CHOLINE ACETYLTRANSFERASE-DEFICIENT MUTANTS OF THE NEMATODE *CAENORHABDITIS ELEGANS*

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ABSTRACT

We have identified five independent allelic mutations, defining the gene *cha-1*, that result in decreased choline acetyltransferase (ChAT) activity in *Caenorhabditis elegans*. Four of the mutant alleles, when homozygous, lead to ChAT reductions of >98%, as well as recessive phenotypes of uncoordinated behavior, small size, slow growth and resistance to cholinesterase inhibitors. Animals homozygous for the fifth allele retain approximately 10% of the wild-type enzyme level; purified enzyme from this mutant has altered K_m values for both choline and acetyl-CoA and is more thermolabile than the wild-type enzyme. These qualitative alterations, together with gene dosage data, argue that *cha-1* is the structural gene for ChAT. *cha-1* has been mapped to the left arm of linkage group IV and is within 0.02 map unit of the gene *unc-17*, mutant alleles of which lead to all of the phenotypes of *cha-1* mutants except for the ChAT deficiency. Extensive complementation studies of *cha-1* and *unc-17* alleles reveal a complex complementation pattern, suggesting that both loci may be part of a single complex gene.

GENETIC analysis can be of great value in studies on the mechanism and control of neural transmission. We have used the genetic advantages of the small soil nematode *Caenorhabditis elegans* to explore the roles of neurotransmitters and the enzymes of their metabolism on neural development and function. Our efforts have been focused on the neurotransmitter acetylcholine (ACh), partly because considerable evidence now indicates that ACh is an excitatory transmitter at nematode neuromuscular junctions (for references, see RUSSELL 1981 and JOHNSON and STRETTON 1980) and partly because evidence from *Drosophila melanogaster* suggests that mutations affecting the enzymes of ACh metabolism can have profound effects on neural development. In Drosophila, mutations affecting the biosynthetic enzyme choline acetyltransferase (ChAT) and the degradative enzyme acetylcholinesterase (AChE) have been isolated and have served to identify, respectively, the apparent structural genes *cha* (GREENSPAN 1980) and *ace* (HALL and KANKEL 1976). Most mutant alleles of either gene are lethal in early development when homozygous, as might be expected on functional grounds. More surprisingly, mutant patches homozygous for either sort of allele, in an otherwise normal background within a mosaic individual, exhibit marked neuroanatomical disorganization (HALL, GREENSPAN and KANKEL 1979; GREENSPAN 1980; GREENSPAN, FINN and HALL 1980).

This intriguing observation, suggesting dependence of neural structure on ACh-related functions, has been somewhat difficult to pursue in Drosophila because of the relative complexity of the nervous system and because no clear and accessible cholinergic synapses have yet been identified. It has been our hope that similar effects might occur in *C. elegans*, where the nervous system is a great deal simpler (302 neurons in total; SULSTON and HORVITZ 1977; J. WHITE, personal communication), and where homology with the larger nematode *Ascaris lumbricoides* permits cholinergic motor neurons to be identified with relative certainty (see JOHNSON and STRETTON 1977). In pursuit of this possibility, we have begun to analyze ChAT and AChE, both biochemically and genetically, in *C. elegans*.

Previous communications have described the multiple forms of AChE from *C. elegans* (JOHNSON and RUSSELL 1983) and the identification of two genes, *ace-1* and *ace-2*, which control the expression of two different classes of AChE forms (JOHNSON *et al.* 1981; CULOTTI *et al.* 1981). We have also described the purification and properties of ChAT from wild-type *C. elegans* (RAND and RUSSELL 1984). In the present study, we describe the isolation of mutants in a gene that we call *cha-1*, and we present evidence that *cha-1* is the structural gene for ChAT or a subunit of ChAT. In addition, we present evidence that *cha-1* may be a complex gene.

MATERIALS AND METHODS

Materials: Ethyl methanesulfonate (EMS) was obtained from K & K Laboratories, Inc. (Plainview, New York). Aldicarb [2-methyl-2-(methylthio)propionaldehyde O-(methyl carbamoyl) oxime; 97% pure] was obtained as technical grade from Union Carbide (Salinas, California). [^{3}H]choline (NET-109, 80 Ci/mmol) was purchased from New England Nuclear (Boston, Massachusetts). The ChAT assay data presented in Figure 1 and Tables 2 and 3 were obtained using [^{3}H-methylene]choline (prepared by reaction of sodium boro[^{3}H]hydride and betaine aldehyde) which was generously provided by CARL D. JOHNSON. [^{3}H]acetyl-coenzyme A (no. 23005, 9 Ci/mmol) was obtained from ICN (Irvine, California). Choline kinase was purified from brewers' bottom yeast (Sigma YBB) as previously described (MCCAMAN, DEWHURST and GOLDBERG, 1971; RAND and JOHNSON 1981). Sodium tetraphenylboron (TPB) was obtained from Fisher Scientific (Pittsburgh, Pennsylvania). Sephacryl S-200 and DEAE-Sephacel were purchased from Pharmacia Fine Chemicals (Piscataway, New Jersey). Affi-Gel Blue was obtained from Bio-Rad (Richmond, California). Neostigmine bromide, bovine serum albumin, all buffers and all other biochemicals were obtained from Sigma Chemical Company (St. Louis, Missouri).

Growth and culture: Maintenance and culture of the soil nematode *Caenorhabditis elegans* have been described previously (BRENNER 1974; DUSENBERY, SHERIDAN and RUSSELL 1975). Growth of synchronous populations was measured quantitatively using a nematode sizing counter developed in our laboratory (BYERLY, CASSADA and RUSSELL 1975, 1976). To obtain the large quantities of material necessary for biochemical purification, nematodes were grown in dense liquid suspension at 20° with *E. coli* strain NA22 as food source and harvested essentially as described by SULSTON and BRENNER (1974). Crosses were conducted in standard fashion at 20° (BRENNER 1974; HERMAN and HORVITZ 1980).

Strains: Wild-type *C. elegans* (Bristol) was used throughout this study (BRENNER 1974). All mutations and genetic markers discussed in this paper are on linkage group IV, except for *ace-2 I* and *ace-1 X*. Strains containing the following mutations were generously provided by the Caenorhabditis Genetics Center, Columbia, Missouri: *dpy-13(e184)*, *lin-1(e1275)*, *unc-17(e245)*, *unc-33(e204)*, *unc-77(e625)* and *vab-2(e96)*. The following *unc-17* mutant alleles were kindly provided by JONATHAN HODGKIN, Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom: *e113*, *e245*, *e283*, *e284*, *e327*, *e334*, *e335*, *e359*, *e464*, *e795* and *e876*. DAVID

HIRSH (University of Colorado) sent us the *cha-1* strain DH401 (*b401*), originally designated *tcf*^{r}*-1* (RUSSELL *et al.* 1977). The *mor-2* allele *e1125* was the gift of JAMES LEWIS, Columbia University. The *ace-2* strain GG202 and the *ace-2; ace-1* strain GG201 have been described previously (CULOTTI *et al.* 1981). All alleles with a *p* prefix were isolated in our laboratory, and all strains with a PR prefix were constructed in our laboratory. The *cha-1* strain PR503 (*p503*) was previously designated DH103 (RUSSELL *et al.* 1977). The *osm-3* strain PR802 (*p802*) was previously designated P802 (CULOTTI and RUSSELL 1978). All genetic terminology conforms to the standard *C. elegans* nomenclature (HORVITZ *et al.* 1979).

Mutagenesis and mutant selection: Animals were mutagenized in standard fashion (BRENNER 1974), using 0.05 M EMS. In the first mutagenesis, the *ace-2* strain GG202 was used. The doubly mutant *ace-2; ace-1* strain GG201 was used for the second mutagenesis. In both cases, potentially drug-resistant mutant F_2 animals were selected for improved movement and/or growth in the presence of 0.05 mM aldicarb. Further details are given in RESULTS.

Preparation of extracts for first mutant screen: The nematode strain to be tested was grown on two to five Petri plates and eluted with distilled water (approximate total wet weight was 20–100 mg). The animals were washed twice with water by successive rounds of centrifugation in a tabletop centrifuge, then brought up in a volume of 1 ml, and added dropwise to liquid nitrogen in a chilled mortar. The frozen droplets were ground to a fine powder, which was transferred to a test tube. After thawing, the extract was diluted with an equal volume of 200 mM Tris, pH 7.4, containing 20 mM 2-mercaptoethanol, 2 mM EDTA and 30% glycerol. This extract was used for ChAT assays and also for protein assays by the method of LOWRY *et al.* (1951), and the resulting activity was calculated as ChAT activity per milligram of protein in the crude extract.

Enzyme assays: To measure ChAT activity, we used primarily a sensitive radiometric assay developed in our laboratory (RAND and JOHNSON 1981). Briefly, the method involves incubating the sample with [^{3}H]choline, acetyl-CoA and neostigmine (a cholinesterase inhibitor) in a 4-ml assay/scintillation vial. At the end of the incubation, the reaction is terminated by adding ATP, $MgCl_2$ and a large excess of yeast choline kinase. After 10 min, during which all the unacetylated choline is phosphorylated, a standard, toluene-based scintillation fluid containing 10% isoamyl alcohol and 3 mg/ml of TPB is added; the samples are then shaken and counted in a scintillation counter. Acetyl-[^{3}H]choline formed by ChAT is extracted efficiently by the TPB into the organic phase, whereas the phosphoryl-[^{3}H]choline (derived from the substrate not acetylated by ChAT) remains in the aqueous phase, and its weak beta-disintegrations do not reach the scintifluors (RAND and JOHNSON 1981). Because this assay uses choline as the radioactive substrate with acetyl-CoA at saturating concentrations, it is relatively immune to interference from acetyl-CoA hydrolyzing activities and was, therefore, the assay of choice when ChAT was measured in crude homogenates. This same assay was also used in the determination of choline K_m values and for the thermal inactivation studies; for those experiments, however, purified enzyme (free from AChE) was used, and, therefore, neostigmine was omitted from the assay. For the determination of acetyl-CoA K_m values, we used a modification of FONNUM's method (FONNUM 1975; RAND and JOHNSON 1981), which employs [^{3}H]acetyl-CoA as the labeled substrate.

"Microscale" ChAT assay: This assay was developed to measure ChAT activity in small numbers of animals and was, therefore, useful in screening mutant clones and essential in analyzing progeny from crosses. Individual nematodes (or up to six or eight; see Figure 1) were transferred to 5 μl of 0.1 M Tricine, pH 7.5, containing 10 mM sodium thioglycollate, 1 M EDTA, 1 mM phenanthroline, 2 mg/ml of bovine serum albumin, 0.2% Triton X-100 and 20% glycerol in a chilled 1.5-ml polypropylene centrifuge tube (Eppendorf). The samples were then subjected to five cycles of freezing in liquid nitrogen and thawing, and care was taken that the samples were always kept below 10°. Five microliters of a cocktail consisting of 1 mM acetyl-CoA, 8 mM sodium thioglycollate, 4 mM neostigmine bromide, 200 mM NaI, 20% glycerol and 10–50 μCi/ml of [^{3}H]choline were then added (the choline was dried under a stream of nitrogen before use); the tubes were then capped and incubated for 12–18 hr at 10°. At the end of the incubation period, the reaction was terminated with 100 μl of 0.1 M Tris, pH 8.0, containing 10 mM ATP, 20 mM $MgCl_2$ and approximately 0.04 unit of yeast choline kinase. After 10 min, during which all the unacetylated choline was phosphorylated by the choline kinase, 1.1 ml of scintillation fluid (containing 3 mg/ml of TPB) added, and the tubes were capped, shaken, spun for 30 sec in an Eppendorf microfuge

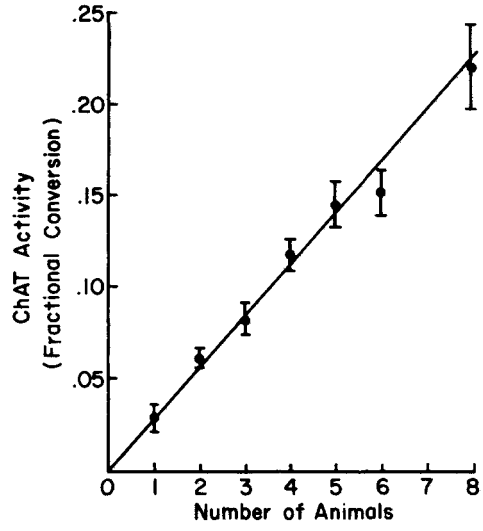

FIGURE 1.—Linearity of microscale ChAT assay. One to eight animals (82 ± 3 hr from hatch; wet weight about 4 μg each) were transferred to assay tubes and incubated at 10° for 16 hr as described in MATERIALS AND METHODS. Points represent means, and error bars represent standard deviations for four to six separate determinations. For this batch of [^{3}H]choline, the blanks were 70 cpm, which corresponded to 0.0035 of the 19,800 cpm of [^{3}H]choline present in each assay. The data point corresponding to one animal per tube represents a mean of 636 cpm (*i.e.*, 566 cpm above blank) or more than eightfold above blank.

and counted in a scintillation counter. ChAT activity is presented as fractional conversion, *i.e.*, the fraction of the choline initially present that was converted to acetylcholine, with blank subtracted (RAND and JOHNSON 1981).

When this assay is used, enzymatic activity is linear with the number of animals assayed (as long as they are synchronous; see Figure 1) and with time to at least 20 hr (data not shown). Preliminary studies had shown that, although ChAT activity per animal increased considerably during larval development, once the animals reached the egg-laying period of adulthood (about 60–110 hr from hatch), ChAT activity per animal was relatively stable (*i.e.*, did not vary more than 30% during this period). Thus, provided we assayed only adults, we were confident that we would be able to identify mutants with significant deficiencies of ChAT.

Purification of ChAT: We have already described the purification of ChAT from wild-type *C. elegans* (RAND and RUSSELL 1984), and the purification of the enzyme from the mutant PR1162 employed the same protocol. The procedure involved gel filtration on Sephacryl S-200, ion-exchange chromatography on DEAE-Sephacel and nucleotide affinity chromatography using Affi-Gel Blue; typical preparations were purified 4000-fold with 12% recovery, and the ChAT was completely free of AChE and acetyl-CoA hydrolases. Bovine serum albumin was added to the purified ChAT to a final concentration of 0.5 mg/ml, and the preparation was dialyzed against 10 mM Tricine, pH 8.0, containing 5 mM sodium thioglycollate, 1 mM EDTA, 1 mM 1,10-phenanthroline and 20% glycerol. Aliquots of the ChAT preparation were flash-frozen in liquid nitrogen and stored at −80°; when thawed, they were diluted to the desired activity with the buffer containing 0.5 mg/ml of bovine serum albumin.

Outcrossing protocol: PR503: The mutation present in PR503 was designated *p503*. PR503 was first crossed to *dpy-13(e184)*, and the linked double mutant (containing both *p503* and the *dpy-13* mutation) was isolated. Since animals homozygous for *p503* had no morphological or behavioral phenotype, this cross was scored using the microscale assay. The mutation causing the ChAT deficiency was then reisolated from the dumpy marker by a cross to wild-type males, and the

resulting strain, homozygous for the *p503* mutation and doubly outcrossed, was designated PR1162.

DH401: Strain DH401, homozygous for the *b401* mutation, was crossed twice to wild-type animals. In each cross, putative mutant homozygous F_2 progeny were identified on the basis of their uncoordinated behavior, and the presence of the *b401* mutation was confirmed by ChAT assay. The doubly outcrossed strain homozygous for *b401* was designated PR1158.

PR1151, PR1153 and PR1155: Each of these strains was first crossed to wild-type males, and F_2 progeny were selected that contained the ChAT defect but were wild-type at the *ace-1* and *ace-2* loci (for the cross of PR1151, only the *ace-2* locus was monitored). After a second cross to wild-type males, the mutants were mated with *dpy-13* males and recombinant F_2 animals, homozygous for both the ChAT defect and *dpy-13*, were isolated. This double mutant strain was then crossed once more to wild-type males, and the ChAT deficiency was separated from the *dpy-13* mutation by recombination. Once again, animals were identified at each stage by their behavioral phenotype, and then confirmed by subsequent ChAT assay. The quadruply outcrossed strains derived from PR1151, PR1153 and PR1155 were designated PR1152, PR1154 and PR1156, respectively, and the ChAT-deficient mutations they contained were named *p1152*, *p1154* and *p1156*, respectively.

PR1159: This strain was selected for further study, even though it contained apparently normal ChAT levels, because its uncoordinated phenotype very strongly resembled that of the ChAT-deficient strains. We show that this strain contains a mutation at the *unc-17* locus. PR1159 was outcrossed using the same protocol described earlier for PR1153 and PR1155 (except that the uncoordinated phenotype only was scored). The quadruply outcrossed strain was designated PR1160, and the mutation it harbored was named *p1160*.

Complementation: In all complementation tests, the hemaphrodite parent contained, in addition to the *cha-1* or *unc-17* allele to be tested, the closely linked marker *dpy-13(e184)*. This permitted us to determine unambiguously the phenotypes of all cross-progeny.

Enzymatic complementation using the *p503* allele of *cha-1*: Since males homozygous for the *p503* allele are behaviorally unimpaired, they could be mated directly to hermaphrodites homozygous for both another putative *cha-1* allele and *dpy-13*. Cross-progeny (*i.e.*, non-Dpy animals) were assayed for ChAT, using the modified ChAT assay described earlier (usually four hermaphrodites per assay in quintuplicate). ChAT activity approximately 50% of wild-type animals was considered to represent complementation (this was the enzyme level usually found in *p503*/+ individuals; see following data), whereas ChAT activity 10% or less of the wild-type represented noncomplementation between *p503* and the allele that was tested.

Behavioral complementation using uncoordinated *cha-1* and/or *unc-17* alleles: Wild-type males were mated to animals homozygous for one of the alleles to be tested (*e.g.*, the *a* allele), and the resulting heterozygous male cross-progeny (*a*/+) were then mated to hermaphrodites homozygous for the second allele (*e.g.*, the *b* allele) and also for *dpy-13*. The cross-progeny (at least 75 and usually 100–200 animals), both male and hermaphrodite, from this cross (identifiable by their non-Dpy phenotype) were examined. If all of the males and all of the non-Dpy hermaphrodites were normally coordinated (*i.e.*, non-Unc), then the two mutations complemented each other. The two alleles were scored as noncomplementing if approximately 50% of the cross-progeny (both males and hermaphrodites) were uncoordinated.

Enzymatic complementation using uncoordinated strains: This was carried out as in behavioral complementation, except that the uncoordinated heteroallelic animals (*i.e.*, genotype *a*/*b*) were assayed for ChAT activity using the microscale assay.

Gene dosage: For the uncoordinated ChAT-deficient strains, enzyme levels in heterozygotes were determined by mating wild-type males to the *cha-1* strains and, after 24 hr, allowing the mated hermaphrodites to lay eggs for a 5-hr period. Four days later, the normally coordinated hermaphrodite cross-progeny were assayed for ChAT activity using the microscale assay.

Heterozygotes containing the mild *p503* allele were constructed by mating males homozygous for *p503* to *dpy-13(e184)* hermaphrodites. Thereafter, the same protocol as used before was followed, except that non-Dpy hermaphrodite cross-progeny (*p503* +/+ *e184*) were assayed for ChAT. As a control for this set of assays, *e184*/+ hermaphrodites were constructed and assayed as well.

Mapping: Two-factor recombination data were obtained by analysis of the self-progeny of *cis*

double heterozygotes. In most cases, only one of the parental phenotypes and one of the recombinant phenotypes were scored. Recombination frequencies were calculated according to the method of BRENNER (1974). Three-factor crosses to determine the relative order of genes were performed in standard fashion (BRENNER 1974; HERMAN and HORVITZ 1980).

RESULTS

Isolation of mutants

Our isolation of ChAT-deficient mutants proceeded in three different stages. At the outset, reasoning that a ChAT deficiency might reasonably be expected to produce behavioral problems, we simply screened a set of 207 uncoordinated strains, a few of which were from other laboratories, but most of which had been previously isolated in our laboratory (COLEMAN *et al.* 1972). When small-scale extracts from these strains were assayed for ChAT activity (MATERIALS AND METHODS), most had normal or near-normal activity levels, but two strains, PR503 and DH401, had very low levels. PR503 was isolated by D. H. HALL in our laboratory as having a marginal uncoordination; in the initial assays, it exhibited ChAT levels 10–15% of those of wild type. DH401 was isolated by S. CARR and D. HIRSH of the Department of Molecular, Cellular, and Developmental Biology at the University of Colorado (unpublished results); it was originally selected by its resistance to the anthelmintic drug trichlorfon, and had less than 2% of wild-type ChAT activity levels. In addition to its drug resistance, DH401 showed marked uncoordination and some other properties to be discussed later.

Our second and third phases of mutant isolation were based on extensions of the observation that DH401 was trichlorfon resistant. Trichlorfon is an inhibitor of AChE, and its major effects are generally assumed to be due to the accumulation of ACh that would normally be hydrolyzed. The trichlorfon resistance of DH401 was, therefore, we thought, understandable: because of its marked defect in the biosynthetic enzyme ChAT, DH401 might be expected to synthesize less ACh and, therefore, might accumulate less ACh when trichlorfon was present to block AChE action. A logical extension of this rationale was that additional mutants resistant to other AChE inhibitors might include some with ChAT defects (as well as others, perhaps, with defects in other aspects of ACh handling, such as vesiculation or exocytosis). In our second and third phases, we specifically sought such inhibitor-resistant mutants as part of two mutant-screening efforts initiated for this and other purposes.

Both of these efforts included attempts to revert mutant alleles previously isolated in the gene *ace-2*, which we have indicated to be a structural gene for one of the two major classes of AChE in *C. elegans* (JOHNSON *et al.* 1981; CULOTTI *et al.* 1981; JOHNSON and RUSSELL 1983). Animals homozygous for mutant alleles of *ace-2* are especially sensitive to the AChE inhibitor aldicarb (CULOTTI *et al.* 1981); they are unable to grow in 0.05 mM aldicarb, whereas wild type can grow and reproduce at aldicarb concentrations at least fourfold higher. *A priori*, we expected two types of mutations that would permit the *ace-2* strain to survive in the presence of aldicarb: (1) reversion of the drug-sensitive *ace-2* mutation or (2) induction of a new, drug-resistance mutation

that was epistatic to the sensitivity of *ace-2*. We, therefore, constructed the double mutant containing both the *ace-2* mutant allele *g72* and the *b401* allele conferring trichlorfon resistance. As we had hoped, this double mutant strain was much more resistant to aldicarb than was the *ace-2* strain alone, which meant that survivors of the aldicarb regimen might include new ChAT mutants as well as potential *ace-2* revertants. We, therefore, selected among the F_2 progeny of mutagenized *ace-2* (strain GG202) animals for the ability to grow in the presence of 0.05 mM aldicarb. Approximately 1,800,000 F_2 progeny of the mutagenized animals were screened (representing approximately 240,000 mutagenized F_1 genomes), and 24 independent lines were derived that were reproducibly more resistant to aldicarb than the starting strain GG202. Individuals from the resistant strains obtained were assayed for ChAT activity, using the microscale assay, and also for AChE activity. Of the 24 strains, most had normal ChAT levels, but one strain, designated PR1151, had <1% of the wild-type ChAT activity.

Our third phase, undertaken partly to isolate additional ChAT mutants, and partly because the second phase had failed to yield any *ace-2* revertants, was similar in design, except that the starting strain was an uncoordinated *ace-2*, *ace-1* double mutant (strain GG201), and the scale was increased about tenfold. Preliminary experiments had already shown that the presence of the *ace-1* mutation affected neither the drug sensitivity of animals homozygous for *ace-2* nor the epistasis of the resistance present in DH401 to the sensitivity of *ace-2* (CULOTTI *et al.* 1981; D. L. KOLSON, J. B. RAND and R. L. RUSSELL, unpublished ressults). From approximately 2,900,000 mutagenized F_1 genomes tested, we obtained 290 lines that were apparently resistant to aldicarb: 205 lines were identified by their ability to move after 2 hr in the presence of the drug, and 85 lines (not necessarily independent of the 205 lines already isolated) were later chosen because of their growth and reproduction after 14–16 days on the drug. All of the 290 lines thus derived were assayed for ChAT using the microscale assay, and two of them proved to have extremely low ChAT levels; these strains were independent of each other and were designated PR1153 and PR1155. One of the remaining aldicarb-resistant strains, designated PR1159, was also retained and included in subsequent genetic analyses because of its extreme phenotype (see following data).

Phenotypes of ChAT-deficient strains

The ChAT-deficient mutants identified in this study are listed in Table 1. The phenotypes which will be described later were present in the ChAT-deficient strains obtained initially and also throughout all stages of the outcrossing protocol described in MATERIALS AND METHODS. Phenotypically, there appear to be two classes of ChAT-deficiency mutations, mild and severe. The mild category includes only *p503*; animals homozygous for this mutation have 10–15% of the wild-type ChAT activity and are behaviorally and developmentally almost wild type. It is just barely possible to distinguish populations of such animals from wild-type populations by quantitative measures of mobility, but such differences are minimal at best (S. GARDNER and J. RAND, unpublished observations).

TABLE 1

ChAT-deficient (cha-1) *strains*

Outcrossed strain	Mutant allele	ChAT in extracts (%)	ChAT/animal microscale (%)
N2	+	100.0 ± 1.0	100.0 ± 3.4
PR1152	*p1152*	0.0 ± 2.8	0.8 ± 3.4
PR1154	*p1154*	0.2 ± 0.5	1.8 ± 3.4
PR1156	*p1156*	1.7 ± 1.4	2.5 ± 3.4
PR1158	*b401*	1.1 ± 1.0	0.0 ± 3.4
PR1162	*p503*	8.3 ± 0.6	9.3 ± 0.3

Outcrossing protocol is described in MATERIALS AND METHODS. ChAT activity in extracts is given as specific activity (ChAT per mg of protein) as a percent of wild-type (N2) specific activity. The microscale assay data are presented as activity per animal as a percent of wild-type activity. For both sets of data, the errors correspond to 25% of the assay blank, which reflects the confidence of the measurement rather than the reproducibility of the activity levels.

The mutations leading to severe phenotypes are *b401*, *p1152*, *p1154* and *p1156*. When homozygous, these all lead to the same spectrum of behavioral, developmental, drug-resistance and enzymatic phenotypes. The behavioral, developmental and (based on preliminary data) drug-resistance phenotypes appear to be recessive traits; heterozygotes are indistinguishable from wild-type animals. The enzyme deficiency, as will be shown later, appears to be a semidominant trait. A full description of the behavioral and developmental alterations resulting from these mutations will be published elsewhere, but a brief summary of these phenotypes is given.

Behavior: The severe mutants all coil into tight spirals, especially as young juveniles. As the animals mature, they coil somewhat less but often lie in a tight, S-shaped double-coiled posture. At all stages of development, such animals have difficulty in propagating the waves of body muscle contraction necessary for locomotion, and they are, therefore, unable to move well. In addition, these mutations lead to a characteristic jerkiness when the animal tries to move backward; this is a specific locomotory defect not found in other classes of uncoordinated mutants (except for *unc-17* mutants; see following data).

Development: The severely ChAT-defective mutants are all slow growing and small. The growth of the juvenile stages seems particularly retarded (see Figure 2), so that the overall generation time for these strains is about 30–70% longer than for wild-type animals. In addition, these strains are smaller than wild type: mutant adults at the stage of maximum egg laying are about 30–40% smaller than their wild-type counterparts (even though they have taken longer to reach this comparable stage).

Drug resistance: The severe ChAT-deficiency mutations, predictably, all lead to resistance to cholinesterase inhibitors, since that is the phenotype by which they were isolated. All four lead to aldicarb resistance; this may be easily scored by the ability to grow in the presence of 0.5 mM aldicarb (or, in a strain homozygous for an *ace-2* mutation, growth in the presence of 0.05 mM aldi-

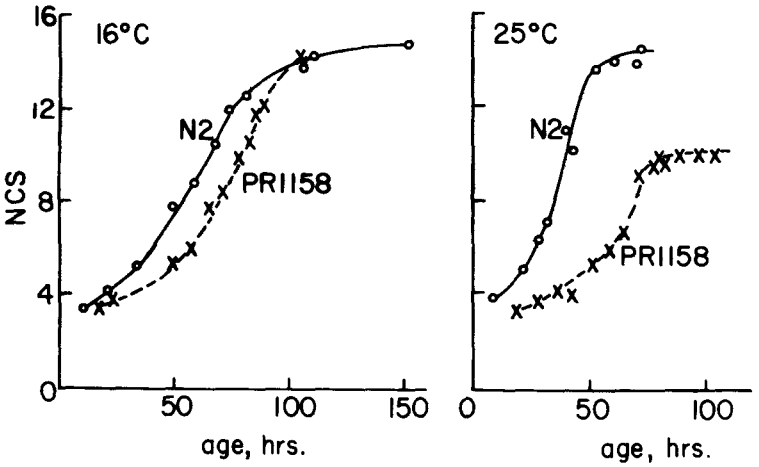

FIGURE 2.—Growth curves of the *cha-1* strain PR1158. Sychronous populations of either wild-type N2 (O) or the strain PR1158, homozygous for the *cha-1* allele *b401*, (X) were obtained by eluting newly hatched (0–2 hr) animals from Petri plates as described by BYERLY, CASSADA and RUSSELL (1976). Animals were grown on solid medium at the indicated temperature, and the size distribution of the populations was measured periodically using a nematode sizing counter. The output of the instrument (NCS on the ordinate) is a metric approximately equivalent to the animals' length. [See BYERLY, CASSADA and RUSSELL (1976) for details on the precise methods and data analysis.]

carb). In addition, animals homozygous for the *b401* mutation are known to be resistant to the inhibitor trichlorfon (the inhibitor against which it was isolated). Since all cholinesterase inhibitors presumably act by permitting accumulation of acetylcholine to toxic levels, and since the resistance of the strains described here is presumably due to undersynthesis of acetylcholine, we expect that these mutations lead to generalized resistance to all cholinesterase inhibitors.

Enzyme deficiency: All four of the severe ChAT-deficiency mutations lead to enzyme levels <2% of wild type. This is true for ChAT specific activities measured in extracts and also for microscale measurements normalized per animal (see Table 1). The exact amount of ChAT activity remaining in these strains is difficult to evaluate, because the apparent activity is so low that it is quite close to the value of the buffer blanks in our assays and is, therefore, subject to some error.

Within the overall framework just described, each mutation had distinctive characteristics. The greatest aldicarb resistance was conferred by *b401* and *p1154*; homozygotes for these mutations were able to grow in the presence of 1.5 mM aldicarb. The *b401* mutation is somewhat temperature sensitive; the uncoordinated behavior, slow growth and small size of *b401* homozygotes are all more extreme at 25° than at 16° (see Figure 2). The *p1152* mutation leads to the most extreme developmental and behavioral impairment. The behavioral and developmental consequences of *p1154* are less extreme than those of the other mutations.

Complementation for enzyme deficiency

The five ChAT-deficiency mutations were tested for allelism to each other by measuring ChAT activity in animals containing all possible pairwise combinations of the mutations in *trans* (see MATERIALS AND METHODS). As shown in Table 2, each of the five mutations fails to complement the other four for ChAT deficiency, indicating that they all have allelic defects within a common gene controlling ChAT activity. We have named this gene *cha-1* (for *ch*oline *a*cetyltransferase deficiency), and we have chosen *p1152* as the *cha-1* reference allele. We will present evidence in this paper that *cha-1* is the structural gene for ChAT (or a subunit of ChAT). In addition, each of the four severely defective mutations failed to complement the other three for nonenzymatic phenotypes; all *trans* double heterozygotes were uncoordinated, slow growing and small as adults. Thus, all four of these mutations have allelic defects producing these nonenzymatic phenotypes, and we argue that these phenotypes are secondary consequences of the ChAT deficiency. By contrast, heterozygous animals containing one *p503* allele and any of the severe alleles were behaviorally and developmentally normal and had enzyme deficiencies intermediate between those of the corresponding mutant homozygotes.

Gene dosage

Animals were constructed that were heterozygous for each of the *cha-1*

TABLE 2

Complementation of ChAT-deficiency mutations

Genotype of heteroallelic animal	ChAT activity	Age at assay (hr)
+/+	0.117 ± 0.009	82 ± 3.0
+/+	0.106 ± 0.033	76 ± 3.0
+/*e184*	0.109 ± 0.035	
p503/p1152	0.003 ± 0.001	83 ± 2.5
p503/p1154	0.005 ± 0.002	
p503/p1156	0.007 ± 0.004	
p503/b401	0.005 ± 0.002	
p1154/p1152	0 ± 0.004	133 ± 4.0
p1154/p1156	0.003 ± 0.004	
p1154/b401	0.002 ± 0.004	
p1156/p1152	0.005 ± 0.002	
p1156/b401	0.002 ± 0.002	
b401/p1152	0.001 ± 0.004	

Heteroallelic hermaphrodites were constructed as described in MATERIALS AND METHODS. Except for the first two lines, which represent wild-type controls, all animals contained the *dpy-13* (*e184*) allele (used as a marker) on the same chromosome as the right member of each pair of alleles. Each assay tube contained four animals; assays were conducted for 16 hr at 10°. ChAT activity is presented as the mean fractional conversion for four to seven separate determinations ± SE. Age of animals is given from hatch. The last six lines represent slow-growing animals; at 133 hr from hatch, they were at a stage of development approximately comparable to that of the controls.

mutant alleles, and these animals were compared with wild-type animals and with animals homozygous for the same mutant allele. Table 3 shows that, for each of the *cha-1* alleles, heterozygotes had ChAT levels approximately the mean of the wild-type and mutant homozygote values. It, therefore, appears that the ChAT activity per animal reflects the sum of the contributions of each of the two *cha-1* alleles present, a result consistent with the notion that *cha-1* is the structural gene for ChAT.

Analysis of mutant ChAT

Of the *cha-1* strains described earlier, only PR1162 (homozygous for the *p503* allele) had enough ChAT activity to analyze biochemically. Therefore, we purified the enzyme from PR1162 as described in MATERIALS AND METHODS and compared its kinetic properties with those of wild-type enzyme prepared by the same protocol (RAND and RUSSELL 1984). We had shown previously that the Michaelis constants of the wild-type enzyme for both substrates were significantly affected by the NaCl concentration in the assay (RAND and JOHNSON 1981; RAND and RUSSELL 1984), so we compared the mutant and wild-type enzymes over a range of NaCl concentrations. The results of these experiments are presented in Figures 3 and 4. The choline K_m of the mutant and wild-type enzymes both increased in an apparently linear manner with increasing salt concentration (Figure 3); however, at all salt concentrations tested, the choline K_m of the mutant enzyme was tenfold higher than that of the wild-type enzyme. The acetyl-CoA K_m of the mutant enzyme was also significantly altered, being 20- to 40-fold higher than the wild-type value at all NaCl concentrations tested (Figure 4).

The thermal sensitivities of these purified ChAT preparations were also compared. Inactivation was conducted at 22° in the presence of acetyl-CoA, which we have shown to stabilize the wild-type enzyme under these conditions

TABLE 3

Gene dosage

	Genotype of animal	ChAT activity	% of control
A	+/+	0.115 ± 0.003	100.0
	p1154/+	0.046 ± 0.004	40.0
	p1156/+	0.053 ± 0.006	46.1
B	+/+	0.120 ± 0.015	100.0
	b401/+	0.063 ± 0.012	52.5
	p1152/+	0.064 ± 0.016	53.3
	p1154/+	0.052 ± 0.011	43.3
C	+ +/+ *dpy-13*	0.109 ± 0.015	100.0
	p503 +/+ *dpy-13*	0.061 ± 0.011	56.0

ChAT activity is presented as fractional conversion ± SE. Assays were performed in quintuplicate, using four animals per assay; assays were conducted for 16 hr at 10°. A, B and C refer to different experiments conducted on different days. Ages of animals from hatch: A, 85 ± 3 hr; B, 98.5 ± 3 hr; C, 83 ± 2.5 hr.

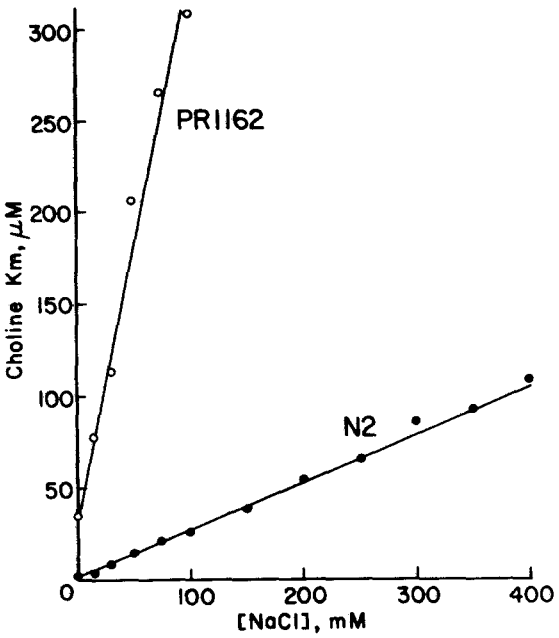

FIGURE 3.—Comparison of choline K_m from wild-type (N2) and *cha-1* mutant (PR1162) ChAT as a function of NaCl concentration. The choline K_m was determined using the [^{3}H]choline ChAT assay and adding different amounts of unlabeled choline to the labeled substrate. The assay buffer concentration was reduced to 10 mM, and NaCl was added to the desired concentration. For each substrate at each NaCl concentration, assays were performed in duplicate at eight to ten different substrate concentrations, which were chosen to bracket the K_m under those conditions. The data for each determination were graphed as an Eadie-Hofstee plot (V *vs.* V/S), and the slope (equal to $-K_m$) was determined by the method of least squares. The correlation coefficient, *r*, for each determination was between −0.956 and −0.995.

(RAND and RUSSELL 1984). As seen in Figure 5, ChAT from the *cha-1* strain PR1162 is significantly more thermolabile than the wild-type enzyme. In addition, the presence of acetyl-CoA during the incubation at 22° had almost no effect on the inactivation kinetics of the mutant enzyme, although it significantly improved the stability of the wild-type enzyme (data not shown). Like the gene dosage result, these qualitative differences between ChAT from PR1162 and wild type are consistent with the notion that *cha-1* is a structural gene for ChAT.

Mapping

S. CARR and D. HIRSH (personal communication) had mapped the *b401* allele of *cha-1* to the left arm of linkage group IV, near the genes *unc-17* and *unc-33*. They were also able to show that *b401* complemented both the *unc-17* allele *e245* and the *unc-33* allele *e204* for the uncoordinated phenotype, which suggested that *b401* was not an allele of either of these previously identified genes. We have confirmed these results. In addition, since it was remotely possible that our *cha-1* mutants might be alleles of one of the other genes known to map in the same general region (and identified and characterized

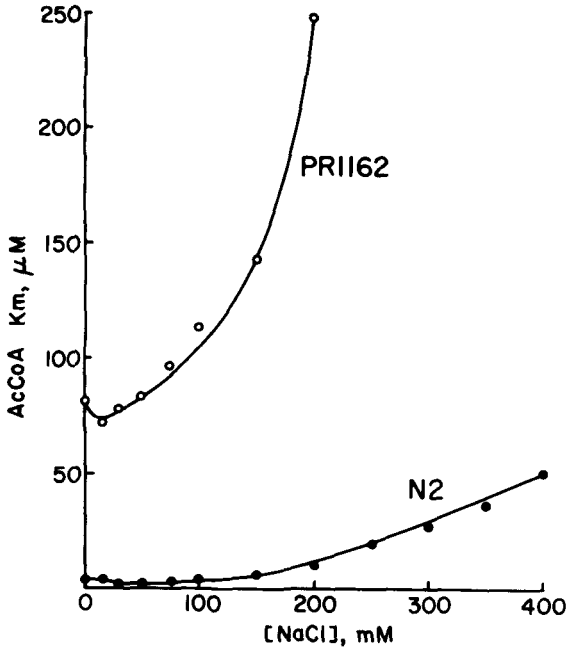

FIGURE 4.—Comparison of acetyl-CoA K_m from wild-type (N2) and *cha-1* mutant (PR1162) ChAT as a function of NaCl concentration. Protocol as for Figure 3, except that the [^{3}H]acetyl-CoA assay was employed.

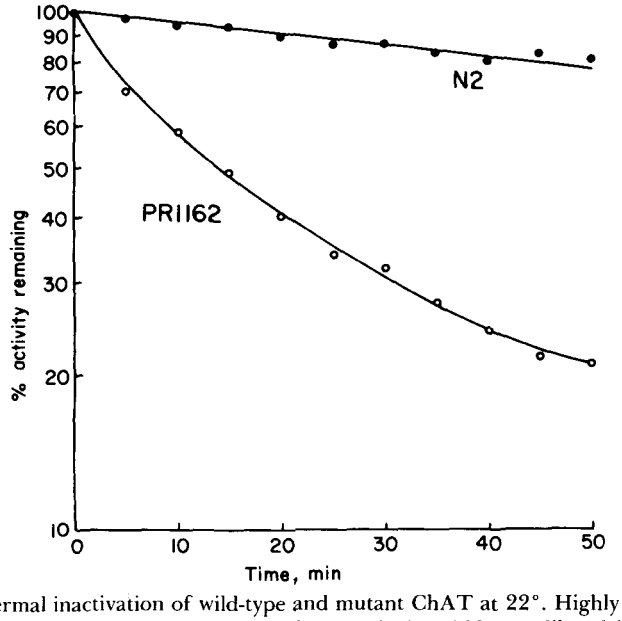

FIGURE 5.—Thermal inactivation of wild-type and mutant ChAT at 22°. Highly purified ChAT from either wild-type (N2) or from the "mild" *cha-1* strain PR1162 was diluted into prewarmed buffer [the same buffer used for enzyme storage (see MATERIALS AND METHODS)] containing 0.5 mg/ml of bovine serum albumin and 0.5 mM acetyl-CoA. Samples were taken in duplicate at the indicated times and assayed for ChAT activity.

on the basis of some alternative phenotype), we measured ChAT activity (using the microscale assay) in such mutants. These included *dpy-13*, *lin-1*, *mor-2*, *unc-77* and *vab-2*, as well as *unc-17(e245)* and *unc-33*, and in all cases, the animals contained normal ChAT levels, supporting the notion that *cha-1* was distinct from these genes.

Since many of the genes in this region of the linkage group had not been precisely mapped or ordered with respect to each other, we included most of the known genes in our mapping experiments (excepting *mor-2* and *unc-77*, which we found difficult to score unambiguously). Two-factor recombination data are presented in Table 4, data from three-factor ordering crosses are given in Table 5 and the map derived from these is shown in Figure 6.

TABLE 4

Two-factor recombination data

Genotype of heterozygous hermaphrodite	Self-progeny		% Recombination
	Parental phenotypes	Recombinant phenotypes	
lin-1 unc-33/+ +	739 WT	24 Lin	4.72
lin-1 unc-17/+ +	1675 WT	52 Lin	4.52
lin-1 dpy-13/+ +	1369 WT	83 Lin	9.20
	418 Lin Dpy	89 Dpy	
unc-33 dpy-13/+ +	1371 WT	38 Dpy	4.05
cha-1(p1152) dpy-13/+ +	1674 WT	39 Dpy	3.42
cha-1(b401) dpy-13/+ +	1315 WT	27 Dpy	3.02
unc-17 dpy-13/+ +	2754 WT	69 Dpy	3.67

All data, except *lin-1 dpy-13*/+ + was based on counts of only one of the parental phenotypes and one of the recombinant phenotypes. The *unc-17* allele used in these determinations was *e245*; alleles of other genes were as specified or as given in MATERIALS AND METHODS. WT, wild-type.

TABLE 5

Three-factor recombination data

Genotype of heterozygous hermaphrodites	Phenotype of selected recombinant self-progeny	Genotype of selected recombinants with respect to *trans* marker
+ *vab-2* +/*unc-33* + *dpy-13*	Dpy	29/48 *vab-2*/+
	Unc	13/37 *vab-2*/+
+ *vab-2* +/*cha-1* + *dpy-13*	Dpy	44/59 *vab-2*/+
+ *osm-3* +/*unc-17* + *dpy-13*	Dpy	17/25 *osm-3*/+
	Unc	9/36 *osm-3*/+
+ *osm-3* +/*vab-2* + *dpy-13*	Vab	1/21 *osm-3*/+
lin-1 + *unc-17* +/+ *unc-33* + *dpy-13*	Lin	74/78 *unc-33*/+
	Dpy	43/49 *unc-17*/+
lin-1 + *cha-1* +/+ *unc-33* + *dpy-13*	Lin	78/83 *unc-33*/+
	Dpy	21/24 *cha-1*/+

The *p1152* allele of *cha-1* was used; it was scored by its uncoordinated phenotype. *unc-17(e113)* was used in the three-factor cross with *osm-3* and *dpy-13*; the *e245* allele of *unc-17* was used elsewhere. The alleles of other genes are given in MATERIALS AND METHODS.

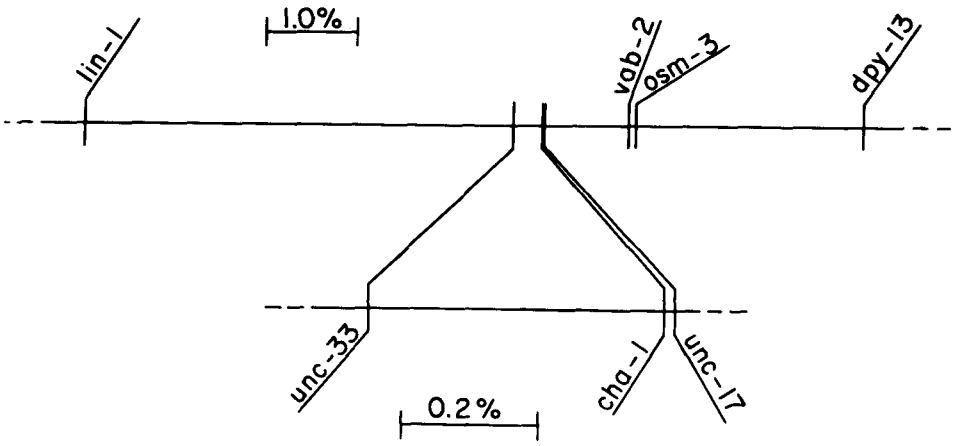

FIGURE 6.—Genetic map of the region of linkage group IV around *cha-1*. The relative order of the genes was determined by three-factor crosses (Table 5), and the distances between adjacent loci was determined using data from two-factor crosses (Table 4) and data from three-factor crosses (Table 5). All genes on this map have been unbambiguously ordered, with the possible exception of *vab-2* and *osm-3*; the order shown for these two genes is the most probable. The positions of *cha-1* and *unc-17* are those determined for the alleles *p1152* and *e245*, respectively (see details in text). The major gene cluster on this linkage group is just to the right of the region shown.

Of particular interest was the extremely tight linkage of *cha-1* to *unc-17*. Mutations in these genes were separated and ordered in the following manner. Animals were constructed of the genotype *lin-1* + *unc-17* +/+ *cha-1* + *dpy-13*, using the *p1152* allele of *cha-1* and the *e245* allele of *unc-17*. More than 20,000 progeny were screened from such quadruple heterozygotes, and we identified and cloned 479 Lin non-Unc and 259 Dpy non-Cha individuals. (The behavioral and slow-growth phenotypes of *cha-1* animals were used to score animals as being Cha or non-Cha.) The Lin non-Unc animals had presumably arisen by a recombination event between *lin-1* and *unc-17*, and the progeny of each of these recombinants were analyzed. Of the 479 Lin non-Unc animals, all but one were shown to contain the *cha-1* mutant allele on the recombinant chromosome; one animal, however, contained the wild-type *cha-1* allele on this chromosome. By the presence of the fourth marker (*dpy-13*) on the recombinant chromosome, we were able to confirm that this one animal had arisen through a single crossover event to the left of *unc-17* and to the right of *cha-1* and rule out other possible interpretations, such as a double crossover or a gene conversion event.

In a similar manner, progeny of the 259 Dpy non-Cha animals (which had presumably arisen through recombination between *cha-1* and *dpy-13*) were analyzed to determine whether they carried the *unc-17* allele on the recombinant chromosome. All but two of these animals were shown to contain the *unc-17* mutant allele; those two carried the wild-type allele at this locus. That these two individuals had arisen by recombination events to the right of *cha-1* and to the left of *unc-17* (and not by gene conversion or double crossovers) was confirmed by the presence of the left-flanking marker, *lin-1*, on both recom-

binant chromosomes. These three independent events led us to conclude that *cha-1* was to the left of *unc-17*.

As a control, a similar experiment was performed using the same four markers in a different configuration. Quadruple heterozygotes *lin-1 cha-1* + +/+ + *unc-17 dpy-13* were constructed, and recombinant progeny from these were analyzed. Of 585 recombination events to the left of *cha-1* (Lin non-Cha animals) and 391 recombination events to the right of *unc-17* (Dpy non-Unc animals), none was between *cha-1* and *unc-17*, thus providing even more support to the assignment of *cha-1(p1152)* to the left of *unc-17(e245)*.

These data not only provide a left-right order for these two mutations, but also let us deduce the approximate distance between them. Thus, the distance between *cha-1(p1152)* and *unc-17(e245)* is approximately $^{1}/_{479}$ the distance between *lin-1* and *unc-17* (5.2%) and $^{2}/_{259}$ the distance between *cha-1* and *dpy-13* (3.4%), which estimates come out to 0.011% and 0.026%, respectively. These numbers are comparable to the estimated size of the *unc-22* gene in *C. elegans* (about 0.01%; MOERMAN and BAILLIE 1979).

Phenotypes of unc-17 *strains*

The extremely close linkage between *cha-1* and *unc-17* was intriguing especially in light of the similarity of phenotypes of *cha-1* and *unc-17* animals. Most mutations at both loci lead to small size, slow growth and the same type of coiling, uncoordinated movement (including jerkiness when trying to move backward). Also, and most interesting of all, mutations at both loci confer resistance to cholinesterase inhibitors; in fact, many of the *unc-17* alleles were isolated on the basis of their resistance to the cholinesterase inhibitor lannate (BRENNER 1974). These phenotypic similarities, coupled with the extremely tight genetic linkage, would ordinarily raise the question of whether *cha-1* and *unc-17* might constitute only a single gene instead of two. However, preliminary ChAT assays of a number of *unc-17* mutants indicated no ChAT deficiency (data not shown), and preliminary complementation results indicated that the *cha-1* alleles *b401* and *p1152* each complemented the *unc-17* allele *e245* for coordinated locomotion.

Complementation of cha-1 *and* unc-17

In an attempt to resolve more clearly the relationship between *cha-1* and *unc-17*, we performed careful complementation tests using almost all possible pairwise combinations of *cha-1* and *unc-17* alleles. A surprising pattern emerged from these tests (Figure 7). There seemed to be three classes of alleles. One class included *p1152*, and all members in this class led to ChAT deficiency; a second class included *e245*, and all members had apparently normal ChAT activity. All members within one of these two classes failed to complement each other, and all members of each class complemented all members of the other class. There was also a third class of alleles, however, consisting of three members (*p1156*, *e113* and *e876*), all of whom failed to complement members of *both* the first and the second class. As a control, all alleles from all three classes were shown to complement the behavioral phenotype of the *e204* allele of *unc-33*, the closest linked *unc* locus to *cha-1* and *unc-17* (see map, Figure 6).

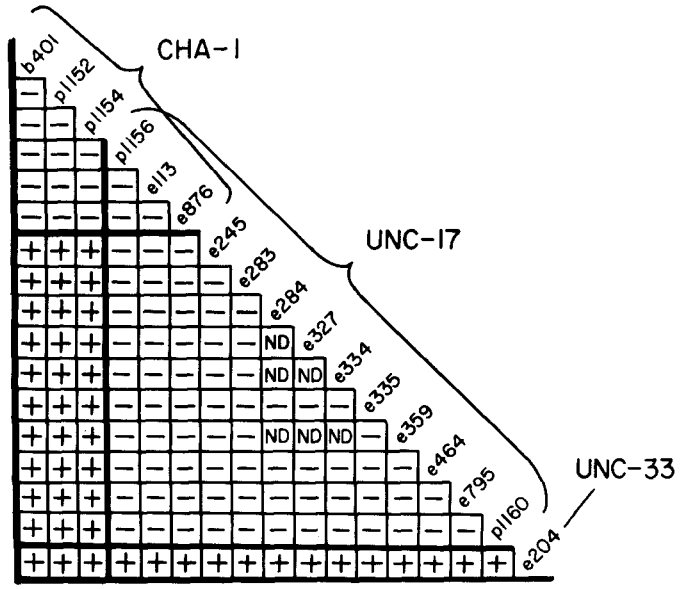

FIGURE 7.—Complementation matrix for the uncoordinated phenotype of *cha-1* and *unc-17* alleles. Since *p503* does not lead to uncoordinated behavior, it is not included here. Complementations were performed as described in MATERIALS AND METHODS. As a control, all alleles were tested for complementation with the *e204* allele of *unc-33*. Brackets indicate the apparent complementation groups. + indicates complementation; − indicates failure to complement; ND indicates not determined.

Thus, there seem to be two discrete complementation groups, one corresponding to *cha-1* and one to *unc-17* and in addition a set of three "overlap" alleles that seem to be members of both complementation groups. Within this overlap group, however, there is a heterogeneity of phenotype. One of the overlap alleles, *p1156*, when homozygous, leads to a ChAT-deficient phenotype; the other two, *e113* and *e876*, lead to ChAT levels at least half those of wild type (data not shown). Given these enzymatic results, it is quite difficult to understand how the *e113* and *e876* overlap alleles, which do have ChAT activity, fail to complement the *cha-1* mutants, whose only deficiencies appear to reside in a ChAT structural gene. In the following discussion we present a possible interpretation of these initially puzzling observations; a central feature of this interpretation is that it treats *cha-1* and *unc-17* as coding elements for separate domains of a single multifunctional polypeptide.

DISCUSSION

cha-1 *is a ChAT structural gene*: We have presented three lines of evidence that suggest that *cha-1* is a structural gene for ChAT (or a subunit of ChAT). First, we have shown that highly purified ChAT obtained from animals homozygous for the leaky *cha-1* allele *p503* has altered kinetic properties compared with the wild-type enzyme. These K_m alterations are of considerable magnitude: the mutant choline K_m is tenfold higher and the mutant acetyl-CoA K_m is 20- to 40-fold higher (depending on the NaCl concentration) than the cor-

responding values for the wild-type enzyme (Figures 3 and 4). Second, as seen in Figure 5, the mutant enzyme is significantly more thermolabile than the wild-type ChAT. Both the K_m and the thermolability data argue that the ChAT protein in *p503* homozygotes is qualitatively altered. Finally, all of the *cha-1* alleles display strict gene dosage; animals containing one copy of the wild-type *cha-1* allele and one mutant allele have ChAT levels intermediate between mutant homozygotes and wild-type homozygotes (Table 3). In a formal sense, this is equivalent to saying that *cha-1* mutants are semidominant for the ChAT deficiency, a result entirely consistent with the notion that *cha-1* is the ChAT structural gene, but difficult to explain if the gene were regulatory rather than structural. Clearly, absolute proof that *cha-1* is the ChAT structural gene would require the complete sequence of the gene and/or the protein, but even in the absence of such data, we feel that the earlier arguments are quite compelling.

Primary ChAT deficiencies produce secondary consequences on behavior, growth and drug resistance: This conclusion is based on three lines of evidence. First, each of the severe *cha-1* mutations leads to the same spectrum of phenotypes, namely, coiling uncoordination, slow growth, small adult size, and resistance to AChE inhibitors (see preceding data). Second, all of these phenotypes cosegregate in crosses, even in crosses designed to force recombination near the *cha-1* locus (see outcrossing protocol in MATERIALS AND METHODS). Finally, the severe *cha-1* alleles all fail to complement each other for the nonenzymatic phenotypes as well as for the ChAT deficiency. Thus, the only alternative to concluding that these nonenzymatic phenotypes derive secondarily from the ChAT deficiency is to assume that in all four independently derived mutants, additional mutations conferring these phenotypes occurred in a very tightly linked gene, the same gene in all four cases. This seems sufficiently unlikely that we discount it as a possibility.

Marked ChAT reductions are required to produce secondary effects: We assume, based on analysis of *cha* mutants in Drosophila (HALL, GREENSPAN and KANKEL 1979; GREENSPAN 1980), that mutations in *C. elegans* leading to a total absence of ChAT would be lethal and that the *cha-1* mutants we have isolated are, therefore, presumably not null. If, in fact, null alleles are lethal, then selection schemes based on resistance to cholinesterase inhibitors would not be expected to yield any null mutants, since survival, growth and development of the strain is a prerequisite to its isolation. It would, therefore, appear that such selection schemes select for a narrow "window" of residual ChAT activity: the enzyme activity must be low enough to give resistance to cholinesterase inhibitors (and, concomitantly, lead to the other behavioral and developmental phenotypes observed), yet the activity must be high enough to permit survival and growth of the animal. Although some of the *cha-1* strains (*e.g.*, PR1154) seem to have negligible ChAT activity in extracts, it is possible that there is significant enzyme activity *in vivo* that is labile to extraction, and we are unable at this point to specify a lower limit of ChAT activity necessary for survival. However, we can set an upper limit on the amount of ChAT activity above which there are no apparent behavioral or developmental effects. Animals containing one copy

of the *p503* allele of *cha-1* and one copy of any of the severe alleles are essentially normal in their behavior and development, yet they contain only about 5–7% of the wild-type ChAT activity. Clearly, this amount of enzyme is enough for normal function under conditions of laboratory culture.

There is also evidence that large reductions in the other enzyme of acetylcholine metabolism, AChE, may be without behavioral consequence. Animals homozygous for either an *ace-1* or *ace-2* mutation and heterozygous at the other locus have AChE levels approximately 25% of wild type (CULOTTI *et al.* 1981), yet they have no apparent behavioral impairment. It is only when there are mutations homozygous at both of these loci, with AChE levels about 5% of wild type, that severe uncoordination is observed. Thus, for both ChAT and AChE, it appears that only a small fraction of the enzyme activity present is actually required for normal behavior and development. We do not know whether such observations are merely artifacts secondary to the "unnatural" culture conditions present in the laboratory or whether they represent a built-in "safety factor" to allow the animal to deal with prolonged stressful conditions. In any event, mutations that eliminate 90% of the wild-type enzyme level may not be behaviorally relevant.

cha-1 *and* unc-17 *may encode different domains of one large polypeptide*: One of the major surprises encountered in these experiments was the complex complementation pattern exhibited by *cha-1* and *unc-17* alleles (Figure 7). The similarity of map position, phenotypes, and especially the resistance of such strains to cholinesterase inhibitors, suggested to us initially that the *cha-1* mutations might be alleles of the previously identified gene *unc-17*. However, the observation that most of the *cha-1* alleles complement most of the *unc-17* alleles (Figure 7) as well as the result that all *unc-17* strains had ChAT activity suggested two genes, although perhaps with related functions.

In either case, the complementation data need to be explained. If *cha-1* and *unc-17* represent two genes, it must be understood why three of the alleles (*p1156*, *e113* and *e876*) seem to be members of both complementation groups. One possible explanation, namely, that all three alleles represent small deletions that eliminate part of each gene (as well as the region between the genes), is unlikely for two reasons. First, two of these alleles, when homozygous, lead to ChAT activity close to that of wild type, which would not be expected from a deletion affecting the ChAT structural gene. Second, we have been able to obtain wild-type recombinants from *p1156/e113* individuals (J. B. RAND, unpublished results), which suggests that, if these two mutations are both deletions, they must not eliminate any genetic region in common, and therefore cannot both overlap the two alleged genes.

We think, therefore, that it is more likely that *cha-1* mutants and *unc-17* mutants are all alleles of a single complex locus; however, if so, it must be understood why *unc-17* strains have ChAT and why most *cha-1* and *unc-17* strains complement each other.

Our current model is that the ChAT molecule consists of two functionally independent domains, one of which contains the ChAT catalytic site and the other of which is involved in some noncatalytic function, such as the proper

subcellular localization of the molecule. The model also requires that these two domains be structurally discrete, in the sense that a mutational alteration occurring in one of these domains would not affect the function of the other. Thus, *cha-1* mutations would affect the catalytic function of the protein, but not its localization function, whereas *unc-17* mutants would have catalytic activity that is improperly localized. If both proper localization and catalytic activity are normally required for proper function, the common secondary consequences of *cha-1* and *unc-17* mutants would make sense.

The fact that most *cha-1* alleles complement most *unc-17* alleles could be explained if the enzyme normally functions *in vivo* as a homodimer; a dimer between one molecule with a localization defect (but normal catalysis) and another with a catalytic defect (but properly localized) could, it is imagined, lead to appropriate localization of at least some catalytic activity. Among the overlap alleles, *p1156* would disrupt both domains, whereas the *e113* and *e876* alleles would be defective both in localization (since they have catalytic activity) and in dimerization (since they fail to complement the enzyme-deficient *cha-1* alleles). We have been unable to generate satisfactory alternative models that account for all these observations.

We are pursuing this model in two ways. First, we are constructing a fine-structure map of the entire region, which will tell us whether the members of the different complementation classes "cluster" on the genetic map or whether they are interspersed. Interspersion of *cha-1* and *unc-17* alleles would argue strongly for one gene, whereas clustering would be consistent with both one-gene and two-gene models. Second, we are studying the biochemical properties of ChAT from *unc-17* strains, to see whether they show any qualitative alterations in the enzyme. We are also investigating the possibility that *unc-17* strains may be deficient in one of the known multiple forms of ChAT (RAND and RUSSELL 1984).

We are grateful to CARL JOHNSON, STEWART SCHERER, ED HEDGECOCK and MAURICE ZWASS for assistance in the initial screening of uncoordinated strains, which resulted in the identification of the first two *cha-1* alleles. We are also grateful to DAVE HALL, who isolated and provided to us strain PR503. The mutageneses that resulted in the isolation of the *p1152*, *p1154*, *p1156* and *p1160* alleles were successful because of DENNIS KOLSON's efforts, for which we are deeply grateful. STEVE CARR and DAVID HIRSH not only generously sent us their trichlorfon-resistant strain DH401, but also provided us with unpublished data concerning the mapping of the mutation, thereby saving us much time and effort. We also thank JIM LEWIS and JONATHAN HODGKIN for providing strains.

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