The University of Manitoba

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

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

Department of Zoology

Winnipeg, Manitoba

May, 1978

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ACKNOWLEDGEMENTS

The author expresses his deep appreciation to: Dr. B.J. Backmann for donating bacterial stocks, Dr. P.K. Isaac for the use of his microscopy facilities, Dr. J.J. Pasternak for communicating unpublished results and to the members of the supervisory committee. I wish to thank Ms. Sandi Chapman for her excellent technical assistance and many hours of useful discussion during the course of this work.

Finally, thanks are extended to Dr. Martin Samoiloff for continued patience, guidance and supervision. In every aspect of our friendship he has earned my deepest respect.

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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 $MgSO_4.7\ H_2O$ 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 $MgCl_2.6\ H_2O$, 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°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°C and stored at 4°C for up to one week.

RESULTS

Chromosome Constitution: 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.

Isolation of Mutants: 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 u13		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 heterozygous 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

Preliminary designation	Gene name	Mutation name	Chromosomal location	Strain
unc t80	unc-1	b1	x	B1
unc i2	unc-1	b2	x	B2
unc i4	unc-2	b3	x	B3
unc d38	unc-3	b4	x	B4
unc u13	unc-4	b5	x	B5
unc f20	unc-5	b6	x	B6
unc u44	unc-1	b7	x	B7
lon i3	lon-1	b8	x	B8
dpy q68	dpy-1	b9	autosome	B9
dpy s225	sma-1	b10	autosome	B10
sma s40	sma-2	b11	autosome	B11

Table V. Complementation tests for allelism of uncoordinated mutants. C - complementing; NC - non-complementing.

♀ \ ♂	b_1	b_2	b_3	b_4	b_5	b_6	b_7
b_1		NC		C	C	C	NC
b_2	NC			C	C	C	NC
b_3	C*	C*			C	C*	C*
b_4	C	C			C	C	C
b_5	C	C		C		C	C
b_6	C	C		C	C		C
b_7	NC	NC		C	C	C	

* CROSSES WITH HETEROZYGOUS FEMALES ONLY - NOT CONFIRMED WITH HOMOZYGOTES.

Mapping: Mutants were mapped by utilizing data from two factor crosses. Repulsion heterozygotes were constructed by crossing homozygous complementing mutants. In mapping uncoordinated mutants, three larvae (unc-a) were crossed to 20 males (unc-b) and all normally moving larvae were selected as repulsion heterozygous females. Thirty females were added to a large plate previously inoculated with 10 wild type males. Recombinants were scored as the number of wild type males among the progeny and numbers were corrected for parental males. The wild type is the only distinguishable recombinant (Fig. 4) and represents 1/2 of the total recombinants. This is a relatively insensitive test for linkage and large numbers of males must be scored. The lon-unc repulsion heterozygotes were constructed by adding three uncoordinated larvae to a plate seeded with long males. Moving progeny, repulsion heterozygous females, were treated similarly to the uncoordinated repulsion heterozygotes. Since both classes of recombinants can be scored (Fig. 4) this is a more reliable test for linkage. Mapping data are summarized in Table VI and the resulting map is presented in Fig. 5.

Table VI. Mapping data from two factor crosses utilizing repulsion heterozygous females.

A

Uncoordinated mutants (unc to unc distance)

Repulsion heterozygous ♀	♂ Progeny wt	♂ Progeny unc	♂ Progeny total	Recombination percent
b1/b2	0	808	808	0.0
b1/b4	23	1704	1727	2.7
b1/b5	73	837	910	16.0
b1/b6	8	1211	1219	1.3
b1/b7	0	1001	1001	0.0
b2/b4	18	1278	1296	2.8
b2/b5	65	918	983	13.2
b2/b6	7	992	999	1.4
b3/b5	0	810	810	0.0
b4/b5	51	937	988	10.3
b4/b6	9	873	882	2.0
b4/b7	9	887	896	2.0
b5/b6	70	1022	1092	12.8
b5/b7	72	934	1006	14.3
b6/b7	9	1202	1211	1.5

B

Long mutants (lon to unc distance)

Repulsion heterozygous ♀	σ Progeny wt	σ Progeny lon unc	σ Progeny unc	σ Progeny lon	σ Progeny total	Recombination percent
b8/b1	42	28	426	508	1004	7.0
b8/b2	55	35	560	568	1218	7.4
b8/b4	12	18	403	483	916	3.3
b8/b5	49	37	631	680	1397	6.2
b8/b6	38	17	490	418	963	5.7
b8/b7	58	36	656	706	1456	6.5

DISCUSSION

Stable visible mutations that are inherited in a classical Mendelian fashion can be produced in Panagrellus redivivus following EMS mutagenesis. The limited number of phenotypes involve changes in morphology or movement of the nematode and are similar to those observed in C. elegans (Brenner, 1974). No novel phenotypes characteristic of Panagrellus were observed. Mutations that severely alter movement or morphology cannot be maintained as homozygotes as males are ineffective at mating and can only be kept by recurrent selection of heterozygotes.

To obtain autosomal recessive mutants requires the animals to be grown until the F_3 after mutagenesis. At this point, the population initiated from a single mating pair would contain approximately 2×10^5 animals (S. Chapman, unpublished). A mutation generated in one of the gametes would appear in the F_3 with a frequency of approximately .0001 or in 20-25 of the F_3 individuals given that the plates could support such a large population.

X-linked recessive mutations will be expressed in the F_2 males following mutagenesis; and under the culturing conditions employed, it is possible to obtain a complete F_2 population. The isolation of individual F_1 larvae from mutagenized parents provides the most precise method of recovering mutants in subsequent generations.

Mutations will appear with predictable ratios allowing an initial assignment to the nature of the mutation (sex linked recessive, autosomal recessive, dominant, etc.). Large numbers of hemizygous males, obtained in the F_2, can be isolated from uncrowded cultures and the number of plates that require subculturing after the initial screen is greatly reduced.

Mutations are isolated with relative ease in C. elegans because of the self-fertilizing hermaphroditic nature of this nematode. Recessive mutations on all chromosomes are isolated in the F_2 after mutagenesis. These mutants will produce completely homozygous progeny. Mutants with extreme phenotypes are maintained with ease since functional males are not required to effect fertilization. Mutations are transferred between mutant strains by utilizing heterozygous males. Reversion of mutant loci in individual hermaphrodites would result in a slow increase in the frequency of the wild type allele in a population. In contrast, reversion of mutant loci in Panagrellus results in an increased reproductive capability of the male and the frequency of the wild type allele increases rapidly. Revertants have been detected in B2, B5, B6 and B7.

The detection of wild type revertants within mutant strains of Panagrellus indicates that b2, b5, b6 and b7 were point mutations. This is consistent with the proposed mechanism of EMS action, a high frequency

of G-C to A-T transitions (Auerbach, 1976). It is unlikely that large chromosomal rearrangements have been induced within the X chromosome of mutant strains since there is reasonable additivity in mapping distances.

Mapping mutants from two factor crosses provides unambiguous assignment of the order of genes on the X chromosome. Three factor crosses with only two discernable phenotypes would provide no additional precision regarding map distances but could be used to order mutants on the chromosome if ambiguities were observed (Fig. 4). This was not necessary for the mutants studied. Map distances from different reference genes (Fig. 5) provide reasonable additivity over the short distances involved. The reason for the low frequency of recombination between genes on the X chromosome is unknown; the situation resembles the clustering of mutant loci on the chromosomes of C. elegans (Brenner, 1974). The low recombination frequencies may reflect a small physical distance between genes or the genes may be associated with a region of the X chromosome with high interference which reduces crossover frequency. The possibility that the genes are within a region of high mutability cannot be disproved at this time.

The X-linked mutants were isolated from 1523 F_1 lines derived from crossing 2840 F_1 larvae with wild type

males. Assuming that each F_1 female larva would be successfully mated, the number of plates that gave no offspring represents the F_1 males. If the mutagen induced lethal mutations on the X chromosome of the female, a proportion of the F_1 males would not survive to be selected in the lines. Thus a deviation from a 1:1 ratio can be used as an index for mutagen effectiveness. The frequency of X chromosomes carrying lethal mutations is $\frac{206}{1523}$ or 0.135. A sex linked lethal assay was performed by isolating gravid F_1 females after mutagenesis and inspecting the progeny. Thirty of 126 tested females produced fewer than 50% male progeny, a crude ratio R = 0.24. The frequency of induced lethal mutations per X chromosome can be calculated from $1-\sqrt{1-R}$ (Brenner, 1974) and equals 0.13. Since the F_1 females carry mutagenized X chromosomes from both parents, the mutagen is equally effective in both sexes.

The 1523 selected lines produced 24 X-linked mutants (Table IV). The induced forward mutation rate for the X chromosome is $1/2 \times \frac{24}{1523}$ or 0.79% for this class of visible mutants. Assumption of an equal distribution over all 5 chromosomes yields a mutation rate of 4.0% for the entire genome and is similar to that observed in C. elegans (Brenner, 1974).

The average frequency of change per locus can be

calculated from the average frequency of observed mutants per locus divided by the total number of tested chromosomes (Demerec, 1934). The 8 X-linked mutants define 6 complementation groups with an average frequency per locus of $\frac{1.33}{3046}$ or 4.4×10^{-4}. The total number of genes on the X chromosome can be estimated assuming that the visible mutants that could be maintained constitute a representative subset of all visible mutants with an induced mutation rate equal to that of the lethal genes. The estimate of the total number of genes is given by (Demerec, 1934):

$$\frac{\text{frequency of change in all loci (or \% sex linked lethals)}}{\text{average frequency of change per single locus}}$$

or $\frac{.13}{0.00044} \approx 300$ genes with indispensable function. If the genes are equally distributed over all five chromosomes, the total number of genes is estimated as 1500 which is similar to the estimate of 2000 in *C. elegans* (Brenner, 1974).

The genome size of *Panagrellus* has been estimated as 0.09 pg by microspectophotometry and 0.097 pg from reassociation kinetics (J. Pasternak, pers. comm.). For largely sheared DNA, foldback, repetitive and single copy sequences comprise 9.3%, 26.1% and 61.3% of the genome respectively. Assuming an average molecular weight per nucleotide of 300, the haploid genome would contain approximately 9.5×10^7 nucleotide pairs (Hood *et al.*, 1975). Assuming an equal distribution of DNA in all 5

chromosomes, the X chromosome would contain 1.90×10^7 base pairs of which 61.3% or 1.2×10^7 base pairs represent unique sequences. This is enough information to code for 1.2×10^4 polypeptides of average molecular weight. The reason for the 30-40 fold excess of unique sequence DNA is not known but is similar to estimates in Drosophila (Manning et al., 1975) and C. elegans (Sulston and Brenner, 1974). The majority of the excess DNA may function to regulate the expression of a much smaller proportion coding for proteins (Lewin, 1974) and a number of interesting models have been proposed to explain this regulation (Georgiev, 1969; Britten and Davidson, 1969; Davidson et al., 1977). Studies of Panagrellus mutants with identifiable gene products (Kriger et al., 1977) have been proposed and may ultimately lead to a characterization of a specific genetic unit.

The corkscrew mutant previously described in Panagrellus (Pollock, 1974; Pollock and Samoiloff, 1976) was an autosomal dominant mutant which first appeared six generations after mutagenesis with N methyl-N-nitroso-N' nitrosoguanidine (NMG). This delayed appearance of a dominant mutant is suspicious and suggests that the mutation was spontaneous in nature and that the mutagen was ineffective. A sex-linked lethal assay utilizing NMG (Pollock, 1974) was performed to test the effectiveness of the mutagen. Twenty-one of 99 plates gave fewer than

50% males, a crude ratio R = 0.21. The frequency of induced lethals per X chromosome under the conditions employed by Pollock (1974) equals 0.11.

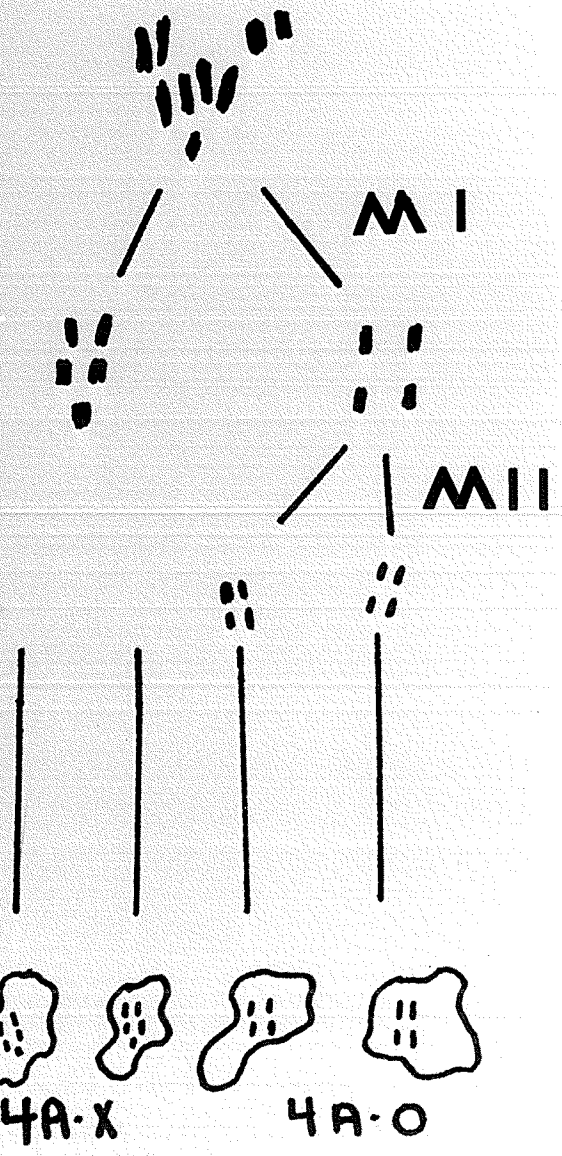
If the mutagen was effective, what could account for the failure to generate stable mutants in the earlier study? Besides the Cs mutant, a large number of variants were isolated which "proved to be unstable and showed damping out effects" (Pollock, 1974). Investigators have obtained similar results with putative mutants of C. elegans (R.L. Russel, pers. comm.) and many of these are isolated as dominant mutants (D.L. Baillie, pers. comm.). Analysis of population growth in Panagrellus (S. Chapman, unpublished) under monoxenic conditions reveals that the F_3 are produced from day 10 to day 17 after fertilization of a single female. The majority of F_3 progeny are produced on day 13 which agrees well with similar observations under xenic conditions (Samoiloff, unpublished). Under Pollock's screening protocol (Pollock, 1974), large numbers of animals were mutagenized, allowed to mate at random for 14 days and inspected visually for visible mutants. By day 14, 88% of the F_3 would be present in the culture but of these, 99% would be L_3 or younger. Thus, it is reasonable that the only mutant obtained was dominant in character and that the other variants behaved similarly to putative dominant mutants in C. elegans.

Mass screening for mutants can only add confusion when mutants are selected by visual inspection. The frequency with which autosomal recessive mutations can be expected in the F_3 is very low. Only a mutation with a very high induced forward mutation rate could be recovered by the procedure employed by Pollock. Once the variants were isolated, mutant A was crossed with mutant B and the future generations were inspected to try to recover the phenotypes. It would have been more precise to cross putative mutants with wild type to get the mutation(s) into a wild type background. Since the majority of putative mutants were behavioural in nature and possibly dominant in character, interactions between mutant loci might account for a part of the damping out effect.

Attempts to define the entire genome specifying development have already begun in C. elegans (Herman et al., 1976; Herman, 1978). Restrictions on the maintenance of visible mutants in Panagrellus would inhibit similar types of analyses. The use of conditional mutants provides the best tool to analyse paralytic mutants in Drosophila melanogaster (Suzuki, 1971), the equivalent of uncoordinated mutants in Panagrellus. A large number of temperature sensitive (ts) mutants has been described in C. elegans (Hirsch and Vanderslice, 1976) and ts alleles of unc-54, the structural gene for myosin (Epstein et al., 1976), have been described (MacLeod

et al., 1977). Unc-54 completely lacks muscular movement except for pharyngeal pumping. The possibility exists that a large number of the most severe phenotypes in Panagrellus may be isolated as conditional mutants.

Figure 1. Meiosis in the male gonad. Intensified Feulgen stain as described in Materials and Methods.



10μm

Figure 2. Morphological mutants: dpy-dumpy, sma-small, lon-long 90X.

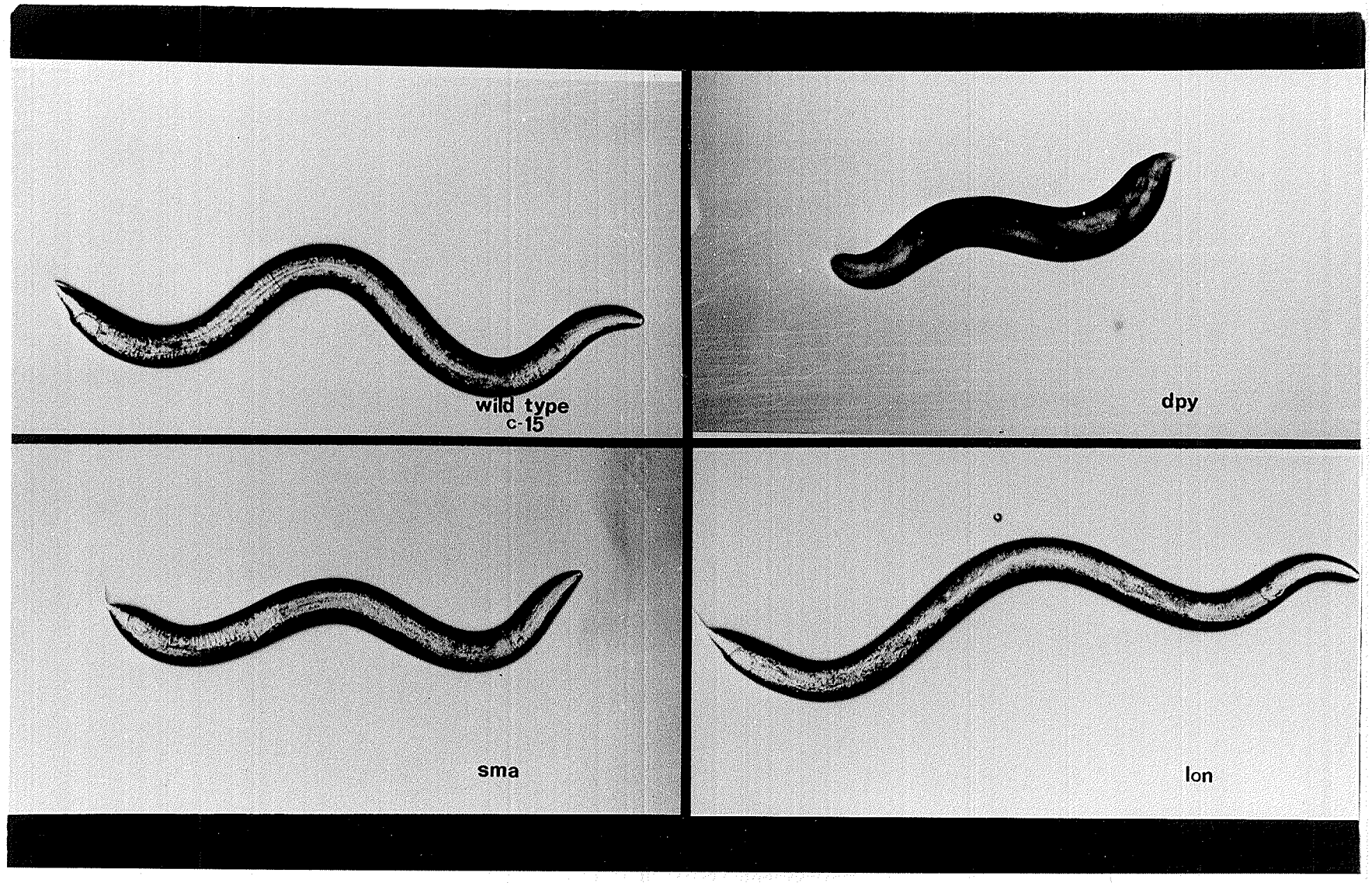

wild type
c-15
dpy
sma
lon

Figure 3. Blistered mutant. Nomarski interference contrast, lactophenol cotton blue. 190X.

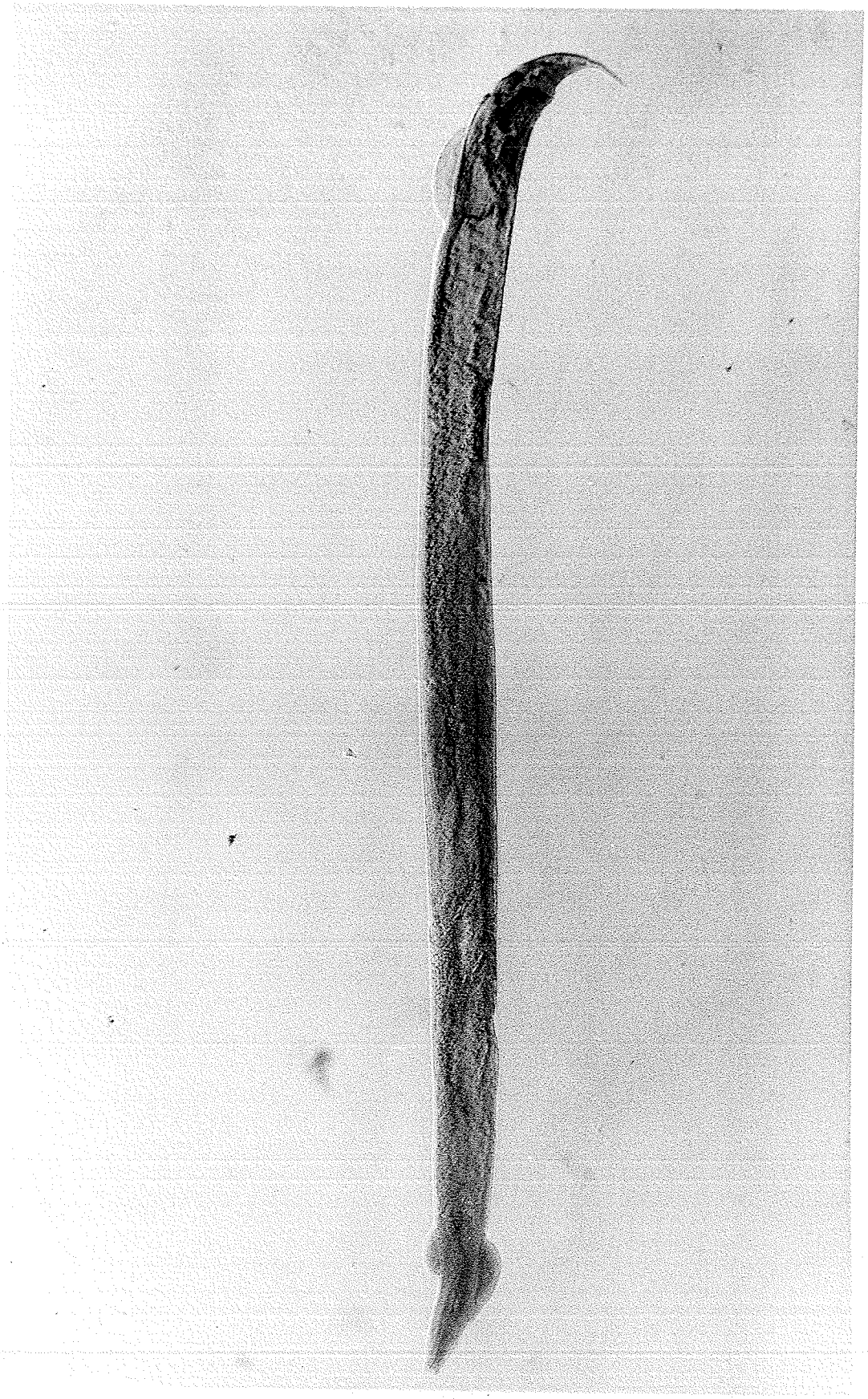


Figure 4. Segregation of chromosomes from heterozygous females. Phenotypes are indicated in parentheses.

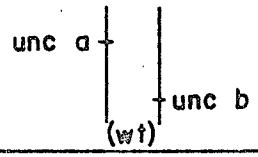
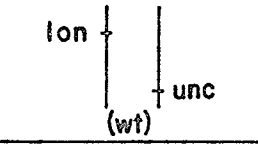
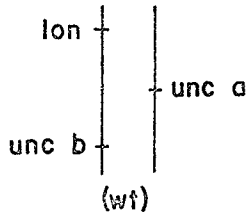
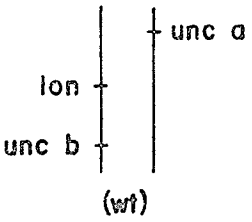
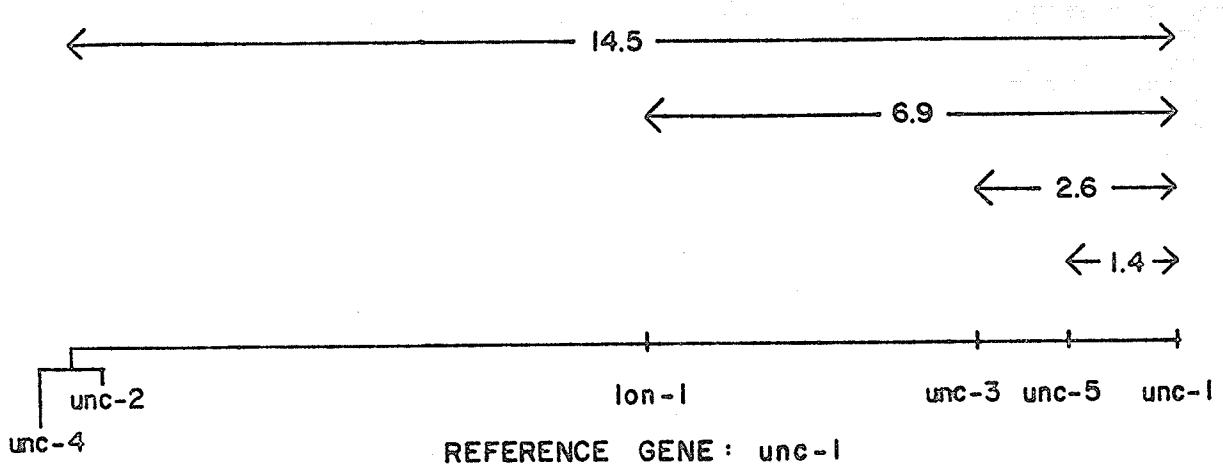
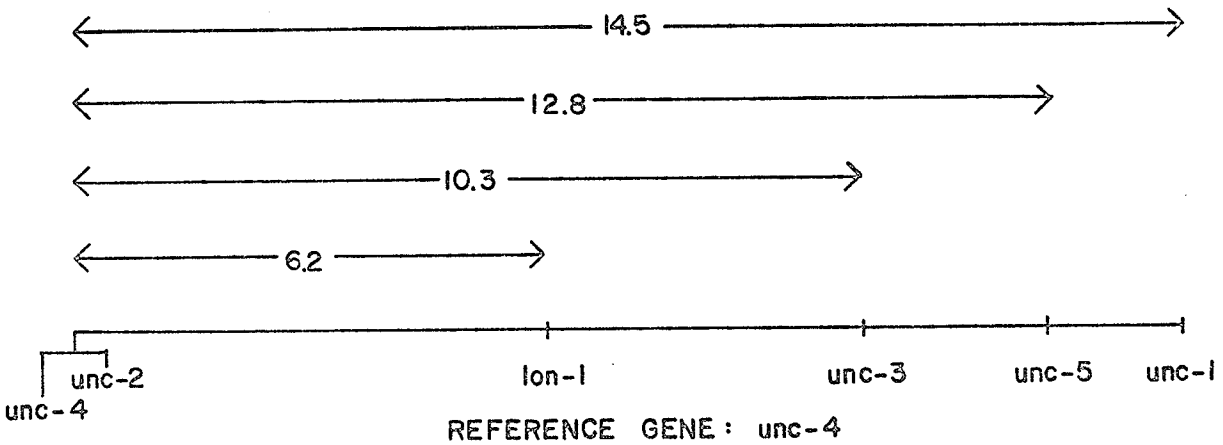
♀	♂	
	PARENTAL	RECOMBINANT
	unc a (unc) unc b (unc)	unc a, b (unc) wt (wt)
	lon (lon) unc (unc)	lon, unc (lon, unc) wt (wt)
	lon unc b (lon, unc) unc a (unc)	SINGLE CROSSOVER lon unc a (lon, unc) unc b (unc) unc a,b (unc) lon (lon) DOUBLE CROSSOVER lon unc a,b (lon, unc) wt (wt)
	lon unc b (lon, unc) unc a (unc)	SINGLE CROSSOVER wt (wt) unc a,b (lon, unc) lon (lon) unc a, b (unc) DOUBLE CROSSOVER unc b (unc) unc a lon (lon, unc)

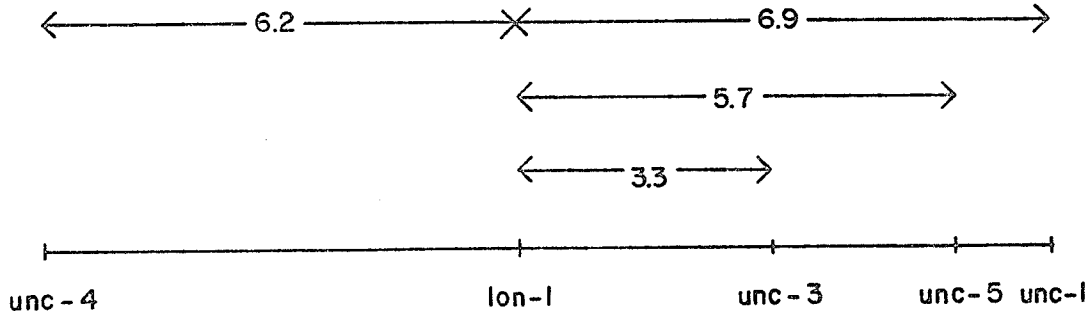
Figure 5. Genetic map of the x chromosome of Panagrellus redivivus. The ordering of genes is obtained from mapping data. 1m.u = 1 map unit (1% recombination)



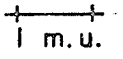
REFERENCE GENE : unc-1



REFERENCE GENE : unc-4



REFERENCE GENE : lon-1



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