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Lethal and Amanitin-Resistance Mutations in the *Caenorhabditis elegans* *ama-1* and *ama-2* Genes

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Manuscript received March 18, 1988
Revised copy accepted June 22, 1988

ABSTRACT

Mutants of *Caenorhabditis elegans* resistant to α-amanitin have been isolated at a frequency of about 1.6×10^{-6} after EMS mutagenesis of the wild-type strain, N2. Four new dominant resistance mutations have been studied genetically. Three are alleles of a previously identified gene, *ama-1 IV*, encoding the largest subunit of RNA polymerase II. The fourth mutation defines a new gene, *ama-2 V*. Unlike the *ama-1* alleles, the *ama-2* mutation exhibits a recessive-lethal phenotype. Growth and reproduction of N2 was inhibited at a concentration of 10 μg/ml amanitin, whereas *ama-2/+* animals were inhibited at 100 μg/ml, and 800 μg/ml was required to inhibit growth of *ama-1/+* larvae. We have also determined that two reference strains used for genetic mapping, *dpy-11(e224)V* and *sma-1(e30)V*, are at least four-fold more sensitive to amanitin that the wild-type strain. Using an amanitin-resistant *ama-1(m118)* or *ama-1(m322)* strain as a parent, we have isolated amanitin-sensitive mutants that carry recessive-lethal *ama-1* alleles. The frequency of EMS-induced lethal *ama-1* mutations is approximately 1.7×10^{-3}, 1000-fold higher than the frequency of amanitin-resistance alleles. Nine of the lethal alleles are apparent null mutations, and they exhibit L1-lethal phenotypes at both 20° and 25°. Six alleles result in partial loss of RNA polymerase II function as determined by their sterile phenotypes at 20°. All but one of these latter mutations exhibit a more severe phenotype at 25°C. We have also selected seven EMS-induced revertants of three different *ama-1* lethals. These revertants restore dominant resistance to amanitin. The selection for revertants also produced eight new dominant amanitin resistance alleles on the balancer chromosome, *nT1*.

THE nematode *Caenorhabditis elegans* is well suited to a genetic analysis of RNA polymerase II, the enzyme responsible for mRNA synthesis in eukaryotes. RNA polymerase II from *C. elegans* has been purified (Sanford, Prenger and Golomb 1985) and found to be similar to the enzyme from other eukaryotes (Lewis and Burgess 1982; Sentenac 1985). It consists of two large subunits with apparent molecular weights of 200,000 and 135,000, and eight smaller subunits ranging in size from 29,000 to 9,500. The gene encoding the largest subunit of this enzyme (*ama-1*) has been identified by selecting mutants resistant to the fungal toxin α-amanitin (amanitin). Strains carrying the dominant *ama-1(m118)* mutation produce an RNA polymerase II that is 150-fold less sensitive to amanitin *in vitro* than the wild-type enzyme (Sanford, Golomb and Riddle 1983). The region of chromosome *IV* surrounding *ama-1* has been genetically characterized, and deficiencies and duplications that include this gene have been identified (Rogalski and Riddle 1988).

An important part of the genetic analysis of RNA polymerase II in *C. elegans* is the isolation of mutations in genes encoding subunits of this enzyme. Such mutations may identify regions in particular polypeptides that are important for various aspects of enzyme structure and function. Mutant enzymes can be analyzed biochemically, and the mutations themselves can be characterized by genetic and molecular methods. Several lethal alleles of the *ama-1* gene have been identified, and the developmental effects of these mutations have been studied (Rogalski and Riddle 1988). Four of the alleles examined appear to be "hypomorphic," encoding altered RNA polymerase II enzymes with reduced activity, and a fifth may be a novel "antimorphic" mutation, resulting in an enzyme that interferes with wild-type activity.

In this paper we describe the isolation of 16 additional lethal alleles of *ama-1* by means of four different screening procedures. Nine of these 16 are apparent null mutations. We have also isolated intragenic revertants of three different lethal *ama-1* mutants. By selecting additional amanitin-resistant mutants in a wild-type genetic background, we have identified several new alleles of *ama-1* and one allele of a new gene, *ama-2 V*, which can mutate to confer dominant resistance to amanitin. Unlike the *ama-1* resistance alleles, the *ama-2(m323)* mutation exhibits a recessive lethal phenotype.

MATERIALS AND METHODS

Culture conditions: Nematodes were grown on NG agar plates spread with *Escherichia coli* strain OP50 (Brenner

1974). They were tested for resistance to amanitin by placing adult hermaphrodites in microtiter wells (Costar 96-well dishes) containing 50 μl of a 1% (w/w) suspension of OP50 in S medium (SULSTON and BRENNER 1974), and 20 μg/ml or 40 μg/ml α-amanitin (Sigma). Resistant hermaphrodites produced progeny that grew and reproduced in the presence of amanitin, whereas the progeny of sensitive hermaphrodites arrested development as first-stage (L1) larvae (SANFORD, GOLOMB and RIDDLE 1983). Several sensitive (wild-type) and resistant *ama-1(m118)* hermaphrotides were included in each test as controls. Stock solutions of 2 mg/ml amanitin were made up in 70% ethanol.

Genetic markers: The nomenclature used in this paper conforms to the recommendations of HORVITZ *et al.* (1979). All mutant strains are derivatives of the wild-type Bristol strain, N2 (BRENNER 1974). A complete list of lethal *ama-1* alleles is given in RESULTS, Table 2. The term "lethal" is used to describe sterile (genetically lethal) mutants as well as those arrested in development. An allele containing a lethal mutation, such as *m328*, derived from an *ama-1(m118)* parent was designated *ama-1(m118m328)* when the secondary mutation was within the *ama-1* gene, as determined by loss of resistance to amanitin and by complementation testing. Other mutations used are listed below by linkage group (LG).

LGI: *dpy-5(e61),unc-13(e450)*
LGII: *dpy-10(e128)*
LGIII: *dpy-18(e364)*
LGIV: *unc-33(e204),unc-17(e113* and *e245),dpy-13(e184), ama-1(m118,m313,m314,* and *m322), let-276 (m240), unc-5(e53),unc-8(e15)*
LGV: *dpy-11(e224),unc-23(e324),unc-42(e270),sma-1(e30),ama-2(m323),unc-76(e911)*
LGX: *sup-7(st5)*

The *ama-1* mutations listed above confer dominant resistance to amanitin, the *sup-7* amber suppressor (WATERSTON 1981) dominantly suppresses UAG nonsense mutations, the dumpy marker *dpy-13(e184)* and the uncoordinated *unc-8(e15)* marker are semi-dominant, and the remaining mutations are recessive to their wild-type alleles. The chromosomal rearrangements used in this work were the deficiencies *mDf9* and *mDf10* on LGIV (ROGALSKI and RIDDLE 1988), and *mDf3* and *ctDf1* on LGV (EDGLEY and RIDDLE 1987); the reciprocal translocations *eT1(III;V)* (ROSENBLUTH, CUDDEFORD and BAILLIE 1983), *nT1(IV;V)* (FERGUSON and HORVITZ 1985), or *nT1[let(m435)]*, which carries a recessive-lethal mutation; and the duplication *mDp1(IV;f)* or its derivative, *mDp1[unc-17(e113)](IV;f)*(ROGALSKI and RIDDLE 1988). The *nT1* translocation suppresses recombination on (balances) the right arm of chromosome *IV* from *lin-1* to *dpy-4* and the left arm of chromosome V from *unc-60* to *unc-76* (CLARK *et al.* 1988). Animals homozygous for *nT1* are viable, but slow growing and vulvaless (FERGUSON and HORVITZ 1985).

Amanitin-resistant mutants: All mutagenesis procedures were performed as described by BRENNER (1974), except that hermaphrodites were treated with 0.025 M ethyl methanesulfonate (EMS) from Sigma (ROSENBLUTH, CUDDEFORD and BAILLIE 1983). To obtain amanitin-resistant mutants in the F_1 generation, mutagenized wild-type (N2) hermaphrodites were placed on large (100 mm) Petri plates spread with OP50 and incubated overnight at 15° to lay unmutagenized eggs. The worms were then washed off the plates with S medium, collected in large (18 × 150 mm) glass tubes, rinsed until free of bacteria and resuspended in 2 ml S medium without food. After 2–3 days at 20°, the majority of the F_1 progeny were arrested as L1 larvae. These larvae were separated from the adults, collected in small (13 × 100 mm) glass culture tubes and resuspended in 2 ml of 2.5% (W/W) OP50 in S medium containing 20 μg/ml amanitin. The tubes were incubated with shaking at 20°, and after 1–2 weeks were screened for the presence of adults. Only larvae carrying a mutation conferring resistance to amanitin would be able to grow and reproduce under these conditions. To estimate the total number of F_1 larvae per tube, two 5-μl samples were removed from each tube before the addition of amanitin and spotted onto small Petri plates. These larvae were allowed to grow for 3 days at 20°, and the adults were counted.

To select for mutants in the F_2 generation, EMS-treated hermaphrotides were placed on large Petri plates (22 hermaphrodites/plate) and incubated at 20° for 4–5 days. At this time the plates contained F_2 eggs and larvae as well as F_1 adults. The worms were washed from these plates into large glass tubes (three plates/tube), rinsed until free of bacteria and resuspended in 2 ml of S medium without food for 3 days. The F_2 larvae were collected, sampled for counting and suspended in S medium containing OP50 and 20 μg/ml amanitin as described above.

Putative amanitin-resistant mutants were retested by placing individual adult hermaphrodites into microtiter wells containing amanitin, and screening the wells for the presence of adult progeny after 4 or 5 days at 20°C. Homozygous strains were established from hermaphrodites that segregated only resistant progeny when grown for a generation in the absence of amanitin. The *ama-1(m313), ama-1(m314)* and *ama-1(m322)* mutants were maintained as homozygotes, and the recessive-lethal *ama-2(m323)* mutation was balanced over *nT1* (FERGUSON and HORVITZ 1985).

The amanitin-resistance mutations were tested for dominance by mating males heterozygous for the resistance mutation with sensitive hermaphrodites (*unc-33 IV* or *dpy-13 IV*) for approximately 24 hr, then transferring the hermaphrodites to microtiter wells containing amanitin. The presence of adult cross-progeny in the wells after 4 or 5 days of incubation at 20° indicated that the mutation was dominant.

Linkage mapping of amanitin-resistance mutations: The *m313, m314* and *m322* mutations were tested for linkage to *dpy-13* by mating homozygous resistant hermaphrodites with *dpy-13/+* males to obtain hermaphrodites that were heterozygous for both mutations. At least 15 Dpy and Wild segregants from these individuals were placed in microtiter wells containing amanitin. If the resistance mutation were closely linked to *dpy-13*, none of the Dpy, but all of the Wild hermaphrodites were expected to be resistant to amanitin. If the mutation were unlinked to *dpy-13*, approximately ¾ of both progeny classes would be resistant.

The *ama-2(m323)* mutation was tested for linkage to *dpy-5 I, dpy-10 II, dpy-18 III, dpy-13 IV* and *dpy-11 V*. The procedure differed from that used for the *ama-1* alleles because of the recessive lethal *ama-2* phenotype. Dpy hermaphrodites that had been mated with *ama-2/+* males were placed in wells containing amanitin to select cross-progeny carrying the resistance mutation. Heterozygous hermaphrodites were removed from the wells as L4 larvae and allowed to reproduce in the absence of amanitin. Dpy and non-Dpy self-progeny were then tested to determine whether they were resistant to amanitin. If the two mutations were unlinked, ⅔ of the Dpy and non-Dpy progeny would be resistant. However, if the mutations were linked, most of the Dpy hermaphrodites would be sensitive, whereas most of the non-Dpys would be resistant. Linkage of *ama-2* to *unc-23 V* was tested in a similar manner, except that only

the Unc progeny of *unc-23 +/+ ama-2* hermaphrodites were tested for amanitin resistance.

Three-factor mapping of amanitin-resistance mutations: The *m313* and *m322* mutations were positioned relative to *dpy-13* using the procedure previously described (ROGALSKI and RIDDLE 1988) for mapping *ama-1(m118)*. The *ama-2* gene was positioned relative to *sma-1* and *unc-76* by the following protocol. The progeny of *+ sma-1 + unc-76/dpy-11 + ama-2 +* hermaphrodites were screened for Sma and Unc recombinants. When found, these recombinants were placed in wells containing amanitin to identify those carrying *ama-2*. Both resistant and sensitive Sma and Unc hermaphrodites would be expected if the *ama-2* gene were located between the two markers. Since homozygous *sma-1(e30)* hermaphrodites are hypersensitive to amanitin (see RESULTS), the Sma recombinants were tested for amanitin resistance at a concentration of 5 μg/ml instead of 20 μg/ml.

Two-factor mapping of *ama-2*: The recombination frequencies between *ama-2* and *unc-76*, and between *dpy-11* and *ama-2* were determined at 20° as follows. Ten L4 hermaphrodites of genotype *dpy-11 ama-2 unc-76/+++* were placed on small (60 mm) Petri plates, one per plate, and transferred twice at 24-hr intervals. All the progeny of these hermaphrodites were counted, and recombinants were scored as Unc or Dpy individuals. The formula $p = 1 - \sqrt{1 - (3U/[U + W])}$, where U = number of Unc recombinants and W = number of phenotypically Wild and Dpy progeny, was used to calculate the recombination frequency (p) with *unc-76*. The frequency of recombination with *dpy-11* was calculated using the same formula, substituting Dpy recombinants for Unc recombinants. Results are given with 95% confidence limits.

Deficiency mapping of *ama-2*: The *ama-2(m323)* mutation was tested for complementation with the *mDf3* deficiency (which deletes *unc-23*) by mating males of genotype *unc-23 ama-2/++* with *mDf3/eT1(III;V)* hermaphrodites. Unc progeny were expected only if the *ama-2* lethal were not deleted by the deficiency. In the case of *ctDf1*, *ama-2/+* males were mated with *ctDf1/nT1[unc(n754) let](IV;V)* hermaphrodites. The *nT1* derivative used to balance *ctDf1* carries a dominant *unc* mutation as well as a recessive lethal mutation. Thus, all of the non-Unc progeny from this cross carry *ctDf1*. Non-Unc L4 hermaphrodites were placed in wells containing amanitin. If *ama-2* were deleted by the deficiency, then all of the individuals tested would be sensitive to amanitin since no *ama-2/ctDf1* animals would have survived. However, if *ama-2* were not deleted by *ctDf1*, then half of the Wild progeny would be resistant.

Screen 1, lethals linked to *dpy-13*: Eight of the 16 lethal *ama-1* alleles characterized here were isolated as described previously (ROGALSKI and RIDDLE 1988). Briefly, *dpy-13 ama-1(m118)* hermaphrodites were treated with 0.025 M EMS and mated with N2 males. Semi-Dpy F_1 hermaphrodites were placed individually on plates, allowed to self, and the F_2 generation was screened for the presence or absence of fertile, adult Dpy segregants. The absence of Dpy progeny indicated that a closely linked lethal mutation had been induced on the *dpy-13 ama-1* chromosome. To identify mutations affecting *ama-1*, heterozygotes were tested for loss of dominant amanitin-resistance. In these tests, several adult semi-Dpy hermaphrodites were placed in microtiter wells containing amanitin, and the wells were screened for the absence of adult progeny after 4–5 days at 20°.

Screen 2, lethals linked to *unc-8*: The *ama-1(m118m221)* mutation was induced on a chromosome marked with *unc-8* instead of *dpy-13*. Hermaphrodites of genotype *dpy-13++/+ ama-1(m118) unc-8* were treated with 0.05 M EMS. Their heterozygous self-progeny were placed individually onto small plates, and after 4 days at 25° the plates were screened for the presence or absence of adult Unc progeny. Hermaphrodites carrying a lethal mutation in the *ama-1(m118)* gene were identified by scoring for the loss of amanitin resistance as described above.

Screen 3, lethals from frozen stocks: Four lethal *ama-1* alleles, *m118m328*, *m118m329*, *m118m332* and *m322m335*, were isolated as follows. Hermaphrodites of genotype *unc-17++ unc-5/+dpy-13 ama-1(m118* or *m322) +* were treated with 0.025 M EMS, placed on large Petri plates and incubated overnight at 15° or 20°. The worms were washed off these plates with S medium and collected in large glass tubes, rinsed until free of bacteria and resuspended in S medium for approximately 3 days at 20°. At this time, the majority of the F_1 progeny present had arrested development as L1 larvae. These worm suspensions were frozen in glycerol (BRENNER 1974), stored in liquid nitrogen, and aliquots were thawed as required. The thawed F_1 larvae were allowed to grow, and semi-Dpy L4 larvae or young adults were placed individually onto small Petri plates. The plates were screened for the absence of Dpy progeny as described above.

Screen 4, direct test for amanitin-sensitivity: The remaining three lethal *ama-1* alleles, *m118m396*, *m118m397* and *m118m398*, were generated as in screen 1, except that the F_1 semi-Dpy cross-progeny were tested directly for loss of amanitin resistance by placing them individually into microtiter wells containing amanitin. This bypassed the step in which strains carrying *dpy-13*-linked lethals were identified. A total of 945 wells were screened after 3 days at 20° for those containing arrested larvae. Such arrested growth indicated that the heterozygous hermaphrodite placed in the well was no longer resistant to amanitin, and carried a newly induced mutation in the *ama-1* gene. Strains were established by removing the adults and arrested larvae from the wells, and allowing them to recover and grow on plates in the absence of amanitin. The presence of a *dpy-13*-linked lethal mutation was confirmed by the absence of Dpy segregants, and complementation tests with *ama-1(m118m235)* were performed. Confirmed *ama-1* lethal mutations obtained in the four screens described above were balanced over *nT1(IV;V)* or *nT1[let(m435)](IV;V)*.

Lethal phenotypes: The terminal phenotypes of the lethal *ama-1* mutants were determined as previously described (ROGALSKI and RIDDLE 1988), except that the arrested larvae were classified by measuring their lengths, as described below. Comparison of the body lengths of the lethal mutants after eight days at 20° with the *dpy-13 ama-1(m118)* growth curve (Figure 1) revealed whether they were arrested as L1 or L2 larvae. The arrested larvae obtained at 25° were not measured, except for *m118m365*. The lethal phenotype of *ama-2(m323)* was determined by examining a synchronous population of progeny from *unc-46 dpy-11 ama-2/+++* parents for arrested larvae as previously described for *ama-1* lethals (ROGALSKI and RIDDLE 1988).

To determine the terminal phenotypes of mutants heterozygous for *mDf9* and a lethal *ama-1* allele, males heterozygous for *ama-1* (*e.g.*, *dpy-13 ama-1(m118m328)/++*) were allowed to mate with *mDf9/+* hermaphrodites for at least 24 hr. The mated hermaphrodites were transferred to a separate plate (20/plate), allowed to lay eggs for 2 hr and removed. The plates were screened after 24 hr for the presence of unhatched eggs, and after 48 hr for the presence of arrested larvae or slow growing Dpy larvae. When present, the mutant larvae were transferred to separate plates and observed for several days. The original plates were rescreened after another 3–4 days for the presence of

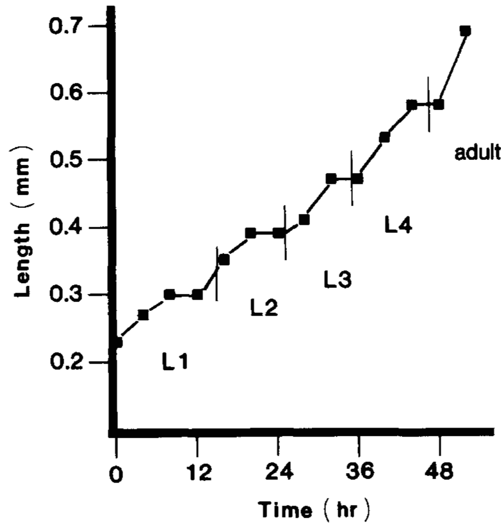

FIGURE 1.—Growth curve of the *dpy-13(e184) ama-1(m118)* strain at 25°. To construct this curve, a synchronous population of L1 larvae were obtained and allowed to develop at 25°. Their lengths were measured at defined intervals from hatching until after the last molt. The molting cycle of this strain was previously determined at 25° (ROGALSKI and RIDDLE 1988), and the times of each molt are indicated by vertical lines on the curve.

males to confirm that cross-progeny were produced. The terminal phenotypes of other heteroallelic mutants were determined in a similar manner. In these experiments, males heterozygous for *dpy-13 ama-1(m118m235)*, *dpy-13 ama-1(m118m236)*, *dpy-13 ama-1(m118m252)* or *dpy-13 ama-1(m322m335)* were mated with *dpy-13 ama-1(m118m328)*/++ hermaphrodites.

Growth of *dpy-13 ama-1(m118)*: To generate the growth curve for the *dpy-13 ama-1(m118)* strain (Figure 1), a sample of 15 animals developing synchronously at 25° was measured at 4-hr intervals for 52 hr. These larvae were killed by placing the tip of a hot soldering iron in the agar beside them, and measured with an ocular micrometer at ×50 magnification. To obtain a synchronous population of L1 larvae, fertilized eggs were collected by alkaline hypochlorite treatment of adult hermaphrodites (EMMONS, KLASS and HIRSH 1979) and suspended in M9 buffer for 12 hr at 20°. At this time, all of the larvae had hatched into the buffer, but further development was arrested due to lack of food. Development resumed when the larvae were placed on plates with OP50.

Suppression tests: Approximately 20 *unc-13 I/+; sup-7 X/O* males were mated with 12 heterozygous *ama-1* lethal hermaphrodites (*e.g.*, *dpy-13 ama-1(m118m328)/nT1*) for 24 hr, and ten of the mated hermaphrodites were placed individually into microtiter wells containing 40 or 80 μg/ml amanitin. The other two mated hermaphrodites were placed on plates and their progeny screened for the presence of males to confirm that the worms had mated. The wells were screened after 4–5 days for the presence of resistant cross-progeny, which would indicate that the *ama-1* lethal mutation was dominantly suppressed. All tests were done at 22.5°, an optimal temperature for growth of strains carrying *sup-7*.

Determination of generation time: Ten to 20 adult hermaphrodites of each strain listed in Table 4 were placed on a small plate to lay eggs for 2 hr and removed. The plate was examined at regular intervals until the F_1 progeny began to lay eggs. The time elapsed between placing the original hermaphrodites on a plate and the appearance of F_2 eggs was considered to be the generation time, plus or minus 2 hr.

Selection for revertants of lethal *ama-1* alleles: Amanitin-resistant revertants of several *ama-1* alleles were obtained using the selection procedure described above for isolating amanitin-resistant mutants in the F_1 generation. In these experiments, the EMS-mutagenized hermaphrodites carried a lethal *ama-1* allele balanced by either *nT1* or its lethal derivative. The translocation was used to prevent intragenic recombination between the lethal and the resistance mutation. The selections were done at either 15°, 20° or 25°, depending on the lethal allele being reverted, and final concentrations of 20 or 40 μg/ml amanitin were used.

Mapping revertants: Revertants of *ama-1* lethal alleles were tested for linkage to *ama-1* using the following procedure. Homozygous Dpy revertants were mated with N2 males, and semi-Dpy cross-progeny were selected and allowed to self in the absence of amanitin. Up to 50 F_2 semi-Dpy segregants were placed in microtiter wells and tested for resistance. If all of the semi-Dpy segregants produced resistant progeny, the site of reversion was considered to be closely linked (less than 3.0 map units) to *ama-1*. If a dominant suppressor of *ama-1* segregated independently of *dpy-13*, ¼ of the semi-Dpy hermaphrodites would be sensitive.

Linkage of amanitin-resistance mutations to *nT1*: Resistant semi-Dpy hermaphrodites obtained in the reversion screens were mated with *dpy-13/+* males. Both the Dpy, *dpy-13+/dpy-13 ama-1(m118mx)*, and Wild, *+/nT1(IV;V)*, cross-progeny were selected as L4 larvae and placed in microtiter wells containing amanitin. If all of the Wild, but none of the Dpy progeny were resistant, the mutation was linked to *nT1*.

Complementation tests: The procedure used for complementation tests between lethal *ama-1* alleles has been described previously (ROGALSKI and RIDDLE 1988). For complementation tests between *ama-2(m323)* and the dominant *nT1*-linked amanitin-resistance mutations obtained in the reversion experiments, hermaphrodites of genotype *unc-42 ama-2/unc-42 +* were mated with males heterozygous for the *nT1*-linked mutation, *e.g.*, *+/nT1[ama(m458)](IV;V)*. The non-Unc progeny issuing from this cross were selected, and their genotypes were determined by progeny testing. The presence of *unc-42 ama-2/nT1[ama](IV;V)* hermaphrodites indicated that the *nT1*-linked mutation complemented the lethal phenotype of *ama-2*.

RESULTS

Amanitin-resistant mutants: The *ama-1* gene, encoding a subunit of RNA polymerase II, was initially defined by the dominant amanitin-resistance mutation *m118* (SANFORD, GOLOMB and RIDDLE 1983). This gene is 0.05 map unit to the right of *dpy-13* on chromosome *IV* (ROGALSKI and RIDDLE 1988). We have selected additional amanitin-resistant mutants from the F_1 or F_2 progeny of EMS-mutagenized N2 hermaphrodites. Four dominant mutations were recovered at a frequency of approximately 1.6×10^{-6}. Three of these mutations are similar to *ama-1(m118)*; they all map close to *dpy-13 IV* and are viable as homozygotes. The fourth mutation, *ama-2(m323)*, is recessive-lethal and maps to chromosome *V*. The *ama-2* homozygotes arrest development during the L2 or L3 stage. No recessive amanitin-resistance mutations were detected in the screen of 5.2×10^6 F_2 progeny of EMS-mutagenized hermaphrodites.

The four resistance mutations were first tested for linkage to *dpy-13 IV*. Hermaphrodites heterozygous for *dpy-13* and each of these mutations were constructed, and their Dpy (*dpy-13/dpy-13*) and Wild (+/+) progeny were tested for resistance to amanitin. The results obtained for the *m313*, *m314* and *m322* mutations indicated that they were linked to *dpy-13*. In each case, all of the Wild segregants were resistant, and all of the Dpy segregants were sensitive. However, in the case of *m323*, almost half of the Dpy and Wild segregants tested were resistant, indicating that *m323* was not linked to *dpy-13*. The new gene defined by *m323* was designated *ama-2*, and it was found to be unlinked to *dpy-5 I*, *dpy-10 II* and *dpy-18 III*. By contrast, none (0/10) of the Dpy and 9/10 of the Wild segregants obtained from *dpy-11* +/+ *ama-2* hermaphrodites were resistant, suggesting that *ama-2* was linked to *dpy-11 V*.

To assess whether the dominant mutations on chromosome *IV* were alleles of *ama-1*, we positioned two of them relative to *dpy-13* by three-factor mapping, using hermaphrodites of genotype *dpy-13* + *unc-5*/+ *ama-1*(*m313* or *m322*) +. The data indicated that both *m313* and *m322* map to the *ama-1* locus. Two of the 188 Dpy recombinants obtained when mapping *m313* were resistant to amanitin, placing this mutation 0.02 map unit to the right of *dpy-13*. Similar results were obtained with *m322*, where 1/110 Dpy recombinants carried *m322*. The *m118* mutation was previously positioned 0.03 map unit to the right of *dpy-13* in a similar mapping experiment (ROGALSKI and RIDDLE 1988). The region of chromosome *IV* around *ama-1* is shown in Figure 2.

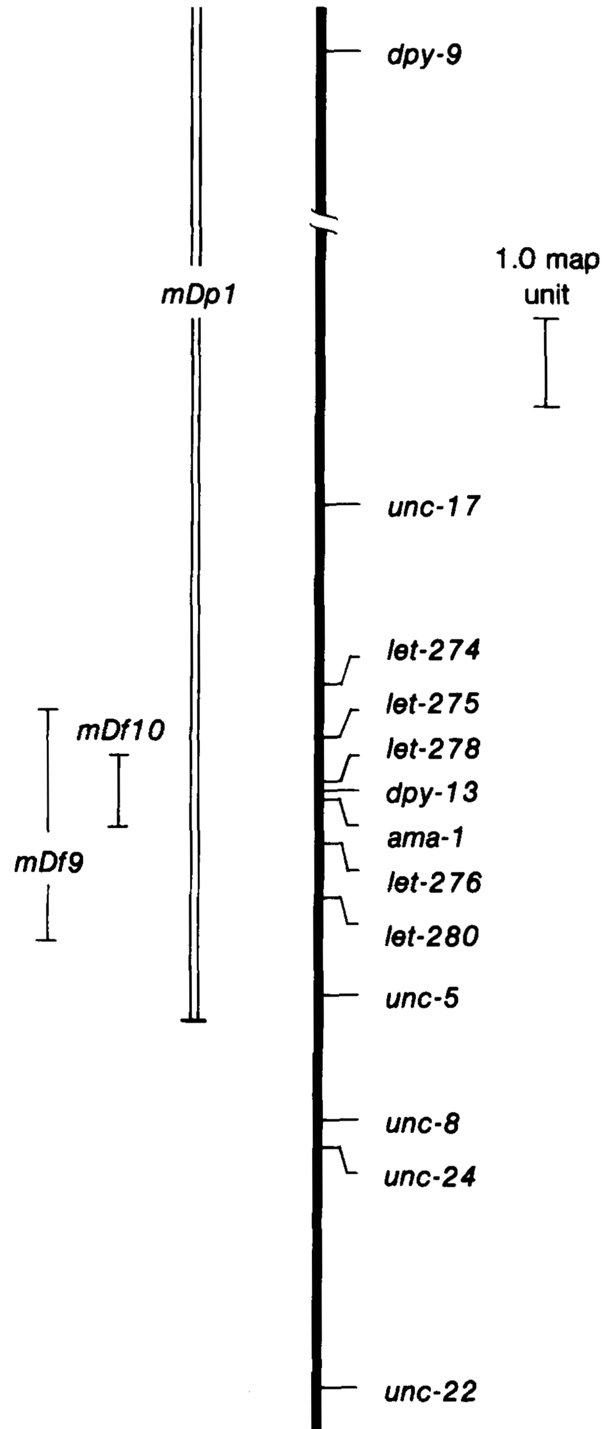

FIGURE 2.—A partial genetic map of the region around *ama-1 IV* showing the positions of the genes, deficiencies (*mDf*) and the duplication (*mDp1*) that were used in this study. In this vertical map, the left arm of LGIV is up and the right arm is down.

Mapping *ama-2*: The *ama-2*(*m323*) gene was found to map between the right end of the deficiency *ctDf1* and *unc-76* (Figure 3). To confirm linkage to chromosome *V*, a strain of genotype *unc-23* +/+ *ama-2* was constructed, and Unc segregants were tested for resistance to amanitin. Of 244 Unc individuals tested, 10 were resistant (of genotype *unc-23 ama-2/unc-23* +) indicating that *ama-2* is closely linked to *unc-23 V*. Hermaphrodites of genotype + *sma-1* + *unc-76/dpy-11* + *ama-2* + were used to position *ama-2* relative to *sma-1* and *unc-76*. Three of nine Sma recombinants, and ten of 20 Unc recombinants were resistant to amanitin, and thus, carried *ama-2*. These data place this gene in the interval between *sma-1* and *unc-76*. Two of the *sma-1 ama-2* recombinant chromosomes and one of the *ama-2 unc-76* recombinant chromosomes were balanced over *nT1* to confirm the presence of the *ama-2* lethal mutation. In all three cases, the lethal and resistance phenotypes segregated together.

The recessive-lethal *m323* phenotype was scored to position *ama-2* by two-factor mapping, using hermaphrodites of genotype *dpy-11 ama-2 unc76*/+++. The results placed *ama-2* 2.2 ± 0.4 map units from *unc-76* (37 Unc and 2487 Wild + Dpy) and 4.5 ± 0.6 map units from *dpy-11* (60 Dpy and 1975 Wild + Unc). The *dpy-11* to *unc-76* distance obtained in this experiment (2.2 + 4.5 = 6.7) agrees well with the genetic map (EDGLEY and RIDDLE 1987). Finally, the *ama-2* lethal complemented the two deficiences in this

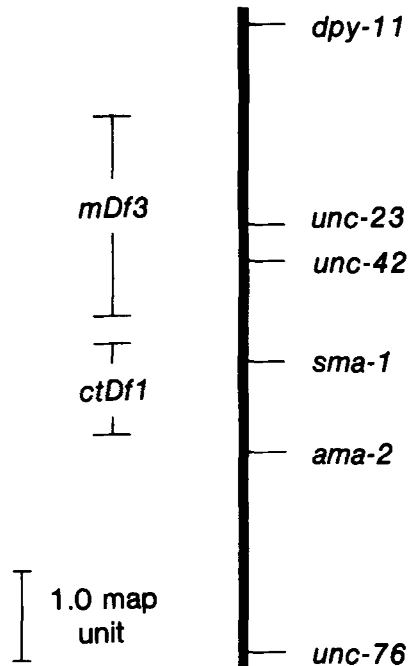

FIGURE 3.—A partial genetic map of LGV showing the position of *ama-2* relative to other genes in the region. The two deficiencies used to position *ama-2* are also shown. In this vertical map, the left arm of LGV is up and the right arm is down.

TABLE 1

Degree of amanitin-resistance in strains carrying *ama-1(m118)* or *ama-2(m323)*

	Concentration of amanitin (μg/ml)[b]					
Genotype of parent[a]	20	50	100	200	400	800
Wild-type	−	NT	NT	NT	NT	NT
ama-1(m118)	+++	NT	+++	+++	+++	++
dpy-13 ama-1(m118) let-276 / *mDf10* +	NT	NT	NT	+++	+++	++
dpy-13 ama-1(m118) let-276 / + + +	NT	NT	NT	+++	++	±
ama-2(m323) / *nT1*	+++	++	±	−	NT	NT

[a] One adult hermaphrodite was placed in each of six microtiter wells in 50 μl of a 1% suspension of *E. coli* in S medium with α-amanitin, and incubated at 20° for 5 days to assess growth.

[b] +++ indicates all adult progeny; ++ indicates ½ adult and ½ L4 progeny; ± indicates progeny developed to the L3 or L4 stages; − indicates progeny did not develop past the L1; NT: not tested.

region, *mDf3* and *ctDf1*. When taken together, the mapping data based on scoring amanitin resistance are fully consistent with the data obtained when the lethal phenotype was scored, and we tentatively conclude that both phenotypes result from the same mutation. However, the possibility that they result from two closely linked mutations has not been eliminated.

Amanitin-resistance phenotypes: Several strains carrying *ama-1(m118)* or *ama-2(m323)* were tested for growth in various concentrations of amanitin (Table 1). These data show that *ama-2*/+ hermaphrodites are not as resistant to amanitin as are *ama-1*/+ animals. Growth and reproduction of the *ama-2*/+ animals was inhibited by concentrations of 100 μg/ml and higher, representing a ten-fold enhancement of resistance over wild type. By contrast, 800 μg/ml was required before inhibition of *ama-1*/+ hermaphrodites was observed, representing nearly a 100-fold enhancement of resistance over wild type. The *ama-1* homozygotes were able to grow and reproduce well in the presence of 800 μg/ml of amanitin, which was the highest concentration used. Thus, it appears that resistance to amanitin is a semidominant trait. However, at the concentrations of amanitin normally used to score *ama-1*, the resistance phenotype is fully dominant. Results with the strain heterozygous for *m118* and a small deficiency, *mDf10*, that deletes the *ama-1* gene (Figure 2) showed that hermaphrodites with one *m118* allele but no wild-type allele were more resistant that *ama-1*/+ animals. No difference in sensitivity was detected between *m118/mDf10* and *m118/m118* animals. We have not yet examined the other *ama-1* resistance alleles to determine their levels of resistance to amanitin.

Amanitin-sensitive mutants: Results obtained while mapping *ama-2* suggested that hermaphrodites of genotype *dpy-11 ama-2/dpy-11* + and *sma-1 ama-2/sma-1* + were not resistant to 20 μg/ml amanitin, the concentration normally used to score the resistance phenotype. We determined that both the *dpy-11* and *sma-1* strains are between four and tenfold more sensitive to amanitin than N2, and this apparently cancels out the tenfold enhancement of resistance conveyed by *ama-2*. Growth of the *dpy-11* and *sma-1* strains was completely inhibited at an amanitin concentration of 5 μg/ml, whereas 20 μg/ml was required to inhibit N2 completely. Hence, the *sma-1 ama-2+/sma-1 + unc-76* recombinants obtained in the experiment described above were selected at a concentration of 5 μg/ml amanitin. The *unc-23*, *unc-42* and *unc-76* mutants, which were also used to position *ama-2*, were inhibited at a concentration of 10 μg/ml amanitin, but this slightly enhanced sensitivity may be partially due to the slower growth of some *unc* mutants in the microtiter wells.

Screens for lethal *ama-1* mutants: We previously isolated five recessive lethal and sterile mutations in *ama-1* (ROGALSKI and RIDDLE 1988). These EMS-induced alleles were identified as second-site mutations in the *ama-1(m118)* gene by scoring for the loss of the dominant amanitin-resistance phenotype of *m118*. Sixteen additional lethal alleles were isolated in this study using the previously described protocol (screen 1, MATERIALS AND METHODS), as well as newly developed procedures (screens 2–4, MATERIALS AND METHODS). The majority of the new mutations were isolated on a *dpy-13 ama-1(m118)* chromosome. The

two exceptions are *dpy-13 ama-1(m322m335)* and *ama-1(m118m221) unc-8.*

All of the 16 newly isolated amanitin-sensitive lethal mutations failed to complement the lethal *ama-1* alleles, *m118m235* and *m118m328*. The failure of the *m322m335* lethal mutation (a derivative of *m322*) to complement these alleles (both derivatives of *m118*) confirms that the dominant amanitin-resistance mutation *m322* is an allele of *ama-1*. The resistance alleles cannot be tested directly for complementation because of their dominance, but testing recessive-lethal derivatives of two different resistance alleles provides an indirect method of complementation. It should be noted that most of the *ama-1* lethals have not been tested for complementation with other nearby essential genes. However, these mutations map as points within the fine-structure genetic map of *ama-1* (BULLERJAHN and RIDDLE 1988), so it does not appear that any of the mutations are deficiencies that delete adjacent genes. The majority of these lethal mutations were isolated in screens large enough to determine a reliable mutation frequency. If we include our previous data (ROGALSKI and RIDDLE 1988), 17 *ama-1* lethal alleles were obtained after screening 9883 chromosomes. From these data (which exclude the four mutants generated in screen 3 from frozen stocks) we calculate an EMS-induced mutation frequency of 1.7×10^{-3} for *ama-1* lethals, which is three to four times the average target size for *C. elegans* genes (BRENNER 1974).

Terminal phenotypes of *ama-1* mutants: The terminal phenotypes of 21 lethal *ama-1* mutants are listed in Table 2. Five of these alleles were previously characterized (ROGALSKI and RIDDLE 1988), and their phenotypes can be summarized as follows. Mutant animals homozygous for *m118m252* arrested development late in embryogenesis when incubated at 20°, but arrested development during the L1 stage when grown at 25°. Two other alleles exhibited temperature-sensitive sterile phenotypes. Both *m118m238* and *m118m251* hermaphrodites were fertile at 20° but were sterile or nearly so at 25°. An effect of growth temperature on the terminal phenotypes of the two remaining alleles also was observed. At 20° all *m118m236* mutants completed larval development and became adults, but were never observed to produce eggs. In contrast, at 25° these mutants were L1 lethals. The *m118m235* mutant exhibited a variable phenotype at 20°. Some individuals arrested development as larvae, whereas other became adults and occasionally laid defective eggs. None of these mutants developed past the late L2 or L3 stages at the higher temperature.

By contrast with the initial set of five mutants, the majority of the new mutations (9 of 16) resulted in developmental arrest during the L1 stage, as determined by their terminal body lengths. (All of the arrested mutant larvae were between 0.25 and 0.3 mm in length.) The terminal phenotype of these alleles was not affected by growth temperature, and this is consistent with their being null, "loss of function" mutations (see below). One allele was obtained that arrested development during the L2 stage (between 0.3 and 0.4 mm in length) at 20° and the L1 stage (between 0.25 and 0.3 mm in length) at 25°. The remaining six mutants were able to complete larval development at 20°. Four of these exhibited phenotypes similar to the *m118m235* and *m118m236* mutants described above, whereas the other two were maternal-effect embryonic lethals. All but one of these hypomorphs exhibited a more severe phenotype at 25° than at 20° (Table 2).

Mutant hermaphrodites of genotype *dpy-13 ama-1* that either failed to lay eggs or laid eggs that did not hatch were mated with N2 males to determine whether any of the mutants could produce viable progeny by fertilization with wild-type sperm. Only in the case of *m118m371* (one of the least severe *ama-1* alleles) were viable cross-progeny produced, and these crosses averaged only three progeny per hermaphrodite. These results show that, in general, the *ama-1* sterile mutants do not produce functional oocytes.

Null *ama-1* alleles: Our previous data suggested that the null phenotype of *ama-1* was developmental arrest during the L1 stage. Mutants homozygous for a small deficiency, *mDf10*, that deletes the *ama-1* gene were able to complete embryogenesis, but arrested development as L1 larvae (ROGALSKI and RIDDLE 1988). Thus, the nine L1-lethal *ama-1* alleles isolated in this study are most likely nulls. To determine whether any of the L1-lethal alleles were amber nonsense mutations, we tested for suppression by the amber suppressor, *sup-7(st5)X* (WATERSTON 1981; BOLTEN *et al.* 1984). None of the nine L1-lethal alleles of *ama-1* were dominantly suppressed by *sup-7*, suggesting that they are not amber mutations, or if they are, that the insertion of tryptophan by the mutant *sup-7* tRNA does not significantly restore resistant polymerase activity.

Phenotypes of heteroallelic mutants: Several *ama-1* alleles were placed in *trans* to a deficiency that deletes *ama-1* to determine the effect of removing one copy of the mutant gene (Table 3). In general, the phenotypes conveyed by hypomorphic mutations are expected to become more severe when they are placed over a deficiency, whereas the phenotypes of null alleles should not change. The phenotypes of the three L1-lethal alleles tested, in fact, did not change. By contrast, deleting one copy of the *m118m235* (hypomorphic) allele resulted in a more severe phenotype. Instead of the variable (mid-larval to adult) phenotype exhibited by homozygous hermaphrodites, the

TABLE 2

Terminal phenotypes of *ama-1* alleles[a]

Allele	Lethal Screen	Temperature 20°	25°
m118m252[b]		Embryonic	L1
m118m221	2	L1	L1
m118m328	3	L1	L1
m118m329	3	L1	L1
m118m332	3	L1	L1
m118m364	1	L1	L1
m118m367	1	L1	L1
m118m370	1	L1	L1
m118m397	4	L1	L1
m118m398	4	L1	L1
m118m365	1	L2	L1
m118m235[b]		Mid larval to adult (ME)	Mid larval
m322m335	3	Mid larval to adult (ME)	Mid larval
m118m369	1	Mid larval to adult (ST)	Mid larval
m118m372	1	Mid larval to adult (ME)	Mid larval to adult (ME)
m118m236[b]		Adult (ST)	L1
m118m368	1	Adult (ST)	L1
m118m396	4	Adult (ME)	Mid larval
m118m371	1	Adult (ME)	Mid larval to adult (ST)
m118m238[b]		Adult (F)	Adult (ME)
m118m251[b]		Adult (F)	Adult (ME)

[a] Alleles are listed in order of decreasing severity of phenotype; ME, maternal effect embryonic lethal (homozygote lays eggs that do not hatch); ST, does not lay eggs; F, fertile, producing 70–90 progeny.
[b] Isolated previously (Rogalski and Riddle 1988).

TABLE 3

Terminal phenotypes of *ama-1* heteroallelic strains

Genotype[a]	Terminal phenotype (20°)[b]
mDf9/mDf9	Embryonic
ama-1(m118m252)	Embryonic
ama-1(m118m252)/ama-1(m118m328)	L1
ama-1(m118m328)	L1
ama-1(m118m328)/mDf9	L1
ama-1(m118m329)	L1
ama-1(m118m329)/mDf9	L1
ama-1(m118m332)	L1
ama-1(m118m332)/mDf9	L1
ama-1(m118m235)	Mid larval to adult (ME)
ama-1(m118m235)/mDf9	Mid larval
ama-1(m118m235)/ama-1(m118m328)	Mid larval
ama-1(m118m236)	Adult (ST)
ama-1(m118m236)/mDf9	Adult (ST)
ama-1(m118m236)/ama-1(m118m328)	Adult (ST)
ama-1(m322m335)	Mid larval to adult (ME)
ama-1(m322m335)/mDf9	Mid larval to adult (ST)
ama-1(m322m335)/ama-1(m118m328)	Mid larval to adult (ST)

[a] All *ama-1* chromosomes carry the linked *dpy-13(e184)* marker; *mDf9* deletes *dpy-13* and *ama-1*.
[b] Abbreviations are as described in Table 2.

m118m235/mDf9 mutants all arrested development as L3 or L4 larvae. This effect is similar to that seen by raising the incubation temperature of the homozygous mutants (Table 2). A smaller effect was observed in the case of *m322m335*. None of the adult *322m335/mDf9* hermaphrodites were observed to lay eggs, whereas some of the *m322m335* homozygous adults did produce fertilized, albeit defective, eggs. In contrast to the above two cases, no apparent difference in phenotype between homozygous and hemizygous mutants was observed with the *m118m236* hypomorphic allele. Additional heteroallelic animals were constructed, in which each of the three hypomorphic mutations were placed in *trans* with one of the null alleles. In all three combinations, the *m118m328* null allele behaved exactly like the *mDf9* deficiency (Table 3).

Embryonic-lethal *ama-1* allele: The *m118m252* allele may produce a novel protein product that interferes with the wild-type polymerase function. The *ama-1(m118m252)* homozygotes did not hatch at 20°, whereas *ama-1(m118m328)* null mutants hatched but arrested development during the L1 stage (Table 2). Animals heteroallelic for these two mutations exhibited a somewhat intermediate phenotype (Table 3). Most completed larval development and hatched. However, the L1 larvae produced were not as healthy as the *m118m328* larvae, and they died soon after hatching. This phenotype was essentially the same whether the maternal allele was *m118m252* or *m118m328*.

To investigate further the nature of the embryonic-lethal mutation, the generation time of strains carrying one or two copies of *m118m252* was determined, and compared with that of strains carrying one or two copies of the L1-lethal allele *m118m328*. Animals car-

TABLE 4

Growth characteristics of strains carrying *ama-1(m118m252)*

Genotype[a]	Generation time (hr) 20°	25°
dpy-13 ama-1(m118m328) / *nT1*	72	
dpy-13 ama-1(m118m252) / *nT1*	76	
dpy-13 ama-1(m118m328) unc-5; mDp1[unc-17(e113)ama-1(+)]	76	55
unc-17 dpy-13 ama-1(m118m398) +; mDp1[unc-17(e113)ama-1(+)] / *+ dpy-13 ama-1(m118m252)* unc-5	76	
dpy-13 ama-1(m118m252) unc-5; mDp1[unc-17(e113)ama-1(+)]	100	68

[a] The *ama-1(m118m252)* allele conveys an embryonic-lethal phenotype at 20°, and an L1-lethal phenotype at 25°. The other *ama-1* alleles convery an L1-lethal phenotype at both temperatures. The *nT1* translocation, used as a balancer, carried *dpy-13*(+) and *ama-1*(+). The duplication, *mDp1[unc-17]*, also carries these alleles, in addition to *unc-17(e113)*.

rying two copies of *m118m252* or *m118m328* were constructed using the free duplication *mDp1*. They are viable due to the presence of the wild-type allele of *ama-1* on *mDp1*. We calculated the generation time by measuring the time elapsed between hatching and subsequent egg-laying at 20° (Table 4). When there was only one copy of *m118m252* present (*m118m252*/+ or *m118m252/m118m398*/+) the generation time was 76 hr, which is essentially normal (BYERLY, CASSADA and RUSSELL 1976). However, when there were two copies in the strain (*m118m252/m118m252*/+) the generation time at 20° was slowed by 24 hr (32%). A smaller effect was seen at 25°, where the strain with two copies of *m118m252* was approximately 13 hr (24%) slower than the strain with two copies of *m118m328* (Table 4). The generation time did not differ significantly between strains with one or two copies of the L1-lethal allele (*m118m328*/+ *vs.* *m118m328/m118m328*/+). These results show that animals with only one functional *ama-1*(+) allele develop at normal rates, and that increased amounts of the *m118m252* product relative to the wild-type product slow development significantly. The slightly reduced deleterious effect of the *m252* mutation at 25° is consistent with the observation that the terminal phenotype of *m118m252* homozygotes is less severe at 25° (Table 2).

Revertants of *ama-1*: Reversion experiments were performed with 17 of the lethal *ama-1* alleles to identify intragenic revertants and unlinked suppressors of these mutations. A total of 15 amanitin-resistant revertants was obtained in a screen of 2.5×10^7 F_1 progeny of EMS-mutagenized animals (Table 5). Analysis of these revertants revealed that eight carried new amanitin-resistance mutations linked to *nT1* and seven carried apparent intragenic revertants. All of the revertants are dominantly resistant to amanitin.

Revertants were detected as rare amanitin-resistant progeny of mutagenized *dpy-13 ama-1(m118mx)/nT1(IV)*; +/*nT1(V)* hermaphrodites. In two selections (reversion of *m118m221* and *m118m364*), a derivative of *nT1* that carries a recessive lethal mutation in a gene other than *ama-1*, *let(m435)*, was used as the balancer chromosome. Normally, such hermaphrodites do not produce amanitin-resistant progeny, since they carry one nonfunctional *ama-1* allele on the *dpy-13* chromosome and one amanitin-sensitive, wild-type allele on the balancer chromosome. Thus, mutations that restored the dominant amanitin-resistance of *ama-1(m118)* were selected. Also, resistant animals were obtained when a new *ama-1* mutation was induced on the *nT(IV)* chromosome.

Seven of the 15 amanitin-resistant revertants were isolated as viable Dpy hermaphrodites, or semi-Dpy hermaphrodites that segregated viable Dpy progeny (Table 5). Homozygous Dpy animals were tested to determine whether the site of reversion was linked to *ama-1* by mating with wild-type males, and testing the F2 semi-Dpy segregants for resistance to amanitin. If suppression of the *ama-1(m118m236)*, *ama-1(m118m367)* or *ama-1(m118m370)* alleles was the result of an unlinked mutation, we would expect ¼ of these semi-Dpy individuals to be sensitive to amanitin. However, all of the hermaphrodites that were tested (usually 50) were resistant, indicating that the sites of reversion were closely linked (less than 3.0 map units) to *ama-1*. Thus, we conclude that all seven strains may be intragenic revertants. Four of the *ama-1(m118m236)* revertants did not completely restore wild-type polymerase II activity since homozygotes shifted from 20° to 25° as L4 larvae were sterile. Thus, it appears that restoration of amanitin resistance in these strains is not due to loss of the lethal mutation by gene conversion or intragenic recombination but, rather, to a third-site mutation. When

TABLE 5

Results of reversion experiments

Parental *ama-1* allele	No. of F_1 screened ($\times 10^{-6}$)	Amanitin-resistant individuals selected	Resistance mutations	Frequency[a] ($\times 10^{6}$)
Antimorphic allele				
m118m252	2.1	1 semi-Dpy 1 Vul[b]	2 Linked to *nT1*	0.9
Null alleles				
m118m221	1.6	2 semi-Unc	2 Linked to *nT1[let(m435)]*	1.2
m118m328	1.5	1 semi-Dpy	Linked to *nT1*	0.7
m118m329	1.8	0		
m118m332	1.0	1 semi-Dpy	Linked to *nT1*	1.0
m118m364	1.3	1 semi-Dpy	Linked to *nT1[let(m435)]*	0.8
m118m367	3.7	1 Dpy	Intragenic	0.3
m118m370	1.1	1 Dpy	Intragenic	0.9
m118m397	0.4	0		
m118m398	1.4	0		
Hypomorphic alleles				
m118m365	1.9	0		
m118m235[c]	1.2	1 semi-Dpy	Linked to *nT1*	0.8
m322m335[d]	1.2	0		
m118m369[d]	1.4	0		
m118m236[c]	1.4	5 Dpy[e]	5 Intragenic	3.6
m118m368[d]	1.0	0		
m118m396[d]	1.4	0		

[a] The frequency of reversion is the number of resistant individuals selected divided by the number of F_1 chromosomes screened.
[b] Vulvaless phenotype of *nT1* homozygotes.
[c] Selections were done at 15°.
[d] Selections were done at 25°.
[e] Four of the five are temperature-sensitive sterile.

putative intragenic revertants were detected, they occurred at a frequency similar to that for new amanitin resistance mutations, indicating that very specific types of mutation are required for reversion.

Seven amanitin-resistant individuals selected in the reversion experiments were semi-Dpy (or semi-Unc) hermaphrodites, that did not segregate viable Dpy (or Unc) progeny. Thus, amanitin-resistance could have been due to a dominant revertant that was also recessive lethal, or to a new amanitin-resistance mutation linked to *nT1*. To distinguish between these two possibilities, semi-Dpy hermaphrodites were mated with *dpy-13/+* males and the Wild and Dpy cross-progeny were tested for amanitin resistance. Approximately 20 Wild (*+/nT1*) and 20 Dpy (*dpy-13 ama-1(m118mx)/dpy-13+*) individuals were tested, and in each case, all of the Wild cross-progeny were resistant, whereas all of the Dpy cross-progeny were sensitive. These results are consistent with the presence of a new amanitin-resistance allele of *ama-1* on the *nT1(IV)* chromosome. Analogous crosses were performed with the two semi-Unc revertants of *m118m221*, and the same results were obtained. The one remaining revertant was isolated as an *nT1* homozygote. Since this strain has a vulvaless phenotype and cannot mate, it was not characterized further.

Some of these new amanitin-resistance mutations could have been alleles of *ama-2*, since both *ama-1 IV* and *ama-2 V* are balanced by *nT1*. However, all of these *nT1*-linked mutations were homozygous viable, except three that were induced on an *nT1* derivative carrying a recessive lethal mutation (Table 5). In these three cases, the lethal phenotype associated with *ama-2(m323)* was complemented. Hence, it is likely that the eight *nT1*-linked amanitin-resistance mutations obtained in the reversion experiments are all *ama-1* alleles. The frequency at which *nT1*-linked resistance mutations were recovered was about 3×10^{-7}, fivefold lower than the EMS-induced frequency in a wild-type genetic background.

DISCUSSION

In *C. elegans*, the *ama-1* gene encodes the largest subunit of RNA polymerase II, and it is defined by dominant amanitin resistance mutations (SANFORD, GOLOMB and RIDDLE 1983) as well as recessive lethal mutations. Mutations that result in an amanitin resistant RNA polymerase II also have been obtained in several mammalian cell lines (CHAN, WHITMORE and SIMINOVITCH 1972; AMATI *et al.* 1975; SOMERS, PEARSON and INGLES 1975; INGLES *et al.* 1976; WULF and BAUTZ 1976), and *D. melanogaster* (GREENLEAF *et al.* 1979). Using a combination of genetics and molecular biology, GREENLEAF and co-workers were able to show

that the *AmaC4* mutation, which confers dominant amanitin resistance in *Drosophila melanogaster*, is in the structural gene for the largest subunit of RNA polymerase II (SEARLES *et al.* 1982; GREENLEAF 1983). Three other amanitin resistant mutants were also found, but they did not produce an RNA polymerase II activity that differed from the wild type *in vitro* (GREENLEAF *et al.* 1979).

The amanitin resistance mutations obtained thus far in *C. elegans* define two genes, *ama-1 IV* and *ama-2 V*. The *ama-2* gene has been positioned on LGV between the right end of the deficiency *ctDf1* and *unc-76*. The mapping data obtained for the other amanitin-resistance mutations indicate that they are alleles of *ama-1*. We have shown that the *m322* mutation is in the *ama-1* gene, since the *m322m335* lethal derivative failed to complement *ama-1(m118m235)* and *ama-1(m118m328)*. Although we have not isolated lethal derivatives of the dominant *m313* or *m314* mutations, we assume that they are also *ama-1* alleles, based on their close linkage to *dpy-13*. Another eight amanitin-resistance mutations were induced on the *nT1* translocated chromosomes, and it is likely that these mutations are also in the *ama-1* gene. All of the resistance alleles are dominant to wild type; no recessive mutations were detected in the screen of F_2 progeny of mutagenized hermaphrodites. The frequency of EMS-induced amanitin resistance alleles in *ama-1* is more than 1000-fold lower than that of lethal alleles, indicating that the target is very small. The *ama-1* gene is about 8.7 kb in length, 5.9 kb of which is coding sequence (D. BIRD and D. L. RIDDLE, unpublished results). Thus, not more than one or two nucleotides appear to be able to mutate to confer a viable amanitin resistant phenotype. Sequence analysis of the mutant alleles should determined which nucleotides are involved in defining the amanitin binding site of this subunit.

Considering that *C. elegans* RNA polymerase II activity *in vitro* is 50% inhibited by 0.01 μg/ml amanitin and maximally inhibited at a concentration of 0.1 μg/ml (SANFORD, GOLOMB and RIDDLE 1983), the concentration of 20 μg/ml required to fully inhibit growth of wild-type worms is relatively high. The 200-fold difference may represent a permeability barrier, or possibly there is degradation of the amanitin *in vivo*. Very similar differences between *in vitro* and *in vivo* sensitivity to amanitin were observed in Drosophila (GREENLEAF *et al.* 1979).

When the level of *in vivo* amanitin resistance was compared among strain carrying various combinations of the *m118* and wild-type alleles of *ama-1*, we observed that strains with only one copy of *ama-1(m118)* but no wild-type copy (*i.e.*, *m118/mDf10*) were more resistant than the *m118/+* strain. In fact, the hemizygous hermaphrodites were as resistant as those homozygous for *m118*. These results suggest that the wild-type enzyme in the *m118/+* strain interferes with the function of the resistant enzyme in the presence of amanitin. In a heterozygote containing both sensitive and resistant polymerase, the sensitive enzymes may obstruct transcription by the resistant ones. This is consistent with the observation that amanitin inhibits RNA chain elongation by RNA polymerase II (COCHET-MEILHAC and CHAMBON 1974). Alternatively, the *ama-1(m118)/mDf10* strain may be producing as much resistant enzyme as the *ama-1(m118)* homozygote, and the higher level of resistant enzyme in comparison with *ama-1(m118)/+* may facilitate growth in the presence of the toxin. This latter hypothesis, that *ama-1* expression may be regulated, is currently being examined by measuring *ama-1* mRNA and protein levels in strains with one, two and three copies of this gene.

The *ama-2(m323)* mutation differs from the *ama-1* mutations in two respects. Whereas the *ama-1* resistance alleles are non-lethal and have little or no obvious effect on development or fecundity (ROGALSKI and RIDDLE 1988), the *m323* mutation is recessive-lethal. Also, hermaphrodites heterozygous for *ama-2(m323)* are about tenfold more sensitive to amanitin than those heterozygous for *ama-1(m118)*. The lethal and amanitin-resistance phenotypes of *ama-2(m323)* were never separated from each other in any of the mapping crosses, and thus appear to be due to the same mutation. The product of the *ama-2* gene is not known at the present time, but it appears to be required for larval development since *ama-2(m323)* mutants arrest development as L2 or L3 larvae. It is not yet known whether *ama-2/+* animals produce an amanitin-resistant RNA polymerase II. The *ama-2* gene may be analogous to the mutations in Drosophila that convey low levels of resistance and do not produce RNA polymerase II that differs from wild type *in vitro* (GREENLEAF *et al.* 1979). Such mutations may affect transport of the toxin from the environment into the cell nuclei, or affect the stability of amanitin in the animal. The *ama-2* mutation conveys such a low level of resistance (at most 10-fold more resistant to amanitin than wild type) that relatively small effects of markers used in genetic mapping (namely, *sma*-1 and *dpy-11*) were noticed. In combination with the abnormal sensitivity of these mutants, the *ama-2* mutation restored a level of resistance not much different from wild type. The *sma-1* and *dpy-11* genes both affect body size. However, similar markers used in the *ama-2* linkage test (*dpy-5*, *dpy-10*, *dpy-13* and *dpy-18*) did not noticeably reduce the amanitin resistance of *ama-2*.

The lethal *ama-1* alleles fall into three classes: null (no apparent gene activity), hypomorphic (some residual function) and a single antimorphic (deleterious)

allele. Nine of the 21 mutants exhibit the null phenotype, although none appear to be suppressible amber mutations. As one might expect, expression of these mutations is not affected by incubation temperature or by placing the mutant allele in *trans* to a deficiency. We previously concluded that the *ama-1* null phenotype is L1-lethal, based on the fact that this is the phenotype of animals homozygous for the small deficiency *mDf10* (ROGALSKI and RIDDLE 1988). Thus, there is sufficient maternally encoded RNA polymerase II to allow completion of embryonic development.

Eleven of the *ama-1* mutants appear to be hypomorphs with some residual RNA polymerase II function. With the exception of the *m118m365* mutant, which arrests development during the L2 stage, these mutants are all able to complete larval development at 20°. The *m118m372* (mid larval to sterile adult) mutant exhibits the same phenotype at both 20° and 25°, whereas the terminal phenotypes of the other alleles become more severe at the higher temperature. Generally, mutations with the same 20° phenotype exhibit similar 25° phenotypes. The *m118m236*, *m118m365* and *m118m368* alleles appear to lose all RNA polymerase II function at 25°, since they become L1-lethals at that temperature. Sixteen of the alleles have been positioned on the *ama-1* fine-structure map (BULLERJAHN and RIDDLE 1988). Four of the six hypomorphs that have been positioned are clustered very near the site of the *m118* mutation.

All of the lethal *ama-1* mutant hermaphrodites that complete larval development appear to be defective in the production of functional oocytes, since fertility is either not increased or only slightly increased by mating with wild-type males. Three of these strains do not produce eggs, whereas the other seven produce fertilized eggs that are defective. We have not determined whether these phenotypes are due to a specific defect in oogenesis or to an earlier defect in gonadal development. However, temperature-shift experiments on the *ts* mutant *m118m238* revealed a temperature-sensitive period centered on the initiation of egg laying (ROGALSKI and RIDDLE 1988). The effect of the hypomorphic mutations on oocyte production may be due to the inability of the altered enzymes to meet an increased need for mRNA synthesis during this period. Alternatively, these mutations may affect the interaction of RNA polymerase II with one or more specific transcription factors.

The availability of several deficiencies that delete *ama-1* (ROGALSKI and RIDDLE 1988), allowed us to determine the effect of gene dosage on the phenotypes of hypomorphic *ama-1* mutants. One mutant allele, *m118m235*, exhibited a more severe phenotype when placed in *trans* to the *mDf9* deficiency. However, the other alleles tested showed only a slight change in terminal phenotype (*m322m335*) or no change at all (*m118m236*). Apparently, in the latter two cases, reducing the amount of the mutant product by half was not sufficient to alter the mutant phenotype substantially, whereas reducing the *m118m235* product by half resulted in arrest at an earlier developmental stage. An alternative explanation for the observed results is that the amount of the *m118m236* and *m322m335* products remains the same in mutants with one or two copies of the gene. This latter interpretation requires that transcription or translation be regulated, and that this regulation does not occur in *m118m235* mutants.

The remaining *ama-1* allele appears to encode a thermolabile product that interferes with wild-type maternal RNA polymerase II during embryogenesis. The *m118m252* mutants exhibit an L1-lethal phenotype at 25° and a more severe embryonic-lethal phenotype at 20°. Mutations such as *m252* appear to be rare, since none of the 16 lethal alleles isolated in this study are embryonic lethals. Our further characterization of the *m252* mutation supports the idea that it is an antimorph. Placing the *m252* mutation in *trans* to an L1-lethal allele results in an intermediate phenotype at 20°. Hence, reducing the amount of the *m118m252* product alleviates the severity of the phenotype. Conversely, increasing the dosage of the *m118m252* product relative to wild-type RNA polymerase II adversely affects the growth rate. The generation time at 20° of the *m118m252/m118m252/+* strain is increased by 32% compared to the *m118m252/+* strain. This suggests that a predominance of the *m118m252* product antagonizes the function of the wild-type enzyme. The fact that *m118m252* homozygotes are L1-lethals at 25° suggests that the antimorphic activity may be thermolabile. That is, the *m118m252* protein does not interfere with the maternal polymerase to the extent that embryogenesis is arrested at 25°. An effect of higher temperature also was seen in the gene dosage studies, in which the generation time of the *m118m252/m118m252/+* strain was slowed relative to an *m118m252/+* strain by 32% at 20°, but only by 24% at 25°. Interference of abnormal enzyme with wild-type polymerase could result from competition for promoter sites, obstruction of RNA chain elongation, or even competition for other RNA polymerase II subunits.

The reversion analysis of the *ama-1* lethal alleles yielded seven revertants and eight new apparent *ama-1* mutations on the balancer chromosome. Only three of the lethal *ama-1* alleles were induced to revert. One intragenic revertant of each of two null alleles was obtained, whereas the hypomorphic allele *m118m236* yielded five putative intragenic revertants. The selections for revertants of the L1- and L2-lethal alleles were done at 20°. Two revertants and seven of the eight new amanitin-resistance mutations were re-

covered at this temperature. However, most of the experiments using the hypomorphic *ama-1* alleles were done at 25° because the earlier developmental arrest at this temperature made screening much easier. Although it is possible to recover amanitin-resistant individuals at this temperature using our methods (BULLERJAHN and RIDDLE 1988), no revertants or amanitin-resistance mutations were found in any of these screens. The five revertants of *m118m236* were selected at 15°. This allele exhibits a sterile phenotype at 15°, and presumably produces a more stable RNA polymerase II enzyme at this temperature than it does at 25°, where it exhibits an L1-lethal phenotype. It may be possible to obtain revertants of some of the other hypomorphic alleles by selecting at a lower temperature if the enzymes produced are more stable at 15° than at 25°.

No unlinked suppressors were found. In most experiments, between one and two million worms were screened, but the experimental design only allowed the recovery of dominant revertants. To obtain unlinked suppressors, it may be necessary to revert other alleles yet to be isolated, increase the size of each screen, or select for recessive revertants. Seven of the mutations obtained were not separated from their respective *ama-1* lethal allele and are tentatively judged to be intragenic. These revertants may be third-site mutations that restore activity of the mutant *ama-1* gene, or they could be the result of a true reversion event changing the lethal mutation back to the wild-type sequence. Since *nT1* was used in the selection, the revertants should not be the result of an intragenic recombination event. Four of the revertants exhibit a temperature-sensitive sterile phenotype, suggesting that RNA polymerase II function may not be completely restored. Some of these strains should provide altered enzymes suitable for biochemical analysis. Further genetic analysis of the revertants will be necessary to distinguish between closely linked intergenic suppressors and intragenic revertants. However, the results thus far show that the approach is feasible, and that revertants of at least some lethal alleles can be selected at a frequency comparable to that with which new amanitin resistance mutations are detected.

The majority of the lethal mutations described in this paper have been positioned within the *ama-1* gene by fine-structure mapping (BULLERJAHN and RIDDLE 1988). We plan to sequence appropriate regions of mutant genes to determine the mutational alterations responsible for particular phenotypes.

We thank C. RITTER and C. M. HAASE for invaluable help in isolating *ama-1* alleles. This work was supported by Department of Health and Human Services grant GM36674. T.M.R. was supported by a postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada, and D.L.R. was supported by Research Career Development Award HD00367. Some of the strains used in this study were obtained from the Caenorhabditis Genetics Center, which is supported by contract NO1 RR-4-2111 with the National Institutes of Health Division of Research Resources.

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Communicating editor: R. K. HERMAN