Methods

High-Throughput Isolation of Caenorhabditis elegans Deletion Mutants

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The nematode Caenorhabditis elegans is the first animal whose genome is completely sequenced, providing a rich source of gene information relevant to metazoan biology and human disease. This abundant sequence information permits a broad-based gene inactivation approach in C. elegans, in which chemically mutagenized nematode populations are screened by PCR for deletion mutations in a specific targeted gene. By handling mutagenized worm growth, genomic DNA templates, PCR screens, and mutant recovery all in 96-well microtiter plates, we have scaled up this approach to isolate deletion mutations in >100 genes to date. Four chemical mutagens, including ethyl methane sulfonate, ethlynitrosourea, diepoxyoctane, and ultraviolet-activated trimethylpsoralen, induced detectable deletions at comparable frequencies. The deletions averaged ~1400 bp in size when using a ~3 kb screening window. The vast majority of detected deletions removed portions of one or more exons, likely resulting in loss of gene function. This approach requires only the knowledge of a target gene sequence and a suitable mutagen, and thus provides a scalable systematic approach to gene inactivation for any organism that can be handled in high density arrays.

A rapid method to ascertain gene function by targeted gene inactivation in C. elegans would be highly desirable, but homologous recombination as in the mouse has not yet proven feasible (Plasterk 1995). Microinjection of target-specific RNA, for reasons not completely understood, is a remarkably effective means of transcriptional interruption in this small organism (Fire et al. 1998). However, the extent of RNA-mediated inactivation is difficult to assess in many cases, and such inactivation obviously does not produce a germ-line lesion necessary for genetic crosses, suppressor screens, and other longer-term genetic manipulations. One large-scale approach to germ-line inactivation is to induce random mutations in the animal population, followed by screening for mutations in a target gene of known sequence. The most well-developed method of this so-called target-selected gene inactivation in C. elegans has used random transposon Tc1 insertions to generate a collection of mutants, followed by PCR screening for the presence of Tc1 in a gene of choice (Zwaal et al. 1993). However, because Tc1 insertion alone does not usually result in gene inactivation, it is necessary to subsequently screen individual Tc1 alleles for animals in which the transposon and flanking DNA have been deleted through transposon excision, a relatively infrequent event (Plasterk 1995). An alternative approach is to use chemical mutagens to directly induce deletions in a population, and then to screen by PCR for deleted segments within a selected target region (Yandell et al. 1994). Jansen et al.

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(1997, 1999) have established the broader feasibility of this approach by isolating mutants of the \textit{C. elegans} heterotrimeric G protein gene family.

In this study we describe our results using random chemical mutagenesis and PCR screening to rapidly isolate deletion mutations in a large number of genes encoding proteins with a broad range of functions. \textit{C. elegans} is unique among model animal species in that it can be grown in liquid cultures and also can be stored frozen but viable at \(-80^\circ\text{C}\). We have taken advantage of these properties to devise a rapid and scalable procedure for gene disruption almost entirely on the basis of microtiter plate arrays of whole animals and genomic DNA. We used four different chemical agents to create mutagenized libraries, and found that all four mutagens induce detectable deletions. Almost all of the deletions were significant enough to result in loss of exons, frame shifts, and other molecular lesions likely to cause loss of gene function. We discuss the sensitivity, specificity, limitations, and broader utility of this approach to systematic gene inactivation in \textit{C. elegans}.

**RESULTS**

Library Construction and Screening

To enhance the scalability and speed of mutagenized library construction and screening, we relied on microtiter plate-based culture and analysis wherever possible (Fig. 1). \(F_1\) larvae of mutagenized \(P_0\) animals were distributed directly into microtiter plate wells (~20 larvae representing 40 mutagenized genomes) and grown in liquid cultures to yield \(F_2\) larvae. Approximately one-half of the worms were processed into a corresponding genomic DNA microtiter plate array, with the remainder frozen at \(-80^\circ\text{C}\) as viable animals. The initial round of nested PCR screening for deletion mutations in a targeted gene was performed on microtiter plate pools each containing ~4000 mutagenized genomes. PCR primers were designed to flank ~3 kb of exon-rich regions of the selected ORF, in many cases specifically targeting putative functional domains. Amplicons smaller than the wild-type amplicon were taken to represent candidate deletions. Such amplicons were expected to be preferentially amplified, compen-
sating for the much lower representation of the mutant target template within the genomic DNA pool. Candidate-positive plate pools were resampled to eliminate false positives, which constituted about three-fourths of the first-round candidate deletions. After confirmation that a plate pool contained a deletion, the corresponding DNA array was then screened to identify a specific microtiter well. The corresponding well of the frozen worm library was thawed and individual animals were grown in microtiter wells; portions of these cultures were screened by PCR to identify heterozygous and homozygous mutant strains. A small number of thawed library wells failed to yield the expected deletion mutant, and the failure rate exceeded 50% when <100 viable animals were recovered from a well (data not shown).

To evaluate various chemicals that have been used to induce mutations in C. elegans, we created mutant libraries using different mutagens, including ethylmethane sulfonate (EMS), ethynitrosourea (ENU), diepoxyoctane (DEO), and ultraviolet-activated trimethylpsoralen (UV-TMP). Each library consisted of forty-eight 96-well plate arrays and ~200,000 mutagenized genomes. For any given target, usually 4–8 such libraries (comprising 0.8–1.6 million mutagenized genomes) were screened simultaneously for potential deletions. At this scale, deletion mutants were isolated from almost all loci that were targeted, and in many instances more than one deletion was identified; in the latter cases usually only the largest deletion was thawed from the worm library. We were unable to detect deletions in a small number (<5) of targeted genes, and these failed loci were too few to determine whether they shared any structural characteristics. The average number of animals recovered from frozen wells differed for each mutagen as follows: DEO 191 ± 159, EMS 359 ± 330, ENU 343 ± 249, and UV-TMP 199 ± 166.

Deletion Mutations

To date we have isolated deletion mutations in >100 independent gene targets using this method, and a representative listing is shown in Table 1. Among these are genes encoding a broad range of protein functional groups, including kinases, transcription factors, membrane receptors, metabolic enzymes, proteases, hormones, and nematode homologs of oncogenes and tumor suppressor genes. However, the largest target group comprises genes encoding proteins of unknown function, reflecting our strategic choice of using the C. elegans model to elucidate the function of novel predicted proteins.

The average size of chemically induced deletions was ~1400 bp (range 700–2900 bp), as detected by a PCR-screening window of ~3 kb (Fig. 2). In several instances, smaller deletions (on the order of 400–500 bp) were fortuitously detected on screening of a specific library plate. The average size of deletions isolated from different mutagenized libraries ranged from ~1320 bp for EMS up to 1580 bp for DEO, and a broad size range was observed for each agent (Fig. 2). DNA sequencing revealed that the majority were simple deletions, but about one-fifth of the sequenced deletions contained small (3–27 bp) insertions at the breakpoints. All of the latter deletions derived from libraries mutagenized by DEO, EMS, or ENU. Only one of our isolated deletions (xfl-1, nr2025), isolated from a UV-TMP library, contained a large inverted insertion, which is in contrast with the examples reported previously for gpa deletion mutants obtained by UV-TMP, in which two of three deletions contained large insertions (Jansen et al. 1997). In addition, in our series, both the proximal and distal deletion breakpoints in most cases fell within a region of two or more consecutive identical nucleotides, rendering the exact breakpoint ambiguous.

C. elegans genes are compact, with most introns ~50 bp and exons 80–250 bp in length (Blumenthal and Steward 1997). Thus, deletions of ~500 bp within a targeted C. elegans ORF are likely to significantly compromise gene function. Within an ORF, the position of such disabling deletions can be as follows: (1) the deletion begins or ends in an exon, removing portions of one or more exons, and usually leads to a premature stop codon (Fig. 3a); (2) the deletion begins and ends in introns, but removes one or more exons encoding domains essential for protein function (Fig. 3b); or (3) the deletion removes the entire ORF (Fig. 3c). Less than 2% of the deletion mutations that we isolated removed only noncoding DNA (data not shown), a figure that was optimized by selecting target regions that excluded long stretches of noncoding DNA. Given the characteristics of these molecular lesions, it is likely that most of these intragenic deletions represent severe or null mutations. The resulting phenotypes range from apparently wild-type to dramatic and lethal defects. Of note, less than one-half of the isolated deletion mutants (Table 1) displayed an obvious phenotype (such as lethal, uncoordinated, or sterile), but further analysis is necessary and ongoing in many cases.

Although generally 4–8 libraries were screened for each target, the total number of mutant libraries generated during the period of this study was greater. Additional libraries were produced as template DNA became depleted in the course of multiple target screens, and all of these libraries were included for analysis. In addition, the cumulative number of libraries prepared with each chemical mutagen was different for each agent. To estimate the likelihood of isolating a deletion with different mutagens, we compared the number of deletions relative to the total number of primary library screens for each mutagen (Table 2). By this analy-
<table>
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<th>Cosmid name</th>
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<th>Allele name</th>
<th>Mutagen</th>
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<th>Deletion effect on ORF</th>
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<td>545</td>
<td>exon 7–exon 8</td>
<td>no obvious phenotype</td>
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*Personal communications, unless otherwise noted.*
sis, UV-TMP appeared to generate a higher frequency of deletions compared with the other three chemical mutagens.

**DISCUSSION**

In this paper, we describe our experience with a rapid procedure for isolating deletion mutations in *C. elegans* that uses PCR to screen for deletions in a target gene within a randomly mutagenized population of animals. The growth of mutagenized animals, preparation of genomic DNA templates, PCR screens, and mutant worm recovery are all handled in scalable microtiter liquid plate well formats that maximize speed and utility. All of the mutagens we used resulted in significant deletion mutations in target genes almost certainly sufficient to produce loss of function.

In practice, this protocol yields a deletion in a selected target for roughly every 600,000 mutagenized genomes screened. However, many factors affect the apparent sensitivity of this procedure. Small deletions of <500 bp were not isolated in screening plate pools for two likely reasons. First, such amplicons would be difficult to distinguish from an abundant wild-type band on a gel, unless special and more time-consuming electrophoresis conditions were applied. Second, mutant templates with small deletions would be similar in size to the much more abundant (4000-fold in the primary screening pools) wild-type templates and less likely to be preferentially amplified. The fact that we discovered several smaller (<500 bp) deletions only on screening of plate arrays (100-fold less complex than the plate pools) suggests that smaller deletions may be more abundant than larger deletions. Reducing the complexity of the DNA pools may improve the sensitivity of detecting these smaller deletions.

Larger deletions of >3 kb would also be useful because of their increased likelihood of severely affecting coding regions; furthermore, in certain cases the simultaneous disruption of two adjacent genes (e.g., evolutionarily duplicated genes) by one large deletion is desirable. The upper range of detectable deletions is limited by the size of the PCR deletion screening window, which, in this series, was 2800–3400 bp, and the processivity of the thermostable polymerase and other components of the amplification reaction. Modification of PCR reagents and conditions would allow a larger screening window, and we have recently isolated larger deletions using a target window of up to 5 kb (data not shown). Increasing the sensitivity of deletion detection would also increase the likelihood of obtaining more than one allele for each target in a single screen, which would be desirable for comparative genetic and phenotypic analysis.

This PCR-based approach is highly specific because of the use of nested pairs of screening primers derived from the target gene. We sequenced a number of false-positive deletion amplicons from the primary round of plate pool screening; all were derived from the target gene rather than an extraneous locus (data not shown). Although the genesis of these truncated amplicons is incompletely understood, we observed a comparable number of false-positive amplicons using unmuta-genized genomic DNA as a control template (data not shown), and suspect that they may arise from polymerase slippage across gaps formed by secondary loops in the DNA template.

A wide variety of chemicals and irradiation methods have been used to induce mutations in *C. elegans* (Anderson 1995), and we used four in making our libraries for reverse genetics: EMS, ENU, DEO, and UV-TMP. EMS is the most widely used mutagen for *C. elegans* because of its potency in generating loss-of-function or reduction-of-function alleles in classical phenotypic screens. Many EMS-induced mutations are point mutations (of which the vast majority are G/C–A/T transitions) (Anderson 1995), which, of course, would not be detected in a screen for deletions. However, ~13% of EMS-induced mutations are reported to be deletions or other rearrangements, though most are small (Anderson 1995). ENU appears to generate a rate of mutations similar to EMS; studies of ENU-induced mutations in the *unc-93* gene revealed an increased frequency of A/T–G/C transitions compared with EMS, with a frequency of intragenic deletions of 13% (De Stasio et al. 1997). DEO has not been as broadly used as a *C. elegans* mutagen, but a study of
mutations of the unc-54 gene revealed that DEO generated mutations at a lower rate than EMS, and 27% of DEO-induced mutations were multilocus deletions and deficiencies, as defined by genetic criteria (Anderson and Brenner 1984). Treatment with trimethylpsoralen followed by ultraviolet activation, a procedure that leads to DNA cross-linking, appears to generate a lower frequency of mutations than EMS, as measured by generation of unc-22 and pal-1 mutations, but a relatively high proportion of deletions (Yandell et al. 1994).

For the purposes of a PCR-based reverse genetics screening approach, it is important to identify a mutagen that generates the highest frequency of small deletions (on the order of 100–5000 bp) rather than point mutations that cannot be detected. Furthermore, highly potent inducers of large numbers of point mutations would exacerbate the confounding background of extraneous mutations. In our experience, libraries prepared with each of the four mutagens yielded deletions at similar rates (Table 2). In addition, the average size of detected deletions induced by the different mutagens was not significantly different (Fig. 2). The frequency of deletions obtained from each of the four mutagens far exceeded the spontaneous rate of deletions of this size range, which is predicted to be <1 per 100 million genomes (P. Anderson, pers. comm.). About one-fifth of our chemically induced mutations contained short (≤7 bp) direct sequence repeats or longer (>2 bp) insertions at the deletion termini, in excess of what would be expected from spontaneous deletions (Pulak and Anderson 1988; data not shown). Such insertions may reflect mechanisms of break repair with nonhomologous endjoining, in response to chemically induced breaks in double-stranded DNA (Friedberg et al. 1995). To measure the efficiency of each mutagen in creating detectable deletions, we retrospectively determined the relative yield of deletions from each type of mutagen library (Table 2). By this indirect measure, UV-activated trimethylpsoralen appears to generate the highest frequency of detectable deletions, which confirms prior observations (Yandell et al. 1994). However, fewer animals, on average, were recovered from frozen UV-TMP-treated worm populations compared with EMS or ENU, suggesting that UV-TMP treatment may cause more generally deleterious effects. In our experience, γ irradiation or formaldehyde treatment, which reportedly causes more major rearrangements (Anderson 1995), did not produce significant numbers of deletion mutations in limited experiments (data not shown). Because deletions, rather than mutations per se (the majority of which are point mutations), are the goal of this reverse genetics screening approach, the identification of a specific deletogen chemical would be very helpful.

A disadvantage of random mutagenesis procedures is that substantial numbers of extraneous mutations are generated in the background of any selected target gene, and it can be difficult to titrate the mutagen dose so as to induce large numbers of mutations in the population while also minimizing the number of mutations in any one animal. A strong mutant phenotype may result from the deletion of interest or from mutation of a non-target gene. Similarly, a pleiotropic phenotype may be due to either multiple effects of a single mutation or to mutations in more than one gene. Thus, thorough outcrossing followed by transgenic res-
cue of the observed phenotype with a wild-type copy of the target gene (usually in the form of a cosmid or plasmid clone), is necessary to establish whether a putative loss-of-function phenotype is actually due to the deletion. In addition, reverse genetics may lead to no obvious phenotype at all. Earlier estimates suggested that up to one-half of \textit{C. elegans} genes may cause no obvious phenotype when null (Park and Horvitz 1986). When no phenotype is seen on isolation of the mutant animal, it can be difficult to tell whether this lack of effect is because (1) the randomly generated molecular lesion does not produce loss or reduction of gene function, (2) the gene is functionally redundant, or (3) the phenotype requires specific environmental conditions for its expression or is otherwise too subtle to be distinguished by conventional methods. The complementary use of RNA-mediated interference (Fire et al. 1998) might be helpful in cases in which deletion mutants display a confusing pleiotropy or an apparent wild-type phenotype.

At the current state of development, this method requires ~2 weeks for identification of a deletion and isolation of mutant worms. The rate of deletion identification is about one target per week, per person, without automation. This rapid approach to gene inactivation allows one to simultaneously approach a group of candidate genes, whether they belong to a single gene family or may be functionally related in a putative signaling pathway. Thus, deletion mutants of different, but potentially related, genes may be studied as a group for comparative phenotype analysis, genetic interactions, etc., rather than individually. For example, genetic and phenotypic analysis have revealed overlapping functions between the \textit{C. elegans} presenilins \textit{sel-12} and \textit{hop-1} (Li and Greenwald 1997; Westlund et al. 1999) and in the human Niemann–Pick type-C disease gene homologs \textit{npc-1} and \textit{npc-2} (M. Sym, pers. comm.).

An obvious advantage of this target-selected approach is that specific regions of functional importance can be targeted to maximize the likelihood of obtaining null or severe loss-of-function mutations. On the other hand, this method is less well suited than traditional noncomplementation genetic screens for obtaining weaker (hypo)morphic alleles, which are often useful in genetic pathway analysis.

A procedure such as this one that uses random mutagenesis followed by deletion screening is ideally suited for organisms like \textit{C. elegans}, in which pre-existing genomic sequence information is extensive but homologous recombination or transposon insertion methods are not practical. By improving the sensitivity of detection in more complex mutagenized genome pools, it may be possible to produce a clonal library of individually arrayed mutants, thereby eliminating the need to sort through thawed worms for the deletion. Furthermore, by increasing the density of \textit{C. elegans} growth and DNA microarrays, and automating the PCR set up and analysis, it should be feasible to pursue the systematic disruption of all of the predicted genes in this model animal, as is currently being done, by different methods, for all of the genes in \textit{S. cerevisiae} (Dujon 1998).

**METHODS**

**Bioinformatics**

\textit{C. elegans} homologs of non-nematode genes and proteins were identified by BLAST search (Altschul et al. 1990) of finished \textit{C. elegans} sequences available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and unannotated sequences available at the Sanger Centre (www.sanger.ac.uk/Projects/C_elegans/blast_server.html) and the Washington University Genome Sequencing Center (genome.wustl.edu/gsc/blast/blast_servers.html). Putative ORFs of unannotated sequences were determined with the GeneFinder algorithm available from the Baylor College of Medicine Human Genome Center (dot.imagen bcm.tmc.edu:9331/gene-finder/gf.html). Nested PCR primer pairs were designed to flank a target window of 2.8–3.2 kb with MacVector 6.0. Whenever possible, the target window was chosen to include the most proximal exons, except in those cases in which the first intron was >1 kb in size.

**Construction of Mutagenized Nematode and Corresponding DNA Libraries**

Wild-type Bristol N2 nematodes were cultured by standard methods (Sulston and Hodgkin 1988). A synchronized population of \textit{L}_4 larvae was treated with one of the following four mutagens: 25 mM ethylmethane sulfonate for 4 hr, 0.4 mM ethyl nitrosourea for 4 hr, 1 mM diepoxyoctane for 4 hr, or 30 µg/mL trimethylpsoralen (Yandell et al. 1994), followed by UV irradiation at 3.5 uW/cm² for 15–30 sec (Anderson 1995). The efficiency of mutation was evaluated by quantitating the frequency of \textit{F}_1 resistance to ivermectin (Rand and Johnson 1995); libraries were discarded if the proportion of genomes carrying newly induced ivermectin resistance mutations was <0.5%. \textit{F}_1 eggs of mutagenized worms were collected for 4 hr, then allowed to hatch for 16 hr. Hatched larvae were distributed to 96-well microtiter plates at ~20 worms per well in 50 µL of NGM culture medium containing 1% (by volume) \textit{Escherichia coli} HB101 as food (Sulston and Hodgkin 1988). A library from each chemical mutagenesis comprised 48 microtiter plates and included the arrayed progeny of nearly 10⁵ \textit{F}_1 animals representing 2 × 10⁵ mutagenized genomes. Worms were grown in wells until the food was exhausted, then transferred to 96-well microtiter plates at ~20 worms per well in 50 µL of NGM culture medium containing 1% (by volume) gelatin and 200 µg/mL proteinase K. These worm DNA plates were stored without further purification at ~80°C. Plate pools were digested for 4 hr with 0.4 µL of lysis
buffer containing 100 mM NaCl, 100 mM Tris-Cl (pH 8), 50 
mm EDTA, 1% SDS, 1% β-mercaptoethanol and 100 µg/ml. 
proteinase K (Birren et al. 1997). Plate pool genomic DNA was 
then isolated with the Puregene kit (Gentra Systems) according 
to the manufacturer's protocol, followed by ethanol precipi-
tation and transfer to sealed microtiter plates for storage at 4°C. The remaining half of the worms in the original plates were 
frozen in the wells by adding an equal volume of freeze-
sing solution, mixing, and gradually freezing to ~80°C (Sul-
ston and Hodgkin 1988). Survival following freezing was mea-
sured by counting motile animals thawed from 10 selected 
wells from each of 2 plates, and libraries were discarded if <100 viable animals per well on average were recovered.

PCR Screening for Deletions
Genomic DNA plate pools were screened by nested PCR with 
AmpliTag Gold (Perkin-Elmer) and primer concentrations of 
50 µM for 35 cycles of 94°C for 30 sec, 58°C for 1 min, and 
72°C for 1.5–2.5 min. Aliquots (1 µl) of template DNA from 
two libraries (45 plate pools each) were added to a 96-well PCR 
plate and up to 8 libraries were screened simultaneously for a 
given gene target in a Tetrad thermocycler (MJ Research, Wa-
tertown, MA). Potential deletions detected by gel electropho-
resis of PCR products were confirmed by quadruplicate PCR of 
the original library plate pool. On identification of a positive 
plate, PCR was performed on the unpooled DNA plates to 
identify the positive well. Primary screen data and deletion 
sizes were available for 127 screened targets.

Isolation of Mutant Animals and Deletion Analysis
Deletions were mapped by restriction enzymes on the basis of the 
known genomic DNA cosmid sequence, and the mapping 
information was used to design three new PCR primers, two of 
which flanked the deletion and a third from within the dele-
tion. Worms were thawed from the positive wells onto NGL 
plates and then picked individually into microtiter plate liq-
uid cultures. Following growth and reproduction in liquid 
medium, aliquots of progeny were removed for PCR with 
flanking primers to identify clones bearing the deletion mu-
tation. Positive lines were transferred to agar plates, and single 
worms were tested by multiplex PCR with both the flanking 
and internal primers to distinguish homozygous from hetero-
zygous animals in subsequent generations. Mutant animals 
were subsequently outcrossed with wild-type N2 animals to 
remove other potential mutations.

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