N. gruberi*. Lane 2; *N. fowleri*. PM; Prestained Marker. Lane 3; purified fusion protein of Nfa1. Lane 4; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector.





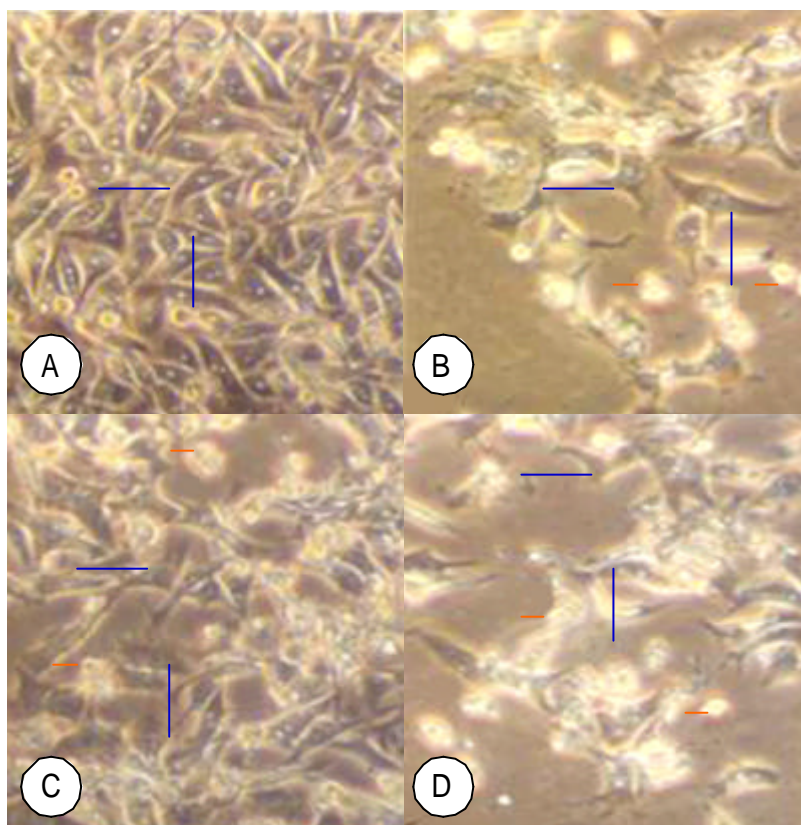
**Fig. 19. Localization of Nfa1 protein in *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector.** *nfa1*-transfected *N. gruberi* has been maintained under G418 for 9 months. Arrows represent pseudopodia in *N. gruberi*. Black arrows (panel A, B, C) are presented under bright microscopy and white arrows (panel A1, B1, C1) are done under fluorescent microscope. A and A1; normal *N. gruberi*. B and B1; *N. fowleri* NF69. C and C1; *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector.

CHO cells cultured with *N. fowleri* trophozoites for 24 hr showed severe destruction (Fig. 20). On the contrary, CHO cells cultured with *N. gruberi* or transgenic *N. gruberi* trophozoites showed less destruction than above experimental group (Fig. 20). However, transgenic *N. gruberi* trophozoites destructed CHO cells more than normal *N. gruberi*. As the results of LDH release assay, red color representing the extent of *in vitro* cytotoxicity was the strongest in wells of CHO cells cultured with *N. fowleri* trophozoites and orderly CHO cells cultured with *N. gruberi* and transgenic *N. gruberi* trophozoites (data not shown). The *in vitro* cytotoxicity of *N. fowleri* was the highest with about 97% in all the experimental groups. The *in vitro* cytotoxicity of *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector was higher with about 21% than that of normal *N. gruberi* with about 9% (Table 4). Therefore, these facts suggested that *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector expressing the Nfa1 protein be a little enough to induce *in vitro* cytotoxic effect. Therefore, an *nfa1* gene could be related with *in vitro* cytotoxicity.

#### **H. Mortality of mice infected with transgenic *N. gruberi***

Ten BALB/c mice infected with *N. fowleri* died after 8–9 days but BALB/c mice infected with untransfected *N. gruberi* or *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector survived over one month (Table 5). Therefore, *N. gruberi*

expressing the Nfa1 protein was not enough to induce the PAME in BALB/c mice.



**Fig. 20. Light microscopic findings showing *in vitro* cytotoxicity.** CHO cells (blue arrows) cultured only in EMEM media (A), cocultured with *N. fowleri* trophozoites (red arrow heads) (B), cocultured with *N. gruberi* trophozoites (red arrow heads) (C), and cocultured with *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector (red arrow heads) for 24 hr (D).  $\times 200$ .

**Table 4. Measurement of *in vitro* cytotoxicity of *N. fowleri*, *N. gruberi*, and transgenic *N. gruberi* against CHO cells by LDH release assay**

Groups	LDH release* (Absorbance)	Cytotoxicity (%)
CHO**	0.485 ± 0.022	—
CHO + <i>N. fowleri</i> trophozoites***	0.956 ± 0.019	97.35 ± 5.05
CHO + <i>N. gruberi</i> trophozoites***	0.531 ± 0.022	9.5 ± 0.4
CHO + Transgenic <i>N. gruberi</i> trophozoites***	0.590 ± 0.009	21.8 ± 3.7****

\* LDH release was measured at 490 nm.

\*\* Each 3 × 10<sup>4</sup> cells

\*\*\* Each 3 × 10<sup>4</sup> trophozoites

\*\*\*\* t-test, P<0.05

Experiments were performed in triplicate and the data was shown with mean ± standard variation value. Transgenic *N. gruberi* is *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. The formula of *in vitro* cytotoxicity was as follows: (%) = (Absorbance of experimental group - Absorbance of control group) × 100 / Absorbance of control group.

**Table 5. Mortality of mice\* infected with *N. fowleri*, *N. gruberi*, and transgenic *N. gruberi*, respectively**

Groups	Days of death post inoculation	Mortality
<i>N. fowleri</i> trophozoites**	8 – 9 days***	100%
<i>N. gruberi</i> trophozoites**	All alive	0%
Transgenic <i>N. gruberi</i> trophozoites**	All alive	0%

\* N = 10

\*\* Each  $1 \times 10^4$  trophozoites

\*\*\* PAME was observed with autopsy

BALB/c mice were infected intranasally with each amoeba after anesthesia.

Transgenic *N. gruberi* is *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector.

## IV. DISCUSSION

PAME caused by *N. fowleri* is an acute, fulminant, and rapidly progressing fatal illness that usually affects children and young adults. The olfactory neuroepithelium is the route of invasion in PAME due to *N. fowleri*. Invasions of the olfactory mucosa and the olfactory bulbs, with hemorrhagic necrosis of both cerebral gray and white matters and an acute inflammatory infiltrate, are the histopathologic characteristics.<sup>4</sup> These organisms have been long recognized as attractive models for a variety of studies in basic cellular and molecular biology. They have a relatively large size, rapid growth in axenic culture, active motility and phagocytosis, and they exhibit unicellular differentiation. Despite the attractiveness of *Naegleria*, it has been underutilized as a model system. So far there has been no evidence for sexual reproduction. Therefore, classical mapping and genetic analysis is limited. On the mechanism of pathogenicity of *N. fowleri* concerned with host-tissue invasion, the adherence of the amoeba to host cells is most important, and the specific pseudopodial projection, so called as amoebastome, is formed.<sup>3</sup> Other cytotoxic toxins and cytolytic proteinases have been proposed that amoeba destroy target cells.<sup>3,4,46</sup> Additionally, Horstmann et al. reported that at least three functions of the amoebae could be considered essential for pathogenic tissue invasion: (i) adherence of the *Entamoebae* to host cells, predominantly mediated by a galactose- and N-

acetylgalactosamine-inhibitable lectin, (ii) killing of host cells by a pore-forming peptide known as amoebapore, and (iii) proteolysis of the host's extracellular matrix mediated by cysteine proteinases.<sup>45</sup> At the molecular level, a 17 kDa of unique membrane protein was expressed from *Mp2CL5* cloned from a cDNA expression library of *N. fowleri*, the surface membrane cytolysin was found to be specifically activated by diluted samples of lysosomal fractions, and the possible role of this surface membrane cytotoxin in the pathogenicity of *N. fowleri* was discussed. We previously reported that the Nfa1 protein expressed from *nfa1* gene was located in pseudopodia and around vacuoles. It powerfully supported that the *nfa1* gene might be related with the mechanism of pathogenicity of *N. fowleri*.<sup>34</sup> The amino acid sequences of *nfa1* gene were closely related to that of hemerythrin or myohemerythrin gene of some marine invertebrates.<sup>34,48</sup> Although many molecular and genetic studies were carried out to elucidate the mechanism of pathogenicity of amoebae, the various molecules could only be studied by *in vitro* analysis and their functional importance could not be addressed *in vivo*.<sup>45,46</sup> To overcome such problems and understand the mechanism of pathogenicity, a sufficient stable transfection system preferentially is required. Transient and stable transfection system in free-living amoeba, *Acanthamoeba* spp., were previously described.<sup>25,37</sup> *Entamoeba histolytica* has been successfully transfected using electroporation.<sup>49</sup> In addition to these, recent developments of transgenic technologies in several pathogenic protozoa<sup>50-55</sup> have extended to study the biology of these primitive organisms at the molecular and cellular levels. Transfection using pantropic

retroviral vectors was reported to study genetic analysis in species lacking transformation systems.<sup>56</sup> Although it has been reported in other protozoa, unfortunately it has not been yet reported in *Naegleria* spp. The application of *Naegleria* for molecular studies of eukaryotic genes has been limited because of those disadvantages above.

In the present study, we showed that *in vivo* transfection system could be established to understand *in vitro* cytotoxicity and *in vivo* pathogenicity of *N. fowleri*. On the basis of developing the transfection system to elucidate the function of newly cloned genes, we transfected the *nfa1* gene cloned from pathogenic *N. fowleri* into nonpathogenic *N. gruberi*. Although nonpathogenic *Naegleria* spp. and other pathogenic *Acanthamoeba* spp. except nonpathogenic *A. royreba* had the *nfa1* gene (Fig. 13), because nonpathogenic *N. gruberi* belong to same genus with pathogenic *N. fowleri* did not express the Nfa1 protein,<sup>34</sup> it was used as a target organism.

The strong intensity of GFP fluorescence was observed in *N. gruberi* transfected with Ubi/pEGFP-C2/*nfa1* vector or Ubi/pEGFP-C2/*nfa1*UTR vector (data not shown). However, Ubi/pEGFP-C2/*nfa1* vector was not only integrated into chromosome of *N. gruberi* but also Ubi/pEGFP-C2/*nfa1*UTR vector was integrated with about 200 bp at the front of GFP and *nfa1* gene. In pEGFP-C2/*nfa1*UTR vector, an *nfa1* gene should be transcribed and translated under its 5' UTR. This 5' UTR could putatively influence the mRNA secondary structure and stability, efficacy of translation initiation, or binding of sequence-specific mRNA-binding proteins.<sup>57</sup> Inclusion of the 3' UTR sequences, which have a role in stabilizing transcripts, has



been reported to participate in gene expression.<sup>58,59</sup> We also used an ubiquitin promoter instead CMV promoter. Ubiquitin is a ubiquitous protein present in all eukaryotic cells.<sup>60</sup> It is involved in several basic cellular functions: marking proteins for rapid degradation, mediation of gene transcription, DNA repair, cell cycle progression, stress responses and the modulation of the immune response.<sup>60</sup> In all these processes ubiquitin is tagged to specific cellular target proteins through the action of an ATP-dependent conjugation system.<sup>60,61</sup> Ubiquitin is encoded by a gene family consisting of single ubiquitin genes and polyubiquitin genes. Single ubiquitin genes code for fusions of an amino-terminal ubiquitin to ribosomal proteins of the small or large subunit. Polyubiquitin genes code for polyubiquitin precursors that consist of a tandem repeat of ubiquitin units followed by a single to several additional amino acids.<sup>62,63</sup> Intraspecies variation is also observed in several organisms.<sup>64</sup> The ubiquitin gene was shown to be regulated during *Acanthamoeba* development from the actively growing stage towards the dormant cyst stage. Ubiquitin transcripts decrease to negligible levels as amoebic growth declines.<sup>65</sup> Polyubiquitin promoters from plant and human sources are constitutively efficient.<sup>66-</sup><sup>68</sup> On the basis of a paper of Hu et al<sup>25</sup> and Yin et al<sup>37</sup>, in Ubi/pEGFP-C2/*nfa1* and Ubi/pEGFP-C2/*nfa1*UTR vector used in this study, a CMV promoter was replaced with an ubiquitin promoter derived from *Acanthamoeba* sp. (donated from Prof. Kong at Kyung-pook National University) in order to transcribe the *nfa1* gene in transgenic *N. gruberi*, because the CMV promoter could not transcribe the *nfa1* gene. The ubiquitin promoter of *Acanthamoeba* might also function in *N. gruberi* but

unfortunately Ubi/pEGFP-C2/*nfa1*UTR vector was integrated with deletion of 200 bp between GFP and *nfa1*UTR. Furthermore, pEGFP-C2/Ubi/*nfa1* vector cloned for the purpose of acting at the *nfa1* gene wasn't integrated into amoebic genome, and no fragment was amplified using the vector- and *nfa1*UTR-specific primer in *N. gruberi* transfected with pEGFP-C2/Ubi/*nfa1* vector. The reason may be the problem of conditions of PCR or primer binding sites. Thus, we couldn't measure the efficiency of the ubiquitin promoter from *Acanthamoeba* to transcribe the *nfa1* gene in transgenic *N. gruberi*.

As the results of western blotting which was performed to identify an expressed Nfa1 protein from transgenic *N. gruberi*, the 13.1 kDa of protein band was observed and same with that of pathogenic *N. fowleri*. Thus, this study showed that the Nfa1 protein was not expressed from nonpathogenic *N. gruberi*, but well from transgenic *N. gruberi*.

IFA test has been performed for 6 months. The fluorescence was been observed in the pseudopodia of *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector using anti-Nfa1 polyclonal antibody. The intensity of immunofluorescence was higher in *N. fowleri* than *N. gruberi*.

Finally, we focused at the *in vitro* cytotoxicity and *in vivo* pathogenicity of *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. The CHO cells cultured with *N. fowleri* were destructed mostly with about 97.35%. On the other hand, *in vitro* cytotoxicity of *N. gruberi* or *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector relatively low with 9.5% and 21.8 %, respectively. However, *in vitro* cytotoxicity of

*N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector was higher with the difference of about 12.3% than untransfected *N. gruberi*. The fact that transgenic *N. gruberi* has more cytotoxic effect than control corresponds with that Nfa1 protein can induce the *in vitro* cytotoxic effect. Unfortunately, Nfa1 protein seemed not to be enough for *in vivo* pathogenicity using BALB/c mice. The mice infected with *N. fowleri* died fastly but those infected with transfected *N. gruberi* and normal *N. gruberi* survived for a long time. In *in vitro* cytotoxicity and *in vivo* pathogenicity, I used the same amount of  $3 \times 10^4$  trophozoites of transgenic *N. gruberi* with that of *N. fowleri*. If the fact that the expression of Nfa1 protein was higher in *N. fowleri* than transgenic *N. gruberi* was considered, the *in vitro* cytotoxicity of transgenic *N. gruberi* might be lower than *N. fowleri* and transgenic *N. gruberi* might not induce *in vivo* pathogenicity like *N. fowleri*. At the same time, these data strongly suggests that additional factor(s) other than Nfa1 protein is (are) required for the presentation of *in vivo* pathogenicity. At present, it has been reported that additional factors be proteases, lectin binding proteins, other enzymes, etc cited at introduction above.

## V. CONCLUSION

I have experimented whether the *nfa1* gene could be related with *in vitro* cytotoxicity or *in vivo* pathogenicity. Thus, I aimed at transfecting an *nfa1* gene cloned from pathogenic *N. fowleri* into nonpathogenic *N. gruberi*. In this study, I could get many significant results acquired through the development of *in vivo* transfection system. Firstly, the pEGFP-C2/*nfa1*UTR vector could be transfected transiently and stably in nonpathogenic *N. gruberi* using SuperFect<sup>TM</sup> reagent. Secondly, the *neo* gene could be used as a selectable marker for this transfection system. Thirdly, GFP under CMV promoter could be expressed in this system. Fourthly, although it was obscure that 5' UTR of an *nfa1*UTR gene acts as a promoter, the *nfa1* gene could be expressed under the presence of 5' UTR. Fifthly, the Nfa1 protein expressed from the *nfa1* gene could be obtained from *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector. Finally, *N. gruberi* transfected with pEGFP-C2/*nfa1*UTR vector could induce *in vitro* cytotoxicity against CHO cells but not *in vivo* pathogenicity in BALB/c mice.

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(*Naegleria fowleri*)

nfa1 in vivo transfection

( : )

*Naegleria fowleri* nfa1 360

, Nfa1 (13.1 kDa)

*N. gruberi* nfa1 가 , Nfa1

western blotting . A.

royreba *Acanthamoebae* nfa1 가

nfa1 가

가

, nfa1 *N. gruberi*

transfection nfa1 가

. Transfection CMV promoter GFP

transfection pEGFP-C2 pEGFP-C2/nfa1,

pEGFP-C2/nfa1UTR (nfa1UTR; 5' UTR, nfa1 ORF, 3' UTR ),  
 ubiquitin promoter가 가 . SuperFect™  
 transfection , transfection N. gruberi GFP  
 G418  
 selection 9 . Transfection nfa1  
 가 pEGFP-C2/nfa1UTR 가 N. gruberi genomic  
 nfa1 primers  
 . 가 N. gruberi nfa1  
 GFP .  
 Nfa1 western blotting  
 13.1 kDa . , pEGFP-C2/nfa1UTR 가  
 N. gruberi CHO  
 BALB/c .  
 , N. gruberi transfection  
 . , N. gruberi stable tranfection  
 nfa1 Nfa1 N.  
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: Naegleria fowleri, Naegleria gruberi, nfa1 , in vivo  
 transfection,