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Expression of human β-amyloid peptide in transgenic *Caenorhabditis elegans*

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ABSTRACT **Transgenic *Caenorhabditis elegans* nematodes have been engineered to express potentially amyloidic human proteins. These animals contain constructs in which the muscle-specific *unc-54* promoter/enhancer of *C. elegans* drives the expression of the appropriate coding regions derived from human cDNA clones. Animals containing constructs expressing the 42-amino acid β-amyloid peptide (derived from human amyloid precursor protein cDNA) produce muscle-specific deposits immunoreactive with anti-β-amyloid polyclonal and monoclonal antibodies. A subset of these deposits also bind the amyloid-specific dye thioflavin S, indicating that these deposits have the tinctural characteristics of classic amyloid. Co-expression of β-peptide and transthyretin, a protein implicated in preventing the formation of insoluble β-amyloid, leads to a dramatic reduction in the number of dye-reactive deposits. These results suggest that this invertebrate model may be useful for *in vivo* investigation of factors that modulate amyloid formation.**

Amyloid is a general term that refers to insoluble protein deposits with a fibrillar, β-pleated structure. These deposits are usually detected by their ability to bind specific dyes such as Congo red or thioflavin S (1). Many different proteins can form amyloid, and amyloid deposits are found in a variety of apparently unrelated diseases. The most intensely studied of these diseases is Alzheimer disease (AD), where characteristic brain plaques contain β-amyloid. The primary biochemical component of β-amyloid is a 39- to 43-amino acid peptide derived from the β-amyloid precursor protein (APP) (2). The identification of germline mutations in patients with familial forms of early-onset AD provides compelling evidence for a central role of β-amyloid in AD pathogenesis (3–6). Similarly, a number of other (autosomal dominant) familial amyloid diseases have been associated with specific mutations in the genes encoding the corresponding amyloid-forming protein. One such disease is familial amyloid polyneuropathy, in which mutations in the plasma protein transthyretin lead to the formation of cytotoxic intracellular deposits in peripheral neurons and visceral cells (7). Neither the mechanism(s) of amyloid toxicity nor the physiological factors required for amyloid formation have been established.

The formation of insoluble, aggregated amyloid may be a necessary prerequisite for the pathogenesis observed in the diseases described above. *In vitro* studies with cultured neuronal cells using synthetic β-peptide strongly suggest that aggregated peptide is required for cell toxicity (8–10). In addition, the $Glu^{22} \rightarrow Gln$ mutation of APP found in Dutch-type hereditary cerebral hemorrhage with amyloidosis (HCHWA-D) disease has been shown to enhance the *in vitro* aggregation of the respective β-peptide (11, 12), and peptides corresponding to different mutated regions of the human prion gene associated with familial Creutzfeldt–Jacob disease (another amyloid disease) also show enhanced *in vitro* formation of amyloid fibrils (13). In AD brains, amyloid deposits are associated with other proteins such as apolipoprotein E and antichymotrypsin; it has been postulated that these or similar proteins may serve as "pathological chaperones" that somehow promote the aggregation of β-peptide into β-amyloid (14, 15). Conversely, the presence of soluble β-peptide in extracellular fluids has also led to the suggestion that specific extracellular proteins may sequester β-peptide, thus preventing amyloidosis (16, 17). Transthyretin has been identified as a major β-peptide-binding protein in cerebrospinal fluid that can inhibit β-amyloid formation *in vitro* (18).

Numerous transgenic mouse models have been created to investigate β-amyloid formation and toxicity (19–26). Here I report the construction and initial characterization of an invertebrate model system using the nematode *Caenorhabditis elegans* for similar investigations. Some of the studies described in this paper were presented at the Fourth International Conference on Alzheimer's Disease and Related Disorders, July 29 to August 5, 1994, and are summarized in the proceedings of this symposium (27).

MATERIALS AND METHODS

Construction of Chimeric Genes. The minigene encoding the β-(1–42) peptide was assembled in three steps. The artificial signal peptide coding sequence of vector pPD50.52 was amplified by using primers SP-up (5′-CGGGATTGGCCAAAGGACCC-3′) and SP-down (5′-CCCGGTACCTGCTGGTGCCAGAAAGAT-3′), cleaved with *Nhe* I and *Kpn* I restriction endonucleases, and inserted between the unique *Nhe* I and *Kpn* I sites of vector pPD49.26, resulting in construct pCL2. This procedure results in a reengineering of the signal peptide, such that the signal-peptide cleavage site, as predicted by the consensus of von Heijne (28), occurs immediately after the Gly-Thr dipeptide encoded by the *Kpn* I site. A 146-bp fragment encoding β-(1–42) (including an artificial stop codon) was amplified from human β-amyloid precursor protein cDNA clone p4T4B (29) by using primers β1–42-up (5′-GGGGGTACCGATGCAGAATTCCGACATGA-3′) and β1–42-down (5′-CCCGAGCTCACGCTATGACAACACCGCCAA-3′), cleaved with *Kpn* I and *Sac* I, and inserted between the unique *Kpn* I and *Sac* I sites of pCL2, generating pCL3. The signal peptide/β-(1–42) minigene fragment was removed from this plasmid by digestion with *Nhe* I and *Sac* I and inserted between the unique *Nhe* I and *Sac* I sites of pPD30.38 to construct pCL12. The sequence of the β-(1–42) minigene was confirmed by dideoxy DNA sequencing (coding strand only).

To assemble chimeric genes containing transthyretin, the transthyretin coding sequence was amplified from a human cDNA clone (30) with primers TTR-up (5′-GGGGGTACCATGGCTTCTCATCGTCTGG-3′) and TTR-down (5′-CCCGAGCTCATTCCTTGGGATTGGTG-3′). This amplification product was cleaved with *Kpn* I and *Sac* I and then inserted between the *Kpn* I and *Sac* I sites in the expression

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Abbreviation: AD, Alzheimer disease.

cloning site of plasmid pPD30.38. The $Thr^{60} \rightarrow Ala$ mutation was engineered by a PCR-based method (31) employing mutagenesis primers ala60-up (5'-GCTCACAGCTGAGGAGGAA-3') and ala60-down (5'-TTCCTCCTCAGCTGTGAGC-3') in addition to TTR-up and TTR-down. The mutated fragment was inserted into pPD30.38 as described above. These constructs were also confirmed by DNA sequencing.

Construction of Transgenic Animals. Transgenes were introduced into *C. elegans* by gonad microinjection (32). Marker plasmid pRF4 [*rol-6(su1006)*] was coinjected with the constructs described above (at ≈100 ng/μl for each plasmid) into *him-5(e1490)* animals, and Roller transgenic progeny were recovered. Transmitting lines were established and maintained by selection for the Roller marker phenotype. Strains containing chromosomally integrated transgenes were recovered by irradiation of lines containing extrachromosomal transgenic arrays with 7000 rad (1 rad = 0.01 Gy) of γ-rays from a cesium-66 source. Progeny of irradiated animals were then screened for 100% transmittance of the marker transgene. Complete transmittance of the chimeric constructs was confirmed by immunohistochemistry of large populations of putative integrated lines.

Immunohistochemistry. Whole mount specimens were prepared by fixing animals in 4% paraformaldehyde and permeabilizing with 2-mercaptoethanol and collagenase (33). Transthyretin expression was detected by incubating fixed, permeabilized animals with rabbit anti-transthyretin polyclonal antibody (8 μg/ml; Boehringer Mannheim) for 16 hr at 4°C and then with Texas Red-conjugated goat anti-rabbit Ig antibody (5 μg/ml; Vector) for 2 hr at room temperature. Bodipy-conjugated phallacidin (165 nM) was included in the secondary antibody incubation to visualize muscle fibers. For β-peptide expression, an analogous set of incubations was done with rabbit anti-β polyclonal antibody (raised against synthetic β-amyloid; Boehringer Mannheim) at 10 μg/ml or with an anti-β specific monoclonal antibody (mAb 4.1, a generous gift of B. Cordell, Scios Nova) diluted 1:50. This monoclonal antibody was raised against synthetic β-(1–40) peptide and recognizes an epitope approximately spanning residues 8–15.

RESULTS

Chimeric genes were constructed with coding regions derived from human cDNA clones and regulatory sequences from expression vector pPD30.38. This vector contains *C. elegans unc-54* promoter/enhancer sequences that produce high-level, muscle-specific gene expression (34). To assemble the β-peptide construct, a 189-bp fragment coding for the 42-amino acid version of the β-peptide preceded by a synthetic signal peptide was incorporated into pPD30.38 to produce the *unc-54*/β-(1–42) minigene construct pCL12 (Fig. 1). For the transthyretin constructs, a 461-bp fragment containing the entire human transthyretin coding sequence (including the natural signal peptide and stop codon) was recovered from a transthyretin cDNA clone by PCR and inserted into pPD 30.38, thus generating the *unc-54*/transthyretin construct pCL10. *In vitro* mutagenesis was used to generate an analogous construct, pCL13, that contained the transthyretin coding sequence with a $Thr^{60} \rightarrow Ala$ substitution, an alteration found in some cases of familial amyloid neuropathy.

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GCTAGCAAAA ATG CAT AAG GTT TTG CTG GCA CTG TTC TTT ATC TTT CTG GCA
           met his lys val leu leu ala leu phe phe ile phe leu ala
CCA GCA GGT ACC GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT
pro ala gly thr asp ala glu phe arg his asp ser gly tyr glu val
CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA
his his gln lys leu val phe phe ala glu asp val gly ser asn lys
GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG TGA GCTC
gly ala ile ile gly leu met val gly gly val val ile ala ——
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FIG. 1. Sequence of the β-(1–42) minigene present in construct pCL12. The β-(1–42) peptide amino acid sequence is in italics, and the restriction sites used in constructing this plasmid are underlined.

Transgenic animals were generated by gonad microinjection, using coinjection of plasmid pRF4 to independently identify animals maintaining the injected transgenes. This plasmid contains a mutant *C. elegans* collagen gene [*rol-6(su1006)*] that produces a dominant, distinctive "Roller" phenotype. Transgenic animals produced in this manner maintain the injected DNA as an extrachromosomal, multicopy array of variable mitotic and meiotic stability. The transmitting lines established for this study have meiotic stabilities of 20–70%. Thus, transgenic (Roller) animals produce both transgenic and nontransgenic (non-Roller) progeny. These nontransgenic progeny serve as good internal controls for phenotypic and immunohistochemical comparisons (see below).

Multiple independent transmitting (extrachromosomal) lines were established for the *unc-54*/transthyretin (CL1014 and CL1015), *unc-54*/transthyretin ala60 (CL1020 and CL1021), and *unc-54*/β-(1–42) minigene (CL1016, CL1017, CL1018, CL1019, and CL1028) constructs. These lines were examined for transgene expression by whole-mount immunohistochemistry. Populations derived from transgenic animals were fixed and probed with polyclonal rabbit antisera directed against transthyretin or β-amyloid. Transgenic (Roller) animals were distinguished from their nontransgenic siblings by virtue of their helically twisted body wall muscle, visualized by simultaneously probing with Bodipy-conjugated phallacidin.

Animals transgenic for the *unc-54*/β-(1–42) minigene construct contained muscle-specific deposits of anti-β-peptide immunoreactivity (Fig. 2 *A* and *B*). No anti-β immunoreactivity was observed in control animals. [Although *C. elegans* contains a homolog of the β-amyloid precursor protein gene (35), this sequence does not contain an apparent β-peptide domain and thus would not be expected to show crossreactivity with the anti-β antibody used.] In contrast, animals transgenic for the *unc-54*/transthyretin and *unc-54*/transthyretin ala60 constructs showed strong anti-transthyretin immunoreactivity throughout their bodies, often with intense staining in the coelomocytes (Fig. 2 *C* and *D*). [Adult hermaphrodite animals have six coelomocytes; dye injection experiments indicate that these cells have a scavenging function (36).] The pattern of anti-transthyretin staining is consistent with transthyretin being synthesized in muscle cells and secreted into the body cavity, where it is scavenged by the coelomocytes.

To examine meiotically and mitotically stable lines, two independent lines with chromosomally integrated copies of the *unc-54*/β-(1–42) and *rol-6* transgenes (CL2005 and CL2006) were derived from transmitting line CL1019 by γ irradiation. In both of these strains, all adult animals showed extensive muscle-specific anti-β-immunoreactive deposits. Similarly, a line was established (CL2008) with integrated copies of the *unc-54*/transthyretin and *rol-6* transgenes. All animals in this strain showed the same pattern of anti-transthyretin immunoreactivity as described above for the extrachromosomal strains.

To determine whether the immunoreactive deposits observed in the *unc-54*/β-(1–42) strains display the tinctural properties of classic insoluble β-amyloid, transgenic strains were fixed and stained with thioflavin S, a fluorescent amyloid-specific dye (37). Thioflavin S-reactive deposits were found in all strains containing the *unc-54*/β-(1–42) minigene construct, but not in control wild-type animals or strains containing either the *unc-54*/transthyretin or *unc-54*/transthyretin ala60 constructs. Examination of animals doubly probed with thioflavin S and anti-β antibody indicated that the thioflavin S deposits were coincident with a subset of the anti-β-reactive deposits (Fig. 3).

To investigate whether transthyretin expression can inhibit the amyloid formation observed in transgenic strains expressing the β-peptide, doubly transgenic strains were constructed by mating CL2008 animals to CL2005 and CL2006 animals.

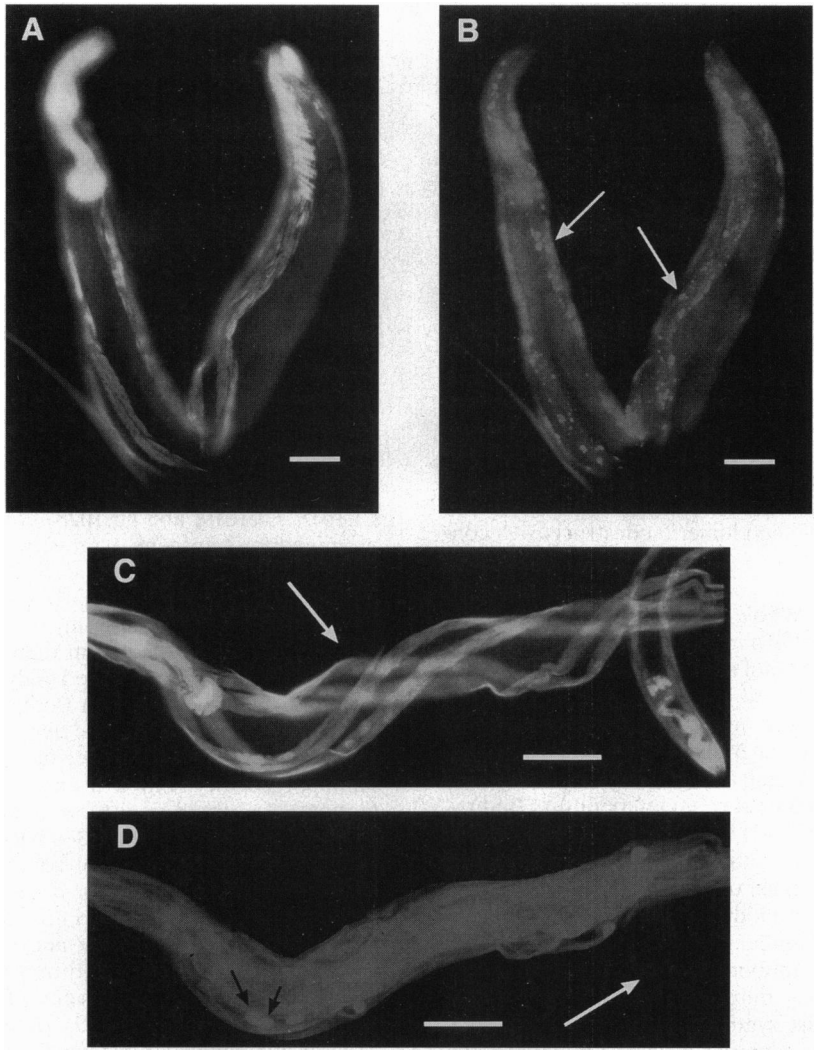

FIG. 2. Immunohistochemistry of transgenic animals. (*A*) *unc-54*/β-(1–42) transgenic male animal (from extrachromosomal line CL1019) with muscle structure visualized by Bodipy-phallacidin. (*B*) Same transgenic animal probed with anti-β-amyloid antibody. Note muscle-specific deposits. (*C*) *unc-54*/transthyretin animals (from extrachromosomal line CL1015) probed with Bodipy-phallacidin, viewed by epifluorescence microscopy. Note helical musculature of transgenic animal (arrow). (*D*) Same CL1015 animals probed with anti-transthyretin antibody. Note intense staining of coelomocytes (black arrows) and complete lack of staining in nontransgenic sibling (white arrow). The intense signal from these coelomocytes bleeds through on the Bodipy epifluorescence channel, producing faint images visible in *C*. These images were generated by digitally scanning color slides and adjusting brightness and contrast with ADOBE PHOTOSHOP (Adobe Systems). (Bars = 40 μm.)

The cross-progeny from these matings will be heterozygous for both the *unc-54*/β-(1–42) and *unc-54*/transthyretin transgenes. These cross-progeny were fixed and stained for thioflavin S-reactive deposits and found to contain a significantly reduced number of thioflavin S-reactive deposits when compared with control animals heterozygous for only the *unc-54*/β-(1–42) transgene (Fig. 4). The pattern of anti-β immunoreactivity in animals coexpressing β-peptide and transthyretin was indistinguishable from that of animals expressing only β-peptide (data not shown).

DISCUSSION

I have demonstrated that the human β-amyloid peptide can be expressed in transgenic *C. elegans*, where it forms deposits displaying the tinctural properties of classic amyloid. In these transgenic animals, a strong muscle-specific promoter was used to express a potentially secretable form of the β-peptide, in an attempt to generate significant extracellular levels of β-peptide. This would mimic to some extent the situation postulated to exist in the human brain and might allow observation of cell-external neurotoxicity. The *unc-54*/β-(1–42) minigene was constructed with a modified signal-peptide sequence that has been shown to allow secretion of a *her-1* protein product ectopically expressed in muscle cells (38). However, β-peptide deposits have not been convincingly detected outside of muscle cells with the antibodies used in this study; amyloid deposition in muscle cells may preclude extracellular secretion of immunologically detectable levels of β-peptide. Alternatively, the small size of the *unc-54*/β-(1–42) minigene translation product might interfere with its efficient secretory routing. Determination of the subcellular location of the β-peptide deposits may help resolve the fate of this peptide in the muscle cells of transgenic animals.

In contrast to animals expressing β-peptide, thioflavin S-positive deposits were not observed in strains expressing wild-type or mutant human transthyretin, and these proteins were readily secreted. The inability of the *unc-54*/transthyretin ala60 construct to produce thioflavin S-reactive deposits may not be surprising if this variant requires comparatively long incubation times at 37°C (i.e., as found in the human disease) to produce amyloid. Over 40 transthyretin variants are known to be associated with familial amyloid neuropathy, and more will need to be tested in the nematode system to determine whether the results with the ala60 variant can be generalized.

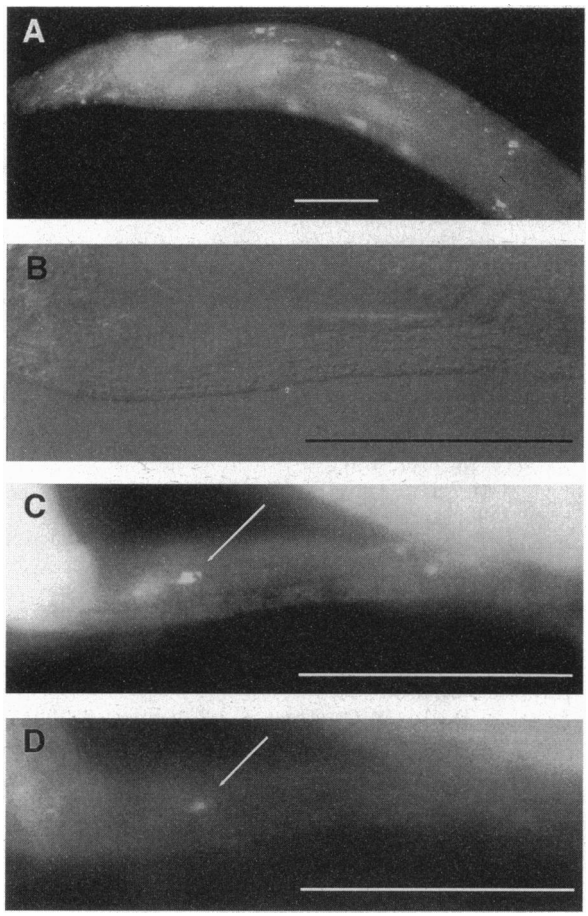

FIG. 3. Thioflavin S and anti-β staining of transgenic animals. (*A*) *unc-54*/β-(1–42) transgenic animal (strain CL1019) stained with thioflavin S. Note numerous thioflavin S-reactive deposits. (*B*) Isolated muscle fragment from *unc-54*/β-(1–42) transgenic animal (integrated strain CL2005) stained with thioflavin S and subsequently probed with anti-β antibody, as shown by differential interference contrast optics. This image shows the muscle fiber morphology used to identify this tissue. (*C*) Epifluorescence image of anti-β staining (long-wavelength filter) in muscle fragment. Note deposits (arrow). (*D*) Thioflavin S-specific fluorescence (short-wavelength filter) in muscle fragment. Note colocalization of thioflavin S-reactive and anti-β-immunoreactive deposits. (There is no crossover in fluorochrome signals with the filters used.) (Bars = 40 μm.)

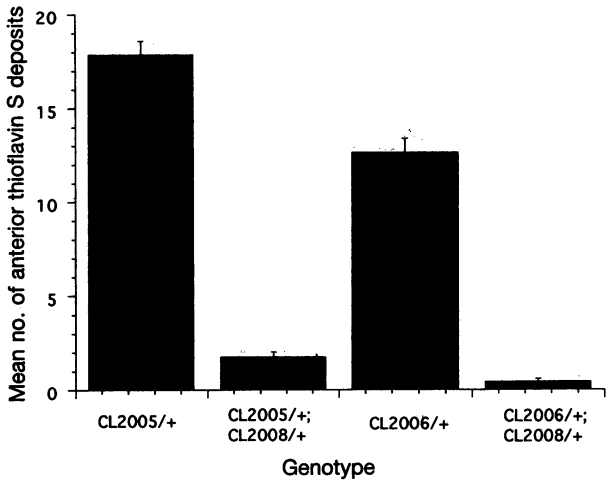

FIG. 4. Inhibition of thioflavin S deposits by coexpression of β-peptide and transthyretin in transgenic animals. CL2008 or wild-type males were mated to CL2005 or CL2006 hermaphrodites at 25.5°C, and male cross-progeny were recovered and stained with thioflavin S. Thioflavin S-reactive deposits anterior of the pharyngeal bulb were scored in individual animals by epifluorescence microscopy ($n > 20$ for each genotype). Mean number of deposits for each genotype is displayed with standard error of the mean.

The formation in this model system of β-peptide deposits with the tinctural characteristics of classic amyloid allows investigation of factors postulated to modulate β-peptide aggregation, many of which have only been examined *in vitro*. The existence of an *in vivo* model may be particularly instructive, because the conformation of the β-peptide is apparently sensitive to local conditions (ref. 39; see also disparate models proposed in refs. 40 and 41), and *in vitro* experiments necessarily require assumptions (often untestable) about the *in vivo* β-peptide environment. I have shown here that coexpression of β-peptide and transthyretin leads to a reduction in dye-reactive deposits. This observation is consistent with previous studies that have demonstrated that transthyretin can inhibit β-amyloid formation *in vitro* (18). However, present data cannot exclude the possibility that the β-peptide/transthyretin interaction observed in this study was due not to the direct interaction of these proteins, but instead to a reduction of β-peptide expression by the transthyretin transgene. Unfortunately, the nature of these single-animal experiments precludes direct quantitative measurement of β-peptide by techniques such as enzyme-linked immunoassays. Although no reduction in β-peptide expression in doubly transgenic animals was observed by immunohistochemistry, this method is not sufficiently quantitative to rule out subtle changes in β-peptide expression levels. Transthyretin variants that fail to bind β-peptide have recently been identified (42); transgenic animals expressing these variants may allow a rigorous *in vivo* demonstration of a specific interaction between β-peptide and transthyretin in this system. The postulated pathological chaperones antichymotrypsin and apolipoprotein E have been shown to promote β-peptide aggregation *in vitro* (43, 44); these studies can be readily replicated in this model system. In addition, mutated β-peptide variants can be expressed in this system to identify residues critical for formation of thioflavin S-reactive deposits.

Transgenic animals containing the *unc-54*/β-(1–42) construct display noticeable phenotypes, including progressive paralysis and vacuoles in larval animals (C.D.L., unpublished work). Although the physiological basis for these phenotypes is uncertain, it is likely that at least some of these phenotypes result from muscle cell toxicity. In this regard, it is interesting that β-amyloid has been detected in vacuolated muscle cells found in the degenerative muscle disease inclusion-body myositis (45). The production of aggregated β-peptide in a model organism with a short generation time and well-characterized genetics also opens the possibility of a genetic approach to studying this aggregation (e.g., by identification of mutations that modulate the formation of dye-reactive deposits).

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