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ISOLATION AND CHARACTERIZATION OF X-LINKED MUTANTS IN THE NEMATODE PANAGRELLUS REDIVIVUS

by

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ABSTRACT

A protocol for obtaining mutants in the nematode
Panagrellus _redivivus_ following mutagenesis with the
alkylating agent ethyl methanesulfonate was established.
The induced mutation rate for the X chromosome is 0.79%
with an average mutation rate per locus of 4.4×10^{-4}.
The total number of genes on the X chromosome coding for
indispensable functions is estimated as approximately 300.

Of the 24 X-linked mutants that were isolated
only 8 could be maintained as homozygotes due to the
severity of the phenotype and the mating inefficiency of
males. The mutants define 6 complementation groups and
map within a limited region of the X chromosome.

Reasons for the failure to obtain stable visible
mutants in earlier studies are discussed.

INTRODUCTION

Collection of mutations which perturb a particular biological process allows the investigator to dissect the process in a stepwise fashion, leading to an understanding of the controlling mechanisms. This approach has been useful in elucidating the mechanisms regulating gene expression in procaryotes (Retznikoff, 1972; Goldberger, 1974). Brenner (1974) demonstrated the utility of a free-living nematode as a model organism for the study of development. Utilizing the self-fertilizing hermaphrodite Caenorhabditis elegans, he isolated 300 ethyl methanesulfonate (EMS) induced visible mutants and defined 77 complementation groups. This laid the groundwork for intensive study, at the cellular and molecular levels, into the mechanisms controlling the temporal sequence and spatial organization observed during development (see Edgar and Wood, 1977; Riddle, 1978).

As an alternative to collecting mutants, the investigator can perturb the process utilizing external agents such as antibiotics, microwave radiation and microbeam surgery. Generally the agents are broad in their action and results are indicative but not conclusive. Brenner and co-workers; Babu (1974); Ward et al. (1975); Herman et al. (1976); Hirsch and Vanderslice (1976); Klass et al. (1976); Hodgkin and Brenner (1977); Lewis and Hodgkin (1977); Sulston and Horvitz (1977);

utilizing the genetic approach, have recently met with great success. Samoiloff and co-workers, utilizing the former approach, have been less successful in developing the dioecous nematode _Panagrellus redivivus_ as a model organism for studying post-embryonic development.

The work on _P. redivivus_ focussed on stage specific post-embryonic events including: growth and ecdysis (Samoiloff and Pasternak, 1968; Samoiloff and Pasternak, 1969; Samoiloff, 1970; Samoiloff, 1973 a,b), gonad development (Boroditsky and Samoiloff, 1973), mating behaviour (Cheng and Samoiloff, 1972; Samoiloff _et al._, 1973; Samoiloff _et al._, 1974; Balakanich and Samoiloff, 1974), chemotaxis and osmotic tolerance (Pollock and Samoiloff, 1976), and the nutritional requirements of development (Abdulrahman and Samoiloff, 1975). In all cases, the processes could be interrupted with various inhibitors of macromolecular synthesis or modified by physical means. The results indicated that bursts of gene activity were required to provide precursor material necessary to complete a particular phase of the life cycle. The experimental data were consistent with a model of nematode development proceeding in quantum steps where the bursts of gene activity coincide with the morphological event of ecdysis. The inherent limitations of this approach meant that the experiments were inconclusive. In most cases, further analysis would have required either sophisticated biochemical techniques or a collection of

mutants to facilitate a genetic approach.

One study reported a single behavioural mutant (Pollock and Samoiloff, 1976), the only mutant isolated after an extensive analysis of many chemically induced behavioural variants (Pollock, 1974). This result is surprising in view of Brenner's success with the hermaphrodite C. elegans and raises questions concerning the utility of Panagrellus for genetic studies.

The present work resolves the following questions:

(1) Can stable visible mutants of Panagrellus be obtained by chemical mutagenesis?

(2) Are the mutations inherited in a classical Mendelian fashion?

(3) What is the range of visible phenotypes possible?

(4) Can the induced mutants be utilized to enhance further genetic studies in Panagrellus?

(5) What are the reasons for the failure to obtain many mutants of Panagrellus in earlier studies?

MATERIALS AND METHODS

Nematode cultures:

NG agar (Brenner, 1974): Prepared by dissolving 3g NaCl, 2.5 g Bactopeptone, 17 g agar and 1 ml cholesterol in ethanol (5 mg/ml) in 975 ml glass distilled water. After autoclaving (15 psi, 15 min), 1 ml $CaCl_2$, (1.0M), 1 ml $MgSO_4$ (1.0M) and 25 ml potassium phosphate buffer (1.0 M) pH 6.0 were added in order. Finally, 5 ml of an aqueous solution saturated with Nystatin (a fungal inhibitor) was added.

M9 buffer: 5 g NaCl, 6 g Na_2HPO_4, 3 g KH_2PO_4 and 0.25 g $MgSO_4 \cdot 7 H_2O$ per litre.

C. elegans Ringer's (Hirsch et al., 1976): 0.821 g anhydrous sodium acetate, 0.018 g NaCl, 0.146 g KCl, 0.103 g $CaCl_2$, 0.080 g $MgCl_2 \cdot 6 H_2O$, 0.206 g Sucrose, 5 ml 0.05 M solution of Hepes buffer (Gibco) in 100 ml glass distilled water.

The nematodes used were from strain C-15 of Panagrellus redivivus obtained as previously described (Pasternak and Samoiloff, 1970). After nine consecutive generations of brother-sister matings, a single gravid female from the F_{10} was used to initiate the stocks used in genetic studies. Stocks were maintained at room temperature ($22^{\circ}C$) by culturing in petri plates containing NG agar. Worms were grown monoxenically on a mutant of Escherichia coli K12 generously donated by Dr. B. Bachmann, Yale University. The bacterium, strain JEF-8 (Coli Genetic

Stock Center #5678) carrying chromosomal markers thr 31,

car B8, rel A, and met B$_1$ has chosen for car B8, a Δ (car

B) - (formerly pyr A locus). The non-reverting mutant is

an auxotroph requiring methionine, arginine and uracil.

The bacterial stock was initiated from a single colony,

grown to saturation in Nutrient Broth (BBL) and tested for

nutritional requirements. A sample was then frozen in 30%

glycerol and stored for future use. Stocks were tested

periodically for requirements. Occasionally bacterial

cultures were contaminated and new stocks were established

from the frozen samples. Before use, NG plates were

seeded with JEF-8, maintained at 37°C overnight and

allowed to cool to room temperature before use. The

growth of the auxotroph on NG agar was slow, preventing

the bacteria from over-growing the worms. The generation

time for P. redivivus under these conditions is 4.0 days.

Small plates (35 mm x 10 mm) inoculated with a

single gravid female can support growth such that a

fertile F$_1$ generation is obtained, The F$_2$ population

overgrows the plate and these F$_2$ larvae do not complete

development. Intermediate-sized plates (60 x 15) will

support growth such that approximately half of the F$_2$

population reach sexual maturity and produce progeny.

A complete F$_2$ population can only be obtained by culturing

on large plates (100 x 15) but the culture becomes
crowded with early F_3 offspring before the entire F_2
population mature. The F_3 larvae do not complete
development under these conditions.

Plates contaminated by molds and bacteria were
rendered monoxenic according to the glutaraldehyde
method (Brenner, 1974). The glutaraldehyde kills the
worms and contaminants but not embryos developing in
utero. These embryos complete embryogenesis, larvae
devour the female and crawl into the bacterial lawn within
36 hours. Ten of these larvae were transferred to a
fresh plate.

Panagrellus redivivus is ovoviviparous. The embryo
and the first larval stage (L_1) develop in utero.
The second (L_2), third (L_3) and fourth (L_4) larval stages
and the adults are free swimming. Each stage is separated
by a molt. It is impossible to distinguish male and
female larvae by morphological parameters. The female
is competent to mate immediately after the final larval
molt. To obtain virgin females it is necessary to grow
individual larvae in isolation and select adult females.
Panagrellus is transparent and mature females are easily
distinguished from adult males. The female is larger
and filled with eggs, the male is smaller with a
conspicuous crescent shaped copulatory apparatus (spicule)
and a well developed ductus deferens. Larvae were grown

in isolation on small NG plates seeded with bacteria.

Mutagenesis:

Mutations were induced with ethyl methanesulfonate (EMS) according to the method of Brenner (1974). A synchronously growing culture was utilized to ensure that all animals were at the same stage of gonad development. Synchrony was obtained by placing 50-100 gravid females on a fresh plate and allowing them to produce larvae for six hours. The females were removed and all remaining larvae developed in good synchrony. After 72 hours the worms were washed off the plate with 3 ml M9 buffer into a scintillation vial. 1 ml of freshly prepared EMS (0.2 M in M9 buffer) was added and the suspension allowed to sit at room temperature for 4 hours. At completion of mutagenesis an equal volume of 5% sodium thiosulfate was added to neutralize the mutagen and five minutes later the suspension was washed into a 250 ml separatory funnel and filled with M9 buffer. The worms were allowed to sediment by gravity and were washed in separatory funnels with M9 buffer, and finally poured into an empty petri plate. Worms were transferred in pairs to NG plates and inspected for progeny.

Handling and Observation:

Worms on agar were manipulated individually with a sharp wooden applicator stick sterilized in alcohol. Mass transfers were achieved by streaking large numbers of animals from crowded plates onto fresh media with

sterile inoculating loops. Worms were manipulated in
fluid with micropipettes prepared as needed. Observations
were made using a Wild M4A transilluminating dissecting
microscope.

Chromosomes were stained by Feulgen's method
(Stowell, 1945) with a slight modification. Whole mounts
of male nematodes were unsatisfactory. The male gonad
was dissected from the male by amputating the anterior
portion of the worm behind the pharynx. The gonad was
dissected on an albuminized slide in C. elegans
Ringer's solution. The tissue was fixed in situ with
Carnoy's fluid after the excess Ringer's solution had
been removed with tissue paper. A clean cover slip was
placed on top and the gonad was squashed. The slide was
frozen on dry ice, the cover slip removed and the
preparation flooded with absolute ethanol. The
preparation was hydrated, hydrolyzed in 1 N HCl (60°C)
for 11 min and stained in Schiff's reagent for 15 min.
After bleaching in sulfurous acid, the staining was
intensified with 0.5% Azur B in 1% sodium metabisulfate
for 6 minutes - a method developed for visualizing Paramecium
cytoplasmic bacteriods (Samoiloff, unpublished). The
preparation was rinsed in distilled water, dehydrated,
counterstained in Fast Green, cleared in xylene and
mounted in DPX.

Whole worms were photographed on thin agar blocks
(Sulston and Horvitz, 1977).

All photographs were taken with a Zeiss universal photomicroscope equipped with Nomarski optics and a flash attachment.

Bacterial cultures:

Bacteria were maintained in liquid Nutrient Broth (BBL) and subcultured bi-weekly. Cultures were grown to saturation at $37^{O}C$ and stored at $4^{O}C$ for up to one week.

RESULTS

<u>Chromosome</u> <u>Constitution</u>: Cytological observations (Fig. 1) confirm a previous report (Hechler, 1970) that spermatogenesis produces two types of sperm with either four or five chromosomes. Oogenesis produces gametes with five chromosomes. The results indicate that the haploid complement of autosomes is four, there is an XO mechanism of sex determination and males are the heterogametic sex. Males are effectively haploid (hemizygous) with respect to the X chromosome and recessive mutations will automatically be expressed as they cannot be masked in a heterozygous form.

<u>Isolation</u> <u>of</u> <u>Mutants</u>: The progeny of two mutagenized parents are the product of two independently mutagenized gametes. It is extremely unlikely that the mutagen would have affected the same locus in both parental gametes, so none of the autosomal mutations will appear in the F_1 in a homozygous form. Two classes of visible mutants in the F_1 are possible: dominant (or semi-dominant) mutations and one-half of the recessive mutations generated on the X chromosome of eggs. In practice only the F_1 female progeny are considered as they contain two independently mutagenized X chromosomes. One-half of the former class and all of the latter class will appear in the F_1 males and would not be isolated. To ensure virginity of F_1 females, each F_1 larva was picked onto a

small NG plate seeded with bacteria and previously inoculated with a single wild type male. Large numbers of F_1 animals (500-1000) were used to establish individual lines. All progeny from a pair of mutagenized parents were designated by a lower case letter and each individual is identified with an arabic numeral. Ten larvae from parental pair a are designated a1, a2, a3, ... a10. Any variant isolated was named according to the phenotype (see below) and given the designation of the line from which it was isolated.

A single X-linked mutation in any F_1 female should be expressed in one half of the F_2 male progeny. All plates with F_2 progeny were screened for X-linked mutations by visual inspection. To obtain autosomal recessive mutants, the animals from F_2 plates with no X-linked mutants were transferred en masse to large plates. A single autosomal recessive mutation should be expressed in 1/16 of the F_3 progeny.

Phenotypes of mutants: Panagrellus mutants are similar in phenotype to those described for C. elegans (Brenner, 1974). A summary of phenotypes isolated is presented in Table 1.

"Uncoordinated" animals with defective movement are the most common class of mutants that can be isolated. The uncoordinated phenotype ranged from slight to extreme paralysis. In some instances the smooth sinusoidal movement of the wild type had a superimposed shaking.

Table I.   Preliminary characterization of mutants

with respect to phenotype, expression and

chromosomal location:

dm - dominant; sd - semi-dominant;

vb - variable expression.

Preliminary mutant designation	Chromosome	Comment
uncoordinated (unc)		
unc s215	x	severe unc; ♂ did not mate
unc f215	x	severe unc; ♂ did not mate
unc b205	x	severe unc; ♂ did not mate
unc c16	x	moderate unc; ♂ effective
unc t80	x	moderate unc; ♂ effective
unc c6	x	moderate unc; ♂ effective
unc t71	x	shaking superimposed; ♂ did not mate
unc a33	x	shaking superimposed; ♂ did not mate
unc k10	x	shaking superimposed; ♂ did not mate
unc f20	x	slight unc; ♂ effective
unc i4	x	moderate unc; ♂ effective
unc m11	x	severe unc; ♂ did not mate
unc d38	x	moderate unc; ♂ effective
unc u13	x	moderate unc; ♂ effective
unc u44	x	moderate unc; ♂ effective
unc e71	x	severe unc; ♂ did not mate
unc i2	x	moderate unc; ♂ effective
unc u1	x	severe unc; ♂ did not mate
unc a49	autosome	severe unc; ♂ did not mate
dumpy (dpy)		
dpy q68	autosome	sd; ♂ effective
dpy j22	x	♂ did not mate
dpy d1	autosome	dm; vb
dpy a49	autosome	extreme dpy; ♂ did not mate
dpy m50	autosome	extreme dpy; ♂ did not mate
dpy s225	autosome	sd
small (sma)	autosome	sd
sma s40	autosome	sd
sma d225	autosome	sd
sma r7	autosome	sd

Preliminary mutant designation	Chromosome	Comment
long (lon)		
lon i3	x	
lon t36	x	
lon t56	x	
blistered (bli)		
bli s100	x	small blister; vb
bli g100	x	small blister; vb
roller (rol)		
rol a50	autosome	very dpy; linked double; ♂ did not mate

"Dumpy" individuals made up the second largest class of mutants. The animals had a short and fattened appearance compared to wild type (Fig. 2).

"Small" - these animals were shorter than the wild type but did not have a fattened appearance (Fig. 2).

"Long" - these animals were longer and thinner than the wild type (Fig. 2).

"Blistered" - these mutants had a fluid filled blister on the cuticle (Fig. 3).

"Roller" - in these mutants, the animals rotated around their long axes as they progressed forward.

Mutant Characterization: Homozygous sex-linked recessive mutations were obtained in the following manner: mutant males from the F_2 plate were crossed to virgin wild type females and virgin female progeny (heterozygotes) were isolated. Large numbers of hemizygous males were obtained by streaking the remaining F_2 animals onto a large plate and allowing them to segregate F_3 progeny. Since one-half of the F_2 females would be heterozygous for the mutation, a large number of F_3 males (1/4 of the total) were mutant. The mutant males were crossed to the virgin heterozygous females and homozygous males and females were isolated. Mutations that were expressed throughout the life cycle permitted isolation of homozygotes with relative ease since mutants (male and female) could be isolated as larvae. When it was impossible to distinguish mutant from wild type larvae, it became necessary to grow

individuals in isolation. Dominant and semi-dominant mutants were isolated as homozygotes by recurrent selection of homozygous females that did not segregate any wild type progeny after three generations. Autosomal recessive mutants were followed by picking mutant gravid females onto a fresh plate and inspecting the progeny. If all of the adults were wild type in appearance the female had mated with a wild type male and all the progeny were heterozygotes. If one half of the progeny were mutant the female had mated with a heterozygous male. None of the females produced only mutant progeny. In both above-mentioned cases, gravid females were then transferred to a fresh plate where they segregated mutants in differing proportions. In all cases of autosomal recessive mutants, the male could not effect mating and a homozygous line could not be established. These mutants were not considered further.

Of the 52 variants initially isolated, 34 segregated the mutant phenotype in subsequent generations. Eleven homozygous lines were established including 3 autosomal and 8 X-linked mutations (Table II). The remaining mutants, 16 sex linked and 7 autosomal, could not be established as homozygous lines. In most cases the males were ineffective at mating but some mutants were discarded because of variable expression of the phenotype.

Table II. Chromosomal location of mutants rendered

homozygous.

Mutants homozygosed	Chromosomal location
unc t80	X
unc i2	X
unc i4	X
unc d38	X
unc u13	X
unc f20	X
unc u44	X
dpy q68	autosome
lon i3	X
sma s225	autosome
sma s40	autosome

Back Crosses: Homozygous virgin female mutants were
crossed to wild type males and the appearance of the
mutation in the progeny was scored.   Sex linked
recessive mutations were defined as those mutations
which appeared in the progeny with expression restricted
to males only.   Mutations which satisfied this criterion
but gave a substantial proportion of wild type males
were considered to have low penetrance and were discarded.
Mutations that expressed in both males and females were
considered semi-dominant since the heterozygote had an
intermediate phenotype.   In all such cases, the males
expressed the intermediate phenotype indicating that
none of the semi-dominant mutants were sex linked.
The semi-dominant mutants were not analyzed further.
A single dominant mutant (a dumpy) was isolated but had
a variable phenotype and was discarded.   Back cross data
are presented in Table III.

Genetic Nomenclature: Homozygous mutants were named
according to the system established for C. elegans
(Horvitz, pers. comm.).   Briefly: gene names refer to
relatively broad phenotypic categories.   The general name
refers to the phenotype originally detected or most
easily scored.   Different genes within the same general
category are distinguished by arabic numerals.   Each
mutation name consists of a lower case letter (b) followed
by an arabic numeral.   Suffixes indicating particular
characteristics of a mutation follow the mutation name.

Table III. Detection of sex linked mutations.
Phenotypes of progeny from homozygous
mutant females (phenotype in parentheses)
mated to wild type males are presented.
Preliminary mutation designations are used.

(r) - recessive

(sd) - semi-dominant

Parental Female	Progeny				Chromosomal Location
(uncoordinated)	unc♀	wt♀	unc♂	wt♂	
unc t80		120	104		X (r)
unc i2		140	116		X (r)
unc i4		239	208		X (r)
und d38		102	95		X (r)
unc ul3		148	171		X (r)
unc f20		164	180		X (r)
unc u44		147	119		X (r)
(Long)	lon♀	wt♀	lon♂	wt♂	
lon i3		132	119		X (r)
(Dumpy)		♀	♂		
dpy q68		174	192		autosomal (sd)
		all intermediate			
(Small)		♀	♂		
dpy s225		128	149		autosomal (sd)
sma s40		169	150		autosomal (sd)
		all intermediate			

These suffixes include: dm, dominant; sd, semi-dominant;
vb, variable phenotype. All mutations are assumed to be
recessive to wild type unless specified. A list of
Panagrellus mutations and gene names is given in Table IV.
The rationale for assigning gene names was derived from
results of complementation tests as described below.

Complementation: Only the sex linked uncoordinated
mutants were complemented. Crosses of two homozygous
mutants often resulted in a low number of progeny
because of the mating inefficiency of uncoordinated
males. The number of progeny could be increased by
utilizing a heterozygous female mated to a large number
of hemizygous males. The heterozygous female was
constructed by crossing three mutant larvae (all of the
uncoordinated mutants isolated expressed the phenotype
as larvae) with 10 wild type males. All normally moving
progeny were heterozygous females and could be distinguished
from males at the larval stages, hence the phenotypes
allowed easy isolation of virgin females. Three heterozy-
gous females (+/unc-a) were crossed with 20 males (unc-b)
and the appearance of mutant females in the progeny
indicated that the mutations did not complement. All
complementation tests were confirmed with homozygotes.
Mutations b1, b2 and b7 were non-complementing and were
considered allelic. All other mutations complemented each
other. Results are summarized in Table V.

Table IV. Genetic Nomenclature. Allelic mutations

were determined by complementation tests.

x - X chromosome

a - autosome