Korean J Biol Sci 5: 65-69, 2001

Isolation of Deletion Mutants by Reverse Genetics in *Caenorhabditis elegans*

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Key Words:
Caenorhabditis elegans
Reverse genetics
Chemical mutagenesis
Deletion mutants
Mutant screening

Obtaining mutant animals is important for studying the function of a particular gene. A chemical mutagenesis was first carried out to generate mutations in *C. elegans*. In this study, we used ultraviolet-activated 4,5',8-trimethylpsoralen to induce small deletion mutations. A library of mutagenized worms was prepared for recovery of candidate animals and stored at 15℃ during screening instead of being made into a frozen stock library. In order to isolate deletion mutations in target genes, a polymerase chain reaction (PCR)-based screening method was used. As a result, two independent mutants with deletions of approximately 1.0 kb and 1.3 kb were isolated. This modified and improved reverse genetic approach was proven to be effective and practical for isolating mutant animals to study gene function at the organismal level.

The nematode *Caenorhabditis elegans* has been one of the most widely used model systems in genetic studies for decades. With the recent completion of the *C. elegans* genome project (The *C. elegans* Sequecing Consortium, 1998), a new era of genetic analysis has been extended from the already successful generation of genetic studies in *C. elegans*. Along with its small size and ease of handling, it has a conveniently short life cycle producing a large number of progeny and makes an excellent organism for mutant screening and other genetic studies.

Obtaining mutant animals is crucial for more specific genetic studies and functional analysis of a gene. Two strategies have been employed in genetic studies: forward and reverse genetics. Forward genetic screens involving mapping of mutant animals with known genetic markers have identified approximately 2,000 of the 19,000 genes existing in *C. elegans* (Barstead, 1999), but with the advent of the genome sequence, reverse genetic studies have been and will be more useful for identification and characterization of novel genes.

In reverse genetics, a mutation can be easily pinpointed to a single gene based on its sequence known prior to the mutagenesis. This is advantageous compared to the laborious classical mutant screening involving extensive characterization and mapping mutations. This reverse genetic technique implements a simple polymerase chain reaction that can identify deletion mutations in the target gene. Though convenient, its drawback is that it can detect only deletion mutations as opposed to classical screening techniques that can identify any kind of mutation.

Mutagenesis has been commonly used to obtain mutant animals. In *C. elegans* the rate of naturally occuring mutations is about 10^{-6} per gene per generation (Anderson, 1995), which is not a practical way of obtaining mutations. One method shown to be effective in obtaining mutant animals is to use endogenous transposable elements. Zwaal et al.(1993) showed that deletion mutants could be obtained by successive screening of animals with a transposable element and then by screening those animals for a deleted transposable element and flanking sequences. Though effective, chemical mutagenesis seems to be a more practical method. The most well known chemical mutagen used in experiments is ethyl methanesulfonate (EMS). It has shown to generate the highest number of mutant animals among commonly used mutagens (Rosenbluth et al., 1983). Another chemical mutagen, 4,5',8-trimethylpsoralen (TMP), has been shown to be very effective in production of deletion mutants (Yandell et al., 1994).

In this paper, we describe a modified technique used to screen for deletion mutants of a specific gene. Since we were specifically screening for deletion mutants, we employed UV-TMP mutagenesis to obtain mutant animals. In addition, maintaining the mutant library at 15℃ instead of storing a frozen library improved the recovery of candidate animals. After the mutagenesis and screening, we have identified two mutants for two different important calcium-binding proteins, calreticulin and calcineurin B.

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Materials and Methods

C. elegans *strains and maintenance*

The wild type Bristol N2 strain was obtained from the *C. elegans* Genetic Center at the University of Minnesota, U.S.A. Animals were originally cultured on 15 cm diameter plates seeded with OP50 strain *Escherichia coli* at 20℃ as previously described (Brenner, 1974; Sulston and Hodgkin, 1988). Synchronous cultures of animals were obtained by collecting the eggs from the original plates and incubating in hypochlorite solution followed by continuous washing in M9 (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl and 1 mM $MgSO_4$) buffer solution. The eggs were then distributed onto 15 cm plates containing OP50 *E. coli*. Animals were allowed to grow for 52 h at 20℃.

Mutagenesis

After the 52 h growth, the animals (P0) were collected in M9 buffer containing 0.5 μg/ml 4,5′,8 trimethylpsoralen (Sigma #T6137) and incubated in the dark at room temperature for 60 min. The animals were then exposed to 360 nm UV light for 20 seconds. The animals were again plated onto 15 cm plates seeded with OP50 *E. coli* and allowed to grow for 24 h. The F_1 eggs were then collected according to the protocol mentioned above and allowed to hatch on the surface of a 15 cm unseeded plate.

Library plating, harvesting, DNA preparation, and sample pooling

The L1-stage F_1 larvae were collected in M9 buffer and concentrated to approximately 500 animals per 0.1 ml. The larvae were then distributed in groups of 500 onto 6 cm heavily seeded OP50 plates. The plates were stored in eleven groups of 96 labeled A through K at 20℃ and allowed to grow for 5 d. The worms were then washed from the plates with sterile distilled water containing streptomycin (100 μg/ml). A portion of the washed worms from each plate was transferred to a 96-well microtiter plate. DNA lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% Tween-20, 0.01% gelatin and 200 μg/ml proteinase K) was added. These plates were then frozen at -80℃ for 30 min and incubated at 65℃ overnight. The 6 cm plates containing the remaining worms were stored at 15℃. For convenience, the samples were pooled into rows and columns. On each 96-well plate (A-K) the samples in each row were equally pooled onto a new 96-well plate so that one plate contained 12 row samples. In the same way, columns were pooled for a total of 8 column samples per plate. The samples were then heated to 95℃ for 20 min to inactivate Proteinase K. Plates were then stored at -20℃.

Primers and PCR

PCR was performed on a MyGenie32 Thermal Cycler (Bioneer, S. Korea). A nested PCR technique involving an outer and inner set of primers was implemented. Primers were designed based on the predicted sequences spanning the full genomic DNA as follows: calreticulin outer upstream primer (5-ACATTCTACTACATTCTGGCTGTGTGATCC-3) and downstream primer (5-ATGAGCATCATTTATTTGGCGGACC-3), calreticulin inner upstream primer (5-AAGCCCTCTGCTGGACTGCTGTCCACC-3) and downstream primer (5-AATGCGAGGAAACGCTTCCCAATTGGC-3); calcineurin B outer upstream primer (5-CAAGGCAATACTGATCTCCAAATGGC-3) and downstream primer (5-TACAAGTGACAGCTACGGGGACATATCGG-3), calcineurin B inner upstream primer (5-CCTGCATAGAGTTTGTAATTATTCAGC-3) and downstream primer (5-CGGAATCAGATGTTCTCCAGCACC-3).

First round sib selection

Once a positive plate was identified, the plate was washed with M9 and the density of animals was determined. The worms were then distributed onto a total of sixteen 12-well microtiter plates supplied with agarose and seeded with OP50. After five days of growth, the worms were collected with streptomycin water and moved to a deep-well microtiter plate. The DNA from the animals was prepared as described above. PCR was again repeated as above.

Second round sib and third round selections

One positive well was chosen and animals were distributed in groups of 10 onto a total of four 24-well plates. After five days of growth, the wells were flooded with streptomycin water and the animals were allowed to lay eggs for approximately one day. A portion of each well was then distributed into a 96-deep well plate and DNA preparation and PCR were performed as above. One positive well was chosen again and transferred to several 6 cm plates. After 2 d of growth single animals were picked and distributed onto a total of eight 24-well plates. After 5 d of growth the worms were again harvested, and DNA preparation and PCR was performed as above.

DNA sequencing of putative deletion bands and determination of homozygosity of deletion mutations

The putative deletion band was directly eluted from agarose gel and cloned into a TA sequencing vector using pGEM-T Easy Vector System I (Promega, Madison, WI). Sequencing was done on an automated sequencer. Sequencing primers used were the same as the inner primer sequences given above. Once the deletion region was identified, a primer was made to determine the homozygosity of the deletion mutant. The upstream (or downstream) primer was designed

within the deleted region as follows: calreticulin (5-TTCCAGTTTGGGCGCTTTCTCCGT-3) and calcineurin B (5-TTGCATTCCGCATCTACGACATGG-3). The downstream primer was the same as the inner downstream primer sequences described above for calreticulin and calcineurin B. PCR was performed and the DNA product was confirmed by gel electrophoresis.

Results

Mutagenesis and Initial Screening

Compared to natural mutagenesis, the possibility to find a mutation in a particular target gene occurs only as high as 50% with the most potent chemical mutagens such as EMS (Barstead, 1999). We screened approximately 500,000 UV-TMP mutagenized worms for specific deletion mutants. However, only mutations occurring in the germ-line would be able to be sustained through generations. Thus, we screened specifically the F_1 progeny of the original mutagenized worms. Since the number of plates that the F_1 progeny was distributed onto was enormous, screening individual plates would be an extremely arduous task. To minimize the work, a scheme of pooling samples by rows and columns was used, and a positive plate could then be easily identified by screening a row and the associated column. A scheme for the mutagenesis and screening of mutants is provided in Fig. 1.

Screening was done by polymerase chain reaction. Since the sequences of all *C. elegans* genes were already known we were able to construct primers for specific genes, namely calreticulin and calcineurin B. The primers would amplify a fragment that spans the entire genomic DNA for both genes so that any deletion within the genome could be cloned (Fig. 2). The size of a wild-type band would be approximately 2.1 kb and 2.2 kb, respectively. If a large deletion mutation had occurred in the region between these two primers, we would detect a smaller size band. Consequently, after the mutagenesis we found putative deletion mutations for both calreticulin and calcineurin B. The sizes of these bands were approximately 1.0 kb and 1.3 kb, respectively (Fig. 3).

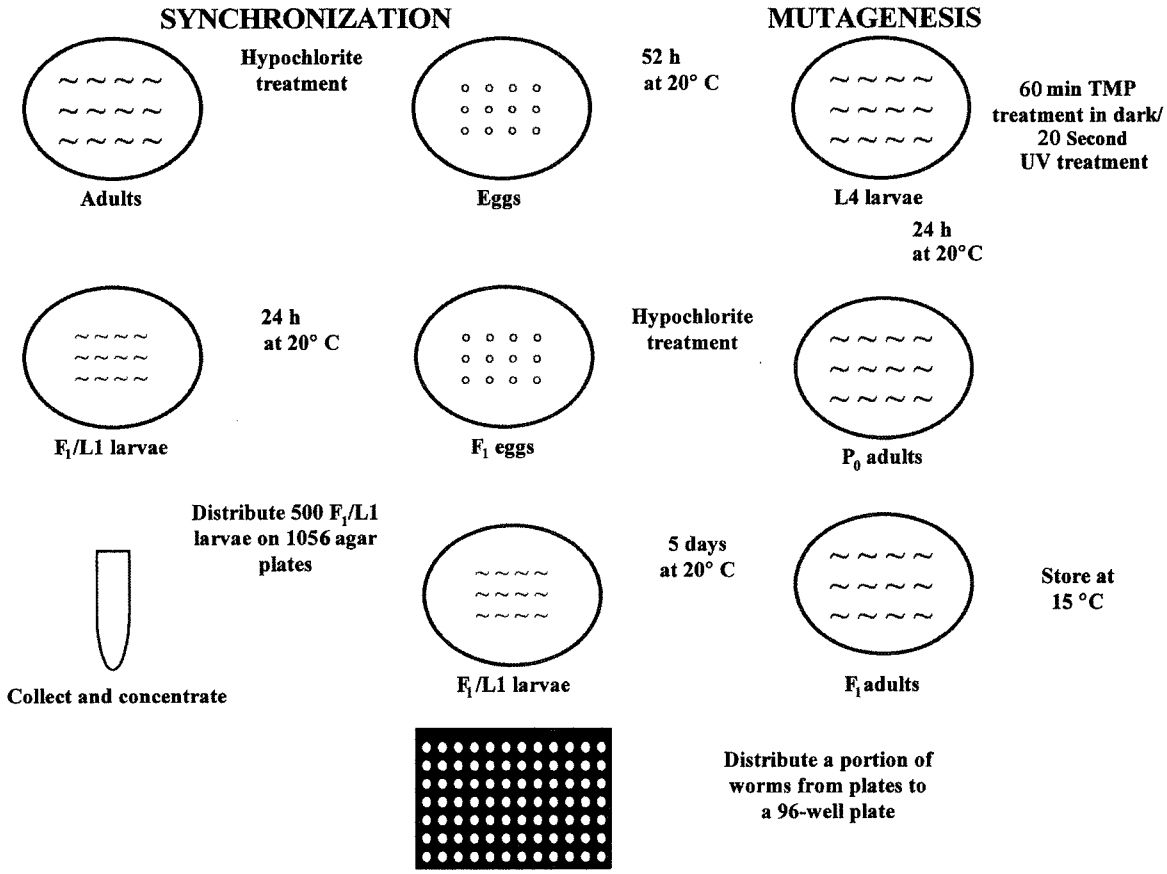

Fig. 1. Mutagenesis and generation of the mutant library scheme. Animals were synchronized for mutagenesis by treating with hypochlorite solution and allowing the remaining eggs to grow to the L4 stage. Mutagenesis was then performed by TMP treatment in the dark followed by exposure to 360 nm UV light. The mutagenized worms were allowed to grow and the F_1 progeny was harvested after hypochlorite treatment. These F_1 eggs were allowed to grow to the L1 stage after which they were collected and concentrated. The animals were then distributed onto 1058 fresh agar plates and allowed to grow for 5 d. After growth, the plates were washed and a portion of the washed worms was transferred onto a 96-well microtiter plate. The DNA from these worms was extracted and used for the initial mutant screening. The plates containing the remaining worms were stored at 15℃.

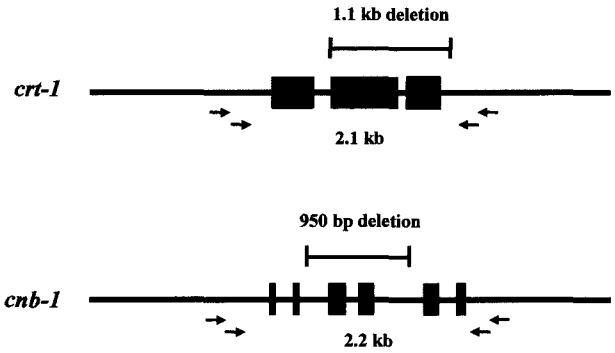

Fig. 2. Nested PCR to identify deletion mutations in calreticulin (*crt-1*) and calcineurin B (*cnb-1*). The intron/exon structure of these genes is shown. Primers are indicated by the short arrows. Primers for both *crt-1* and *cnb-1* were designed to amplify a DNA fragment containing the entire genomic DNA. The size and location of each respective deletion is indicated. The size of the wild-type DNA fragment amplified by PCR is shown below the gene structure.

Sib selection and single worm PCR

Though a putative deletion mutation was found, we had to specifically isolate the animal from which this mutation band was arising. Thus, the remaining worms from the original library plates were distributed into groups of approximately 50 worms, and a second round of PCR was performed. From this first sib selection, a group that showed the deletion band was isolated. The remaining animals from the well of this group were distributed into groups of ten worms each. The worms from this second sib selection were again harvested and a third round of PCR was performed. One group that contained the mutant band was again isolated and the remaining eggs and larvae were allowed to grow. Finally, single worms were distributed, grown, and harvested, and a final round of PCR was performed on the 192 individual worms to isolate specific animals containing the deletion mutation.

DNA sequencing and determination of homozygosity

The size of the resulting deletion bands obtained for calreticulin and calcineurin B was confirmed after the sib selection by PCR. DNA sequencing showed the deletions to be located in the areas of their respected genomes as shown in Fig. 2. Deletions of exactly 1137 bases for calreticulin and 950 bases for calcineurin B were generated by the UV/TMP mutagenesis and detected by successive mutant screening.

Homozygosity of the deletion band in the mutant animal was also determined. A primer designed specifically in the region of the deletion was used to capture a possible wild-type allele in the mutant by PCR. If no band in this deleted region could be found, we could assume the animal to be homozygous. The deletion mutant recovered for calreticulin was determined to be a homozygote while the mutant for calcineurin B was heterozygous.

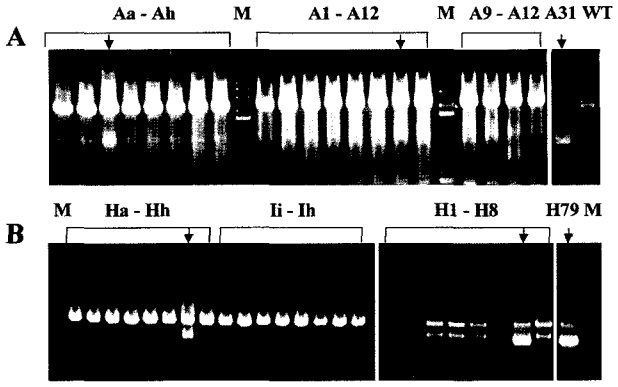

Fig. 3. PCR amplification of part of the deletion library using *crt-1* and *cnb-1* specific primers. Using the row and column technique, a row (labeled A-H) was screened for deletion mutants first. The wild type (WT) for both calreticulin and calcineurin are shown. Mutant band lanes are labeled with short arrows. 1 kb DNA marker is labeled as M. The size of the wild type bands for calreticulin (A) and calcineurin B (B) are 2.1 kb and 2.2 kb, whereas the size of the deletion bands are 1.0 kb and 1.3 kb, respectively. Once the deletion mutant was found, the corresponding column for that row was screened to locate exactly which plate in the library contains the deletion mutation. The calreticulin mutant was found in row Ac, confirmed in column A7, and consequently in plate A31. The calcineurin B mutant was found in row Hg, confirmed in column H7, and consequently in plate H79.

Discussion

Obtaining mutant animals is paramount for functional studies of a particular gene. Though recently double stranded RNA interference has proven to be useful in producing a loss-of-function phenotype in C. elegans, there are many limitations. These include non-expression of null phenotypes in certain tissues, inability to continue RNAi effects far into the germline (Fire et al., 1998), and a limited amount of affected animals to conduct more complex phenotypic studies on (Cho et al., 2000). Thus, the full phenotype of a mutant cannot be completely studied using RNAi. Obtaining mutant animals is the only method at this point to overcome these limitations. Thus, we have conducted a mutagenesis experiment on wild-type C. elegans and have screened for mutant animals on two specific loci, namely, calreticulin and calcineurin B. We have successfully screened for mutations on each of these genes.

Though this process of mutagenesis and screening may be straightforward and relatively simple, there are some areas of caution that should be taken. Firstly, exposure of worms to chemical and radiation should be carefully monitored and controlled. Over-exposure may cause a higher rate of mutation but would obviously kill many of the animals. Improvements in mutation rate in the UV/TMP mutagenesis itself can be made (Genyo-Ando and Mitani, 2000) but should be done cautiously. Secondly, any type of contamination can pose much difficulty in recovery of the target mutant. Steps to avoid contamination, particularly with solutions, should be administered. Also, recovery of a mutant animal can be also difficult. Often the mutant

worm is in a weakened state compared to wild-type animals and cannot survive the rigors of handling during the sib selections. Much care should be taken through these steps to ensure survival of the mutant worm. Along these lines, storage of the mutant library should be taken into consideration. Though others have used frozen mutant libraries for smaller and less complex populations (Zwaal et al., 1993; Jansen et al., 1997; Liu et al., 1999), our libraries are much larger and more complex, and so we store our libraries at 15℃. This will avoid exposing weak mutant worms to rigorous post-freezing recoveries.

We used UV/TMP mutagenesis to spontaneously generate mutations in wild-type *C. elegans* and then screened for deletion mutations only in specific genes. Thus, not only will one find a mutation in their gene of interest, there is likely to be many unwanted random mutations in other parts of the mutated genome. Outcrossing of the target mutation is a necessary step to mitigate this problem. By genetic crossing through the use of marker genes on the target chromosome, we will be able to replace the other chromosomes from the original mutant with wild-type chromosomes while maintaining the mutated target chromosome.

Though slight improvements and adjustments may be made on the mutagenesis and screening strategy outlined in this paper, currently reverse genetics seems to be the most complete and efficient way to study function of a specific gene. By having implemented this strategy we hope to learn the functional importance of calreticulin and calcineurin B in *C. elegans*, and possibly other genes from future mutagenesis and screenings that we plan to conduct.

Acknowledgements

The authors would like to thank the *C. elegans* Genetic Center for strains and all the lab members who participated in this screening work. This work was supported by the grant 1999-2-2-10-001-3 from KOSEF and K-JIST internal grant.

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[Received January 8, 2001; accepted February 3, 2001]