Recruitment and the Role of Nuclear Localization in Polyglutamine-mediated Aggregation

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Abstract. The inherited neurodegenerative diseases caused by an expanded glutamine repeat share the pathologic feature of intranuclear aggregates or inclusions (NI). Here in cell-based studies of the spinocerebellar ataxia type-3 disease protein, ataxin-3, we address two issues central to aggregation: the role of polyglutamine in recruiting proteins into NI and the role of nuclear localization in promoting aggregation. We demonstrate that full-length ataxin-3 is readily recruited from the cytoplasm into NI seeded either by a pathologic ataxin-3 fragment or by a second unrelated glutamine-repeat disease protein, ataxin-1. Experiments with green fluorescence protein/polyglutamine fusion proteins show that a glutamine repeat is sufficient to recruit an otherwise irrelevant protein into NI, and studies of human disease tissue and a *Drosophila* transgenic model provide evidence that specific glutamine-repeat–containing proteins, including TATA-binding protein and Eyes Absent protein, are recruited into NI in vivo. Finally, we show that nuclear localization promotes aggregation: an ataxin-3 fragment containing a nonpathologic repeat of 27 glutamines forms inclusions only when targeted to the nucleus. Our findings establish the importance of the polyglutamine domain in mediating recruitment and suggest that pathogenesis may be linked in part to the sequestering of glutamine-containing cellular proteins. In addition, we demonstrate that the nuclear environment may be critical for seeding polyglutamine aggregates.

Key words: trinucleotide • triplet repeat • Machado-Joseph disease • neurodegeneration • hereditary ataxia

CAG trinucleotide repeat expansion is now known to be the underlying genetic defect in a growing number of neurodegenerative diseases. To date, these include Huntington's disease, dentatorubral-pallidoluysian atrophy, spinobulbar muscular atrophy, and five forms of dominantly inherited spinocerebellar ataxia for which a mutation has been identified, including types 1, 2, 3, 6, and 7 (for reviews see Paulson and Fischbeck, 1996; Hackam et al., 1998). In each disease gene, a CAG repeat expansion occurs within the protein coding region, resulting in an expanded polyglutamine repeat in the otherwise unrelated disease proteins. Evidence supports a model of disease in which polyglutamine expansion confers a toxic gain-of-function property on the protein (Ross, 1997). This novel neurotoxic property likely involves an increased propensity for the disease protein to misfold and aggregate. In glutamine-repeat diseases, a common manifestation of this misfolding and aggregation is the formation of intranuclear inclusions (NI)[1] of the disease protein (Lunkes and Mandel, 1997; Davies et al., 1998). In many glutamine-repeat diseases, NI have been found in neurons that are known to be susceptible to the disease process, suggesting that intranuclear aggregation is central to pathogenesis (DiFiglia et al., 1997; Paulson et al., 1997*b*; Skinner et al., 1997; Holmberg et al., 1998; Igarashi et al., 1998; Li et al., 1998). Moreover, several glutamine-repeat disease proteins are primarily cytoplasmic, yet nuclear inclusions are

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1. *Abbreviations used in this paper:* EYA, Eyes Absent protein; GFP, green fluorescence protein; HA, hemagglutinin; HEK, human embryonic kidney; MJD, Machado-Joseph disease; NI, intranuclear inclusions; NLS, nuclear localization signal; SCA, spinocerebellar ataxin; TBP, TATA-binding protein.

the major pathologic structure observed in these diseases, suggesting that the nuclear environment enhances aggregation.

The fact that NI are found only in select neurons despite widespread expression of the various disease proteins suggests that additional cell-specific factors influence the likelihood of polyglutamine aggregation in a given neuron. Such factors may include the presence of a specific protease or proteases that cleave expanded polyglutamine disease proteins. This processing event would liberate a polyglutamine-containing fragment that is capable of entering the nucleus and forming aggregates. In support of this model, many transgenic and cell culture models of polyglutamine disease have used polyglutamine-containing fragments of the disease protein that are particularly potent at aggregating and causing neuronal dysfunction and degeneration (Ikeda et al., 1996; Mangiarini et al., 1996; Paulson et al., 1997*b*; Cooper et al., 1998; Merry et al., 1998; Warrick et al., 1998). Although no specific protease has yet been identified, it has been documented that many of the polyglutamine disease proteins are substrates for proteolytic processing (Goldberg et al., 1996; Miyashita et al., 1997; Wellington et al., 1998). A working model for aggregation in disease, then, is the seeding of aggregates by proteolytic fragments followed by recruitment of the full-length protein into inclusions.

NI are not pure aggregates of the pathologic polyglutamine-containing protein. Although the full array of molecules comprising NI is still unknown, studies of human tissue and transgenic mice indicate that NI also contain ubiquitin, components of the proteasome complex, and certain molecular chaperones (Cummings et al., 1998; Paulson, H.L., unpublished observations). Given the apparent heterogeneous composition of NI, it is possible that neuronal dysfunction is mediated in part through the recruitment and sequestration of critical cellular proteins. A favored model for the mechanism of aggregation is strong noncovalent, intermolecular interactions between the polyglutamine domains of proteins, perhaps in the form of a hydrogen-bonded "polar zipper" as proposed by Perutz and colleagues (Perutz et al., 1994; Stott et al., 1995; an alternative theory proposed by Kahlem et al., 1996, is covalent cross-linking by transglutaminase). Given these models, intracellular proteins that normally contain glutamine-rich or pure polyglutamine domains might be recruited into NI by virtue of their polyglutamine domains. In an effort to understand disease pathogenesis, it is important to determine the extent to which cellular proteins are recruited into NI, either through glutamine-dependent or -independent mechanisms.

As a model for glutamine-repeat disease proteins we have chosen to study ataxin-3, the defective gene product in spinocerebellar ataxia type 3/Machado-Joseph disease (SCA3/MJD), the most common dominantly inherited ataxia (Matilla et al., 1995; Ranum et al., 1995; Schols et al., 1995; Durr et al., 1996). SCA3/MJD is characterized by selective neural degeneration within the deep basal ganglia, brainstem, cerebellum, and spinal cord, despite widespread expression of mutant ataxin-3 throughout the brain (Sachdev et al., 1982; Yuasa et al., 1986; Takiyama et al., 1994; Paulson et al., 1997*a*). The gene product of the *MJD1* gene, ataxin-3, is a novel protein of unknown function with a molecular mass of ~42 kD (Kawaguchi et al., 1994). Its glutamine repeat lies near the COOH terminus, where it is normally 12–40 glutamine residues in length and is increased in disease to 55–84 residues. Studies of ataxin-3 suggest that its subcellular localization is complex and includes both cytoplasmic and nuclear localization that vary depending upon the cell type and perhaps other cellular factors (Paulson et al., 1997*a*; Wang et al., 1997; Tait et al., 1998; Paulson, H.L., unpublished observations).

We demonstrated previously that a COOH-terminal ataxin-3 fragment containing an expanded glutamine tract forms perinuclear and nuclear inclusions in transfected cells, whereas full-length expanded ataxin-3 does not (Paulson et al., 1997*b*). Here, we define the molecular determinants driving recruitment of full-length disease protein and other proteins into aggregates, and address the role of the nuclear environment in promoting aggregation. We show that polyglutamine plays an important role in mediating recruitment of proteins into NI. The cell-based studies are supported by in vivo data from human disease tissue and a *Drosophila* transgenic model which demonstrate that specific glutamine-repeat proteins are recruited into NI: TATA-binding protein (TBP) in SCA3/MJD tissue, and the nuclear protein Eyes Absent (EYA) in *Drosophila*. Finally, in experiments with nuclear-targeted ataxin-3, we present evidence that the nuclear environment promotes aggregate formation: an ataxin-3 fragment with a nonpathologic repeat does not form inclusions when expressed in the cytoplasm, but does so when it is targeted to the nucleus.

Materials and Methods

Expression Constructs and Transfection

Fig. 1 shows the expression constructs used in this study. Ataxin-1 constructs were provided by H. Orr and E. Burright (Skinner et al., 1997). GFP fusion proteins were provided by W. Strittmatter (Onodera et al., 1997).

A myc or hemagglutinin (HA) epitope tag was placed at the NH_2 terminus of the normal and expanded ataxin-3 sequence using PCR with primers that included a BamHI site to facilitate cloning into expression vectors. Primers were: myc (5′-AGCGGATCCACAGCCATGGAACA-GAAACTCATCTCTGAAGAGGATCTGGAGTCCATCTTCCAC-GAG-3′) and HA (5′-AGCGGATCCACAGCCATGTACCCATAC-GATGTTCCAGATTACGCTGAGTCCATCTTCCACGAG-3′). COOH-terminal FLAG-epitope tagged constructs were generated by PCR with a primer that contained an EcoRI site (5′-TGGAAGTGTGAAATA-GACTGTCTGATGTTCCTGCTGCTACTGTTCATTATCCTTAAG-ATG-3′). Ataxin-3 lacking the COOH terminus of the protein including the glutamine repeat (MJDΔ288-354) was generated by PCR using a 3′ primer that inserts two consecutive stop codons after an alanine residue (Ala287) four amino acids proximal to the glutamine repeat. Truncated ataxin-3 constructs consisting of the glutamine repeat and the remaining COOH terminus were described previously (Paulson et al., 1997*b*). The above ataxin-3 constructs were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). Additional truncated constructs were tagged with a nuclear localization signal (NLS) by PCR and placed into the expression vector pAG-3. In this vector, the expressed protein is tagged at the COOH terminus with a myc-hexahistidine epitope tag (Koppel et al., 1997). For the NLS constructs we used the 5′ primer (5′-GGATCCACCATG-CCCAAGAAGAAGCGGAAGGTCCGAGAAGCCTACTTTGAAA-AACAG-3′) and the 3′ primer (5′-GCGGCCGCTCTGTCAGATA-AAGTGTGAAGG-3′). Human embryonic kidney cells (HEK-293T) were transfected by calcium phosphate as described previously (Paulson et al., 1997*b*). For immunofluorescence, cells were subcultured onto collagen-coated glass coverslips 24 h after transfection, and for Western blotting cells were grown in 35-mm dishes for 36–48 h.

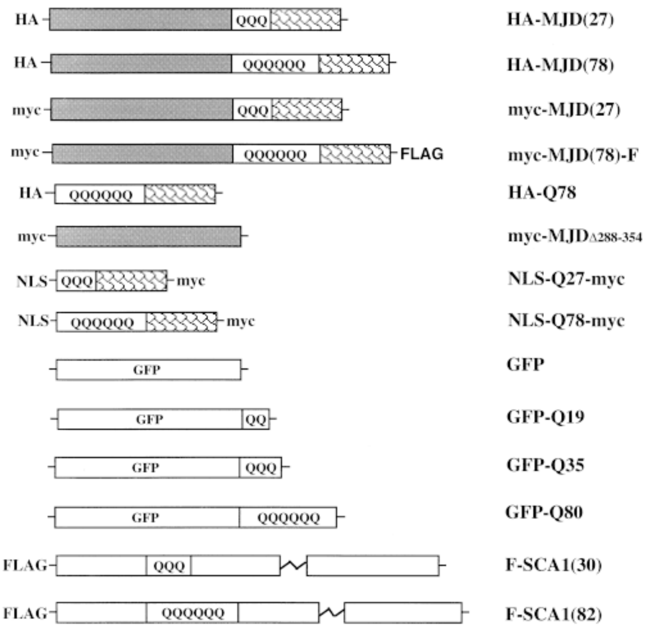

Figure 1. Expression constructs used in this study. Some constructs contain an NH_2-terminal HA, myc, or FLAG epitope tag as indicated, whereas others have an NH_2-terminal NLS and a COOH-terminal myc tag. For the first eight constructs, the gray region represents the NH_2-terminal portion of ataxin-3 up to the glutamine domain and the hatched region represents the COOH-terminal 43 amino acids after the glutamine domain. Individual nomenclature for the constructs is as follows: HA-MJD(27) and HA-MJD(78), full-length ataxin-3 containing 27 or 78 glutamines with NH_2-terminal HA epitope tag; myc-MJD(27), full-length ataxin-3 containing 27 glutamines with NH_2-terminal myc epitope tag; myc-MJD(78)-F, full-length ataxin-3 containing 78 glutamines with NH_2-terminal myc epitope tag and COOH-terminal FLAG epitope tag; HA-Q78, NH_2-terminal HA tagged truncated ataxin-3 containing 12 amino acids NH_2-terminal and 43 amino acids COOH-terminal of the 78 glutamine residues; myc-MJD∆288-354, truncated ataxin-3 containing the first 287 amino acids with an NH_2-terminal myc tag; NLS-Q27-myc and NLS-Q78-myc, COOH-terminal myc tagged, nuclear-targeted fragment of ataxin-3 containing 12 amino acids NH_2-terminal and 43 amino acids COOH-terminal of 27 or 78 glutamine residues; GFP, GFP-Q19, GFP-Q35, or GFP-Q80, GFP either alone of in fusion with 19, 35, or 80 glutamine residues; F-SCA1(30) and F-SCA1(82), full-length ataxin-1 containing 30 or 82 glutamines and an NH_2-terminal FLAG epitope tag.

The fly lines used in these studies were of genotypes *gmr-GAL4+UAS-MJDtr-Q78(S)*, and *dpp-GAL4+UAS-MJDtr-Q78(S)*, *dpp-GAL4+UAS-eya-N/UAS-MJDtr-Q78(S)*, and *dpp-GAL4+UAS-eya-C/UAS-MJDtr-Q78(S)*. *UAS-eya-N* is an Eya COOH-terminal deletion construct containing amino acids 1–487 of the *Drosophila* type I EYA protein (see Bonini et al., 1993). *UAS-eya-C* is an Eya NH_2-terminal deletion construct containing amino acids 1–34 fused to amino acids 449–760 of the type I protein. Fly tissue was stained for immunofluorescence and viewed by confocal microscopy as described (Warrick et al., 1998).

Immunofluorescence and Microscopy

48 h after transfection, cells were prepared for immunofluorescence and confocal microscopy. In brief, cells were washed once in PBS and fixed in 4% paraformaldehyde for 10 min. Cells were rinsed three times with PBS and permeabilized for 10 min in 0.05% Triton X-100 in PBS. Coverslips were then incubated in block buffer (2% goat serum, 0.05% Triton X-100 in PBS) for 30 min. Cells were incubated for 90 min at room temperature with the following primary antibodies diluted in block buffer: 9E10 anti-myc (1:100; Calbiochem, San Diego, CA), Y11 or 12CA5 anti-HA (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), affinity-purified anti-MJD (1:1,000; Paulson et al., 1997*a*), M2 anti-FLAG (1:200; Eastman Kodak, New Haven, CT), the anti-TBP Ab SI-1 (1:200; Santa Cruz Biotechnology); the anti-TBP Ab N-12 (1:200; Santa Cruz Biotechnology), and anti-EYA 10H6 raised to a conserved peptide domain of the *Drosophila* EYA protein (1:10; Bonini et al., 1993). Coverslips were rinsed three times with PBS and incubated with goat anti–rabbit FITC (Jackson Laboratories, West Grove, PA) and/or goat anti–mouse rhodamine (Jackson Laboratories), each at 1:1,000 in PBS for 90 min. Coverslips were rinsed three times with PBS, once with deionized water and mounted on glass slides (Fisher Scientific, Pittsburgh, PA) with vectashield (Vector Laboratories, Burlingame, CA). Standard epifluorescence was performed using an Olympus IX70 microscope and digital images were captured directly into Adobe Photoshop (V.4.0) using a Sony DKC-5000 camera. Confocal images were obtained from a Leica TCS-NT laser confocal microscope (Heidelberg, Germany) and images were processed in Adobe Photoshop.

Western Blots

Pelleted cells were washed in PBS and lysed in 2× SDS sample buffer. Lysates were sonicated and heated for 3 min at 90°C before electrophoresis on 7.5 or 10% SDS-polyacrylamide gels. Gels were then transblotted to PVDF membranes (DuPont NEN, Boston, MA) which were then washed in PBST (0.05% Tween 20 in PBS) and blocked for 1 h in PBS containing 5% nonfat milk (Carnation, Glendale, CA). Blots were incubated in 9E10 (1:1,000), M2 (1:160), or Y11 (1:1,000) for 60 min, rinsed three times in PBST, and incubated in 1:2,000 goat anti–rabbit HRP (Santa Cruz Biotechnology), or 1:4,000 goat anti–mouse HRP (Jackson Laboratories) for 60 min and visualized using chemiluminescence (DuPont NEN) on Kodak film. For human disease tissue, 75 μg of protein from SCA3/MJD pons was lysed in 2× SDS sample buffer, sonicated, and heated for 5 min at 90°C before electrophoresis on a 7.5% SDS-polyacrylamide gel. Transfer of protein onto PVDF and washes were performed as described above. Samples were run in parallel and blots were probed with anti–ataxin-3 antisera (1:15,000) or the anti-TBP antibody SI-1 (1:500) for 60 min, the blots were rinsed three times in PBST and incubated in 1:2,000 goat anti–rabbit HRP for 60 min and visualized using chemiluminescence as described. The blot probed with SI-1 was then washed in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) at 50°C for 30 min, rinsed several times in PBST, and chemiluminescence confirmed that there was no residual signal. This blot was then reprobed with the anti-TBP antibody N-12 (1:500) and visualized as described above.

Immunohistochemistry

Immunohistochemical staining of human disease tissue was performed as described previously (Paulson et al., 1997*a*) except that sections were not counterstained with hematoxylin and, in the experiment shown in Fig. 9, *a–e*, nickel enhancement of the peroxidase reaction was used. The SCA3/MJD brain tissue used was from the case described earlier (Paulson et al., 1997*a*); a tissue block from the midpontine region with abundant NI was chosen for analysis. Sections from the rostral pons of a nondisease control brain were immunostained, then processed and analyzed simultaneously. Two different antibodies were used to detect TBP. The first, SI-1 (sc-273; Santa Cruz Biotechnology), is a polyclonal antibody raised against full-length TBP. The second, N-12 (sc-204; Santa Cruz Biotechnology), is a polyclonal antibody raised against amino acids 12–29 of TBP, a region that does not contain the polyglutamine domain of TBP. Western blot analyses of lysates from normal and SCA3/MJD disease brain confirmed that neither antibody cross-reacted with normal or expanded ataxin-3. Moreover, immunofluorescence studies of transfected cells demonstrated that nuclear aggregates formed by NLS-Q78-myc were not labeled with anti-TBP antisera, indicating that the antibody does not cross-react with an expanded polyglutamine repeat.

To estimate the percentage of NI positive for TBP, all immunostained NI were counted in a sweep across the identical regions of adjacent SCA3/MJD sections stained with anti–ataxin-3 antisera (Paulson et al., 1997*a*) or with anti-TBP antisera SI-1 (Santa Cruz Biotechnology). All neurons with a clearly discernible nucleus were scored for the presence or absence of immunostained NI. 350–380 neurons were analyzed in each complete sweep.

Quantification of Recruitment

To quantitate recruitment of ataxin-3 into ataxin-1 nuclear inclusions, we scored in random microscopic fields all cells that were both rhodamine and FITC positive (i.e., immunofluorescence evidence of expression of both ataxin-1 and ataxin-3) and contained NI of ataxin-1. Small inclusions formed by wild-type ataxin-1 were defined as those <2 μm in size, and large inclusions were >2 μm. Recruitment of HA-MJD(27) or HA-MJD(78) into inclusions formed by nuclear targeted NLS-Q27-myc and NLS-Q78-myc was assessed in a blinded manner and the data were expressed as the percentage of cells with inclusions in which full-length ataxin-3 is recruited into inclusions.

Results

A Pathological Fragment of Ataxin-3 Forms Inclusions and Recruits Full-Length Ataxin-3

Previously, we demonstrated that a COOH-terminal ataxin-3 fragment containing expanded polyglutamine (HA-Q78) forms aggregates as detected by immunofluorescence in transfected cells, whereas full-length expanded ataxin-3, MJD(78), does not (Paulson et al., 1997*b*). Moreover, the aggregates formed by HA-Q78 were biochemically stable to boiling in SDS and migrated on SDS-polyacrylamide gels as high molecular weight complexes that remained in the stacking gel. Coexpression studies suggested that full-length expanded ataxin-3 could be recruited into both the immunofluorescent-detectable and SDS-stable complexes, but this conclusion was based simply on the appearance in aggregates of immunoreactivity to an NH_2-terminal epitope tag (Paulson et al., 1997*b*). To confirm that full-length expanded ataxin-3, and not just an NH_2-terminal fragment, was recruited, we carried out similar coexpression studies

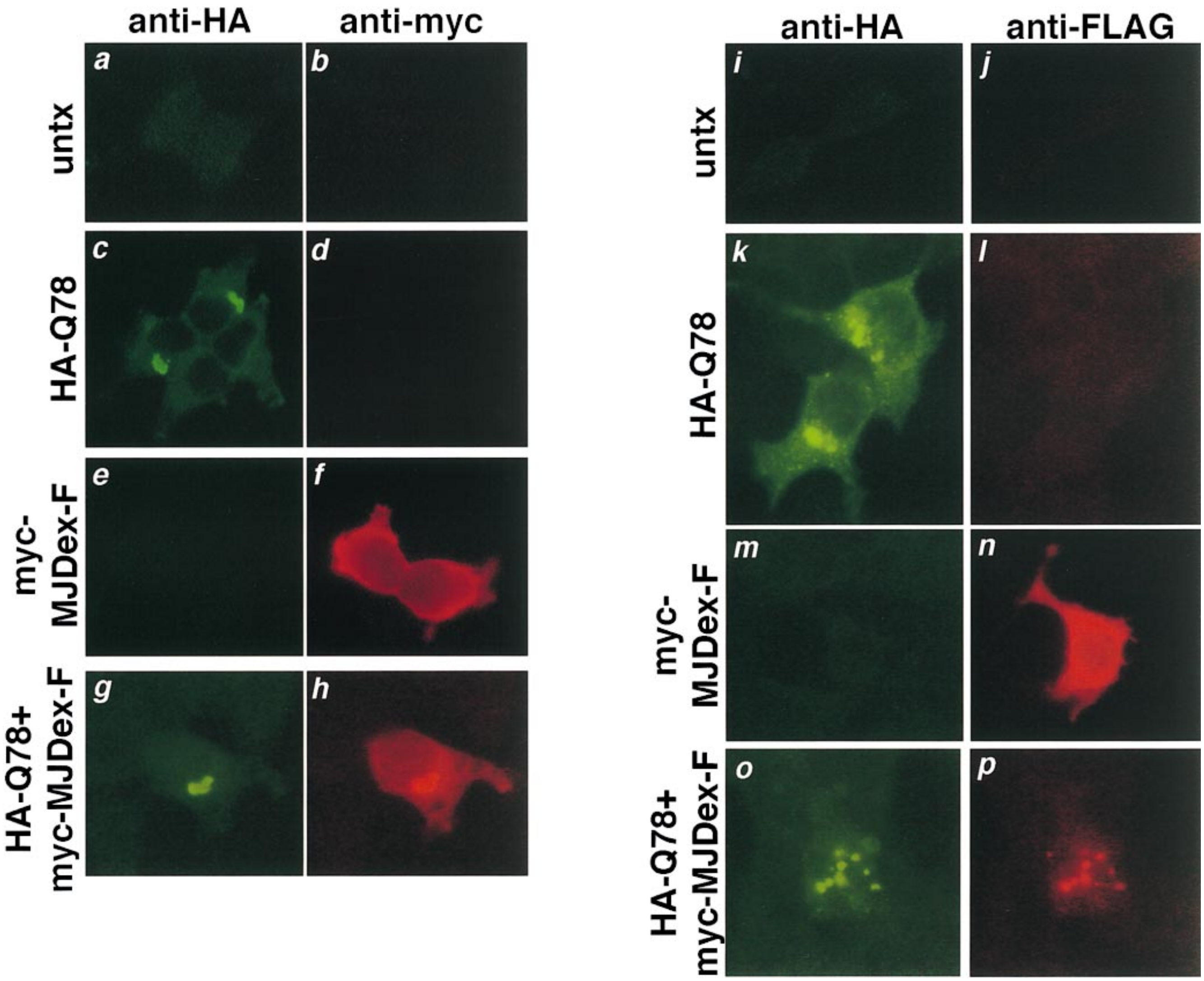

Figure 2. Immunofluorescence studies confirm that the full-length disease protein is recruited into aggregates formed by the polyglutamine-containing ataxin-3 fragment, HA-Q78. When expressed alone in transfected 293T cells, HA-Q78 forms perinuclear inclusions detectable by anti-HA (*c* and *k*). In contrast, full-length mutant ataxin-3 [myc-MJD(78)-F] is diffuse in the cell, detectable with antibodies to epitope tags placed at the NH_2 and COOH termini (myc and FLAG, respectively, shown in *f* and *n*). When coexpressed with HA-Q78, full-length mutant ataxin-3 [myc-MJD(78)-F] is efficiently recruited into the inclusions, again detectable with antibodies to tags at both ends of the protein (*h* and *p* show the myc and FLAG staining, respectively). This demonstrates that the full protein, not simply a proteolytic fragment, is incorporated into polyglutamine inclusions. Background staining in untransfected 293T cells (*untx*) is shown for anti-HA (*a* and *i*), anti-myc (*b*), or anti-FLAG (*j*).

in which both ends of expanded ataxin-3 were epitope-tagged (myc and FLAG). Doubly tagged ataxin-3 [myc-MJD(78)-F] was expressed in HEK-293T cells either by itself or with HA-Q78 (Fig. 2). As shown previously, HA-Q78 expressed alone formed perinuclear inclusions (Fig. 2, *c* and *k*). Full-length expanded ataxin-3 [MJD(78)] did not form aggregates, but instead remained diffusely distributed, primarily in the cytoplasm (Fig. 2, *f* and *n*). However, when coexpressed with HA-Q78, full-length expanded ataxin-3 was efficiently recruited into perinuclear aggregates seeded by HA-Q78 (Fig. 2, *h* and *p*). The recruited protein was detectable with antibodies to both the NH_2- and COOH-terminal epitope tags, suggesting that full-length protein is efficiently recruited into aggregates.

Recruitment of full-length expanded ataxin-3 was confirmed biochemically by immunoblot analysis which showed that the full protein was recruited into the large SDS-insoluble complexes migrating in the stacking gel (Fig. 3). When expressed by itself, full-length expanded ataxin-3 electrophoresed as monomeric protein (Fig. 3, *b* and *c*). However, when coexpressed with HA-Q78, doubly tagged ataxin-3 became incorporated into SDS-insoluble complexes detectable with antibodies to either epitope (Fig. 3, *b* and *c*), again demonstrating that the full protein, not just a fragment, is recruited into aggregates. Note that only expanded ataxin-3 containing 78 glutamines was recruited into SDS-insoluble complexes; neither normal ataxin-3 containing 27 glutamines (Fig. 3) nor ataxin-3 lacking a glutamine repeat (myc-MJDΔ288-354; not shown) was recruited into SDS-insoluble complexes, suggesting that recruitment into SDS-insoluble complexes is dependent both upon the presence and length of the glutamine repeat.

In 293T cells expressing HA-Q78, most inclusions that form are perinuclear rather than intranuclear. This is consistent with the primarily cytoplasmic expression pattern of ataxin-3 in transfected 293T cells. To more closely model the NI found in disease, we targeted expression to the nucleus by adding a NLS to the COOH-terminal fragment of expanded ataxin-3 (NLS-Q78-myc). Most transfected cells expressing NLS-Q78-myc developed predominantly NI (Fig. 4 *a* and Table I). These NI still readily recruited ataxin-3 despite the primarily cytoplasmic localization of the full-length protein (Fig. 4, *a* and *b*). These

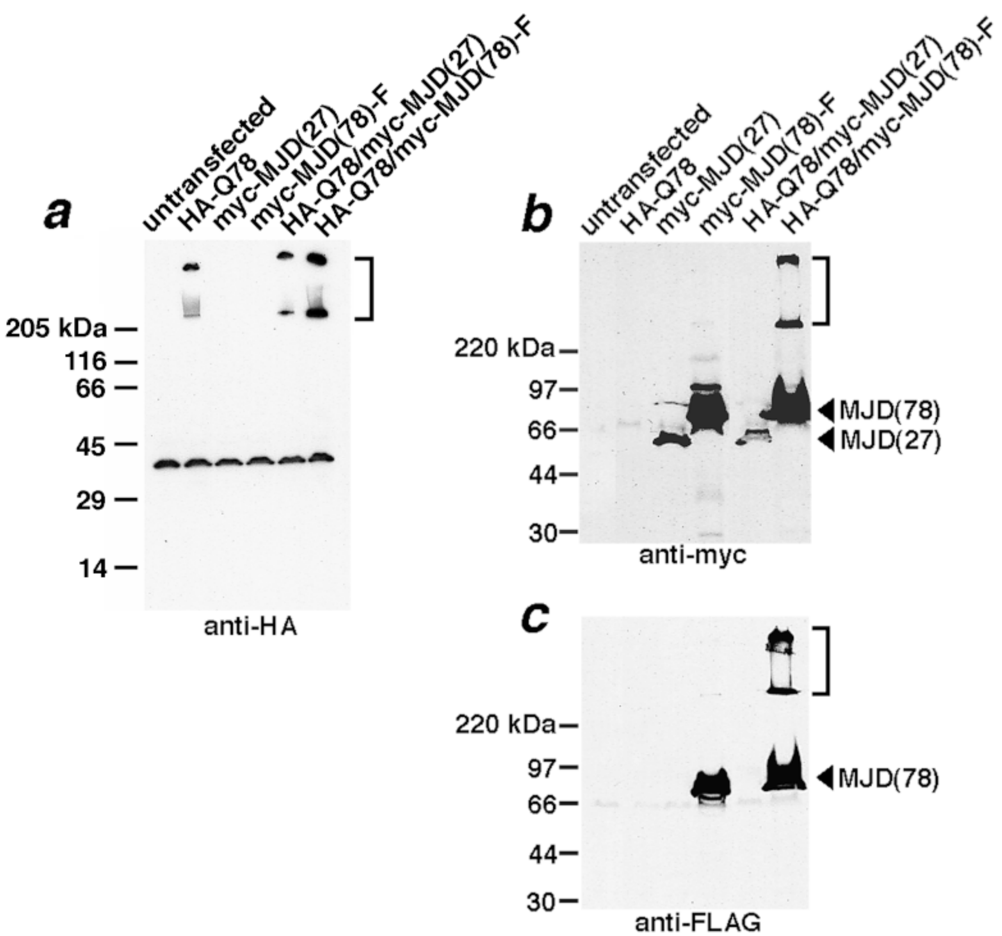

Figure 3. Intact, full-length expanded ataxin-3 is recruited into SDS-insoluble polyglutamine aggregates. Western blots of 293T cells transfected with the indicated constructs (above blots) and probed with anti-HA (*a*), anti-myc (*b*), or anti-FLAG (*c*). Blots represent identical samples run in parallel. (*a*) HA-Q78 migrates as a high molecular weight complex that remains in the stacking gel (*bracket*). On Western blots, anti-HA also cross-reacts with a cellular protein at 35 kD. This protein is not detected under more native conditions using immunofluorescence (see Fig. 2 *a*). (*b*) Coexpression of myc-MJD (78)-F with HA-Q78 leads to recruitment of the NH_2 terminus (myc epitope) of myc-MJD(78)-F into a high molecular weight SDS-insoluble complex in the stacking gel; myc-MJD(27) is not recruited into an SDS-insoluble complex. (*c*) Blots probed with anti-FLAG demonstrate that the COOH terminus of myc-MJD(78)-F is also present in the high molecular weight SDS-insoluble complex. Similar results were obtained when blots were stripped and sequentially probed with each antibody.

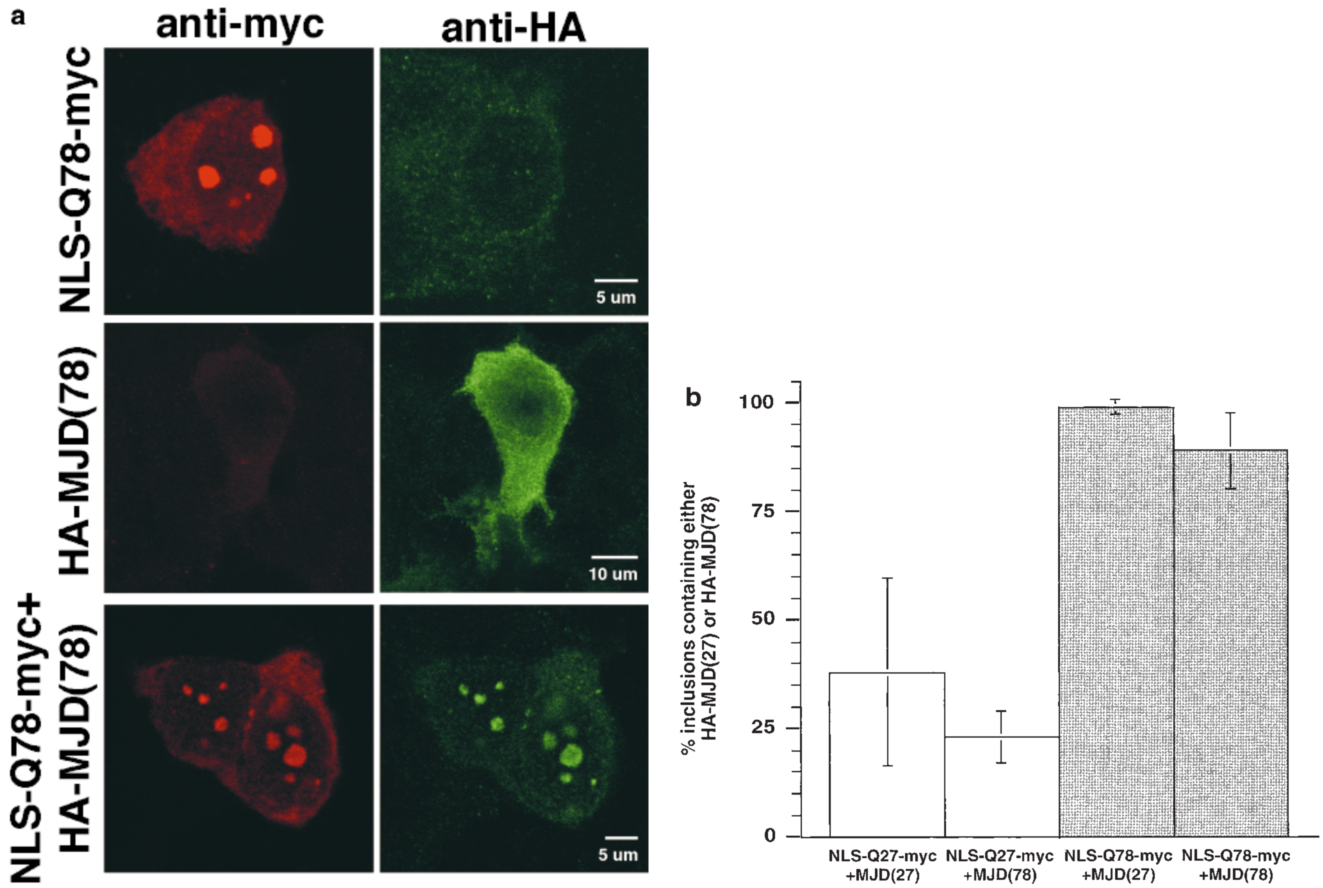

Figure 4. Addition of a NLS to the COOH-terminal fragment of mutant ataxin-3 (NLS-Q78-myc) results in the formation of NI in 293T cells and is capable of redistributing mutant ataxin-3 from a predominately cytoplasmic to nuclear localization. (*a*) Expression of NLS-Q78-myc results in nuclear inclusions which are labeled with anti-myc (*top*). The normally cytoplasmic full-length, expanded repeat ataxin-3 [HA-MJD(78), *middle*] is recruited into these nuclear inclusions (*bottom*). (*b*) Nuclear inclusions formed by NLS-Q78-myc recruit MJD(27) and MJD(78) more efficiently than NLS-Q27-myc (see Fig. 9 *a* for inclusions formed by NLS-Q27-myc). Recruitment was quantified in a blinded manner by counting cotransfected cells with nuclear inclusions in which the ataxin-3 label (anti-HA) colocalized with the inclusions.

data, along with data in Fig. 3, are consistent with a model of disease in which intranuclear aggregates, once initiated, can recruit full-length ataxin-3.

The fact that ataxin-3 recruitment into SDS-insoluble complexes requires an expanded glutamine repeat (see Fig. 3) suggests that polyglutamine is a critical domain in the recruitment process. We also tested an ataxin-3 construct lacking a glutamine repeat (myc-MJDΔ288-354) to address whether other protein domains in ataxin-3 contribute to recruitment. myc-MJDΔ288-354 encodes the NH_2-terminal 80% of the ataxin-3 polypeptide and is missing only the glutamine repeat and the subsequent 43 amino acids of the COOH terminus of the protein. When expressed alone, myc-MJDΔ288-354 localized diffusely throughout the cell, but when coexpressed with HA-Q78 it colocalized to HA-Q78 inclusions detected by immunofluorescence (Fig. 5). However, myc-MJDΔ288-354 is not detected in HA-Q78 SDS-insoluble protein complexes present in stacking gel after gel electrophoresis (not shown). These results suggest that NH_2-terminal regions of ataxin-3 may interact with the COOH terminus of the protein where the glutamine repeat resides, but that while this interaction can be detected under more native conditions of immunofluorescence, it is not stable in the presence of SDS. One candidate region is a predicted coiled–coil domain just upstream of the glutamine repeat, as coiled–coil domains frequently mediate protein–protein interactions (Lupas, 1996). Two-hybrid studies have been initiated recently to examine potential self-association domains of ataxin-3.

Table I. Formation of Nuclear Inclusions by Truncated Ataxin-3 Containing Normal or Expanded Polyglutamine and a Nuclear Localization Signal

Construct transfected	% transfected cells with NI
NLS-Q27-myc	75% (102/136)
NLS-Q78-myc	82% (86/105)

HEK-293T cells were transfected with the above constructs and the percentage of transfected cells containing NI was quantified. Data were pooled from four independent experiments in which random fields were analyzed. The numbers in parentheses represent the ratio of inclusion positive cells to the total number of transfected cells counted.

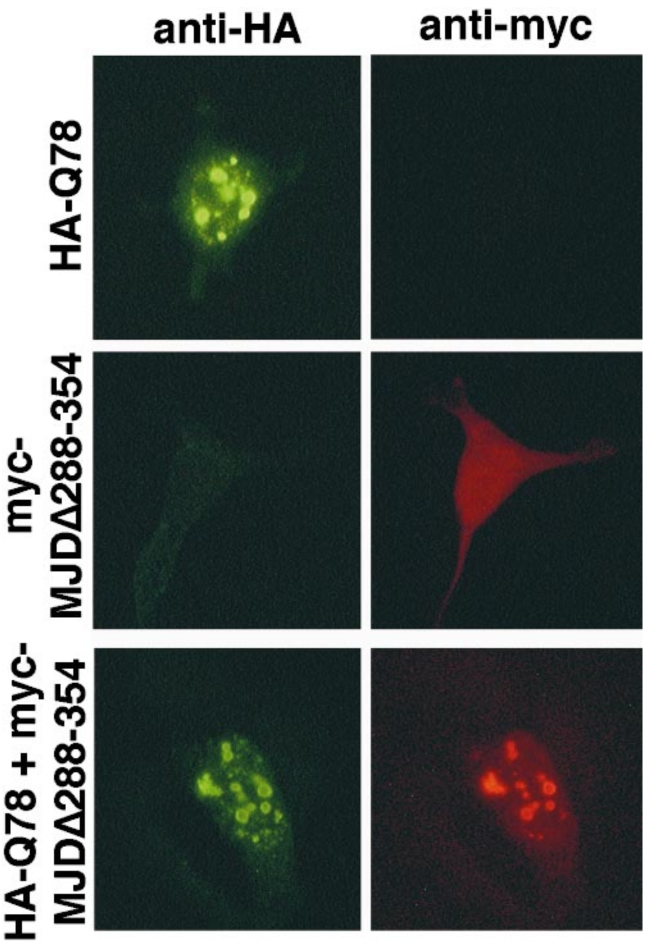

Figure 5. Ataxin-3 lacking a glutamine domain is incorporated into polyglutamine inclusions. Inclusions formed in 293T cells by HA-Q78 are detected by anti-HA antibody (*top*). An NH_2-terminal fragment of ataxin-3 lacking the glutamine domain, myc-MJDΔ288-354, maintains the diffuse localization seen with the full-length protein (*middle*). When coexpressed with HA-Q78, myc-MJDΔ288-354 becomes redistributed into the inclusions seeded by HA-Q78 (*bottom*), suggesting that regions other than the glutamine domain of ataxin-3 may also play a role in recruitment into inclusions.

Recruitment of Ataxin-3 into NI Formed by Another Polyglutamine-Repeat Disease Protein

The preceding results show that although other regions of ataxin-3 may participate in recruitment, the glutamine domain appears to be an important factor in both formation and recruitment into aggregates. Therefore, one would predict that two unrelated glutamine-repeat disease proteins would recruit each other into aggregates. To test this, we coexpressed ataxin-3 with normal or expanded ataxin-1, the disease protein in SCA1. Ataxin-1 is a 95-kD nuclear protein that has no sequence similarity to ataxin-3 outside of the glutamine repeat, and is not known to interact with ataxin-3 (Banif et al., 1994). Skinner et al. (1997) demonstrated previously that full-length ataxin-1 forms NI in transfected Cos cells. In transfected 293T cells, normal ataxin-1 [F-SCA1(30)] formed small NI (Fig. 6 *a*, *middle*), whereas expanded ataxin-1 [F-SCA1(82)] formed large nuclear inclusions (Fig. 6 *b*, *top*), confirming the results of Skinner et al. (1997). When coexpressed with ataxin-1, expanded ataxin-3 [HA-MJD(78)] was recruited into both the small and large nuclear inclusions formed by normal and expanded ataxin-1, respectively (Fig. 6, *a* and *b*, *bottom*). The presence of a glutamine repeat seems to be necessary for this recruitment since myc-MJDΔ288-354 was not recruited into ataxin-1 nuclear inclusions (not shown). This result further supports a model in which the polyglutamine domain is an important component of recruitment into nuclear aggregates.

Addition of a Glutamine Domain onto GFP Induces Recruitment into Aggregates

To directly determine whether a glutamine repeat is sufficient to confer recruitment onto a protein, we performed coexpression experiments with GFP/polyglutamine fusion proteins. Using constructs in which GFP is fused to nonpathologic glutamine repeats of 19 or 35 residues (GFP-Q19 or GFP-Q35) we assessed recruitment into HA-Q78 inclusions. When expressed alone, unmodified GFP, GFP-Q19, and GFP-Q35 were diffusely localized in the cell (Fig. 7, *a* and *b*; data not shown for GFP-Q35). However, when coexpressed with HA-Q78, GFP-Q19 and GFP-Q35 were readily recruited into inclusions (Fig. 7 *b*, *bottom*; data not shown for GFP-Q35), whereas unmodified GFP was not (Fig. 7 *a*, *bottom*).These results demonstrate that a nonpathologic glutamine repeat is sufficient to confer recruitment onto an otherwise irrelevant protein. Despite immunofluorescent evidence of GFP-fusion protein recruitment, Western blot analysis showed that GFP-Q19 and GFP-Q35 were not incorporated into the high molecular weight SDS-insoluble complexes formed by HA-Q78 (Fig. 7 *c*). Fusion of GFP to a long glutamine stretch, GFP-Q80, leads to the formation of a high molecular weight SDS-insoluble protein complex similar to that formed by HA-Q78 (Fig. 7 *c*). This provides further evidence that recruitment into the SDS-insoluble complexes requires an expanded glutamine domain.

Recruitment of Normal Cellular Proteins Containing Polyglutamine into Nuclear Inclusions

To determine if glutamine-mediated recruitment occurs in vivo, we carried out immunostaining for a known glutamine-repeat containing protein, EYA, in a recently developed *Drosophila* transgenic model of glutamine-repeat disease (Warrick et al., 1998). The EYA protein plays an essential role in *Drosophila* eye development and contains a polyglutamine tract near the NH_2 terminus (Bonini et al., 1993). Normally, EYA protein is localized diffusely in the nucleus (Bonini et al., 1998). However, in fly lines with NI, EYA is recruited into NI (Fig. 8 *a*, *arrows*). Ectopic expression of an NH_2-terminal fragment of EYA containing the glutamine domain shows that this too is recruited into NI (Fig. 8 *b*). However, a COOH-terminal fragment of the EYA protein lacking the glutamine repeat, but containing the highly conserved EYA domain, is not recruited into NI (Fig. 8 *c*), suggesting that EYA recruitment is dependent upon the presence of its glutamine repeat. Mammalian orthologues of the EYA protein lack a glutamine repeat,

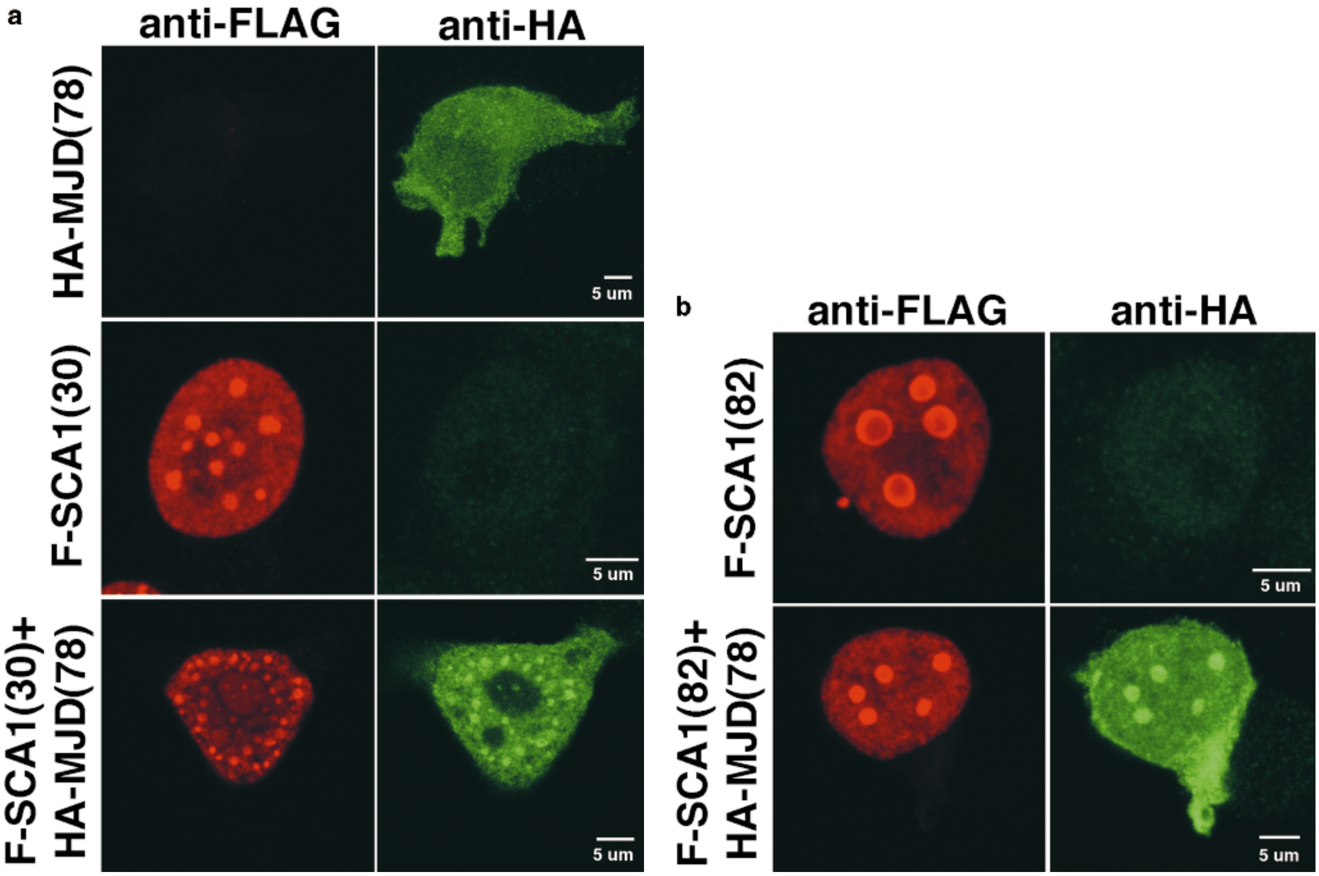

Figure 6. Cytoplasmic ataxin-3 is redistributed into NI formed by normal and expanded repeat ataxin-1. (*a*) FLAG-tagged normal ataxin-1, SCA1(30), forms small intranuclear structures (*middle*) which are able to recruit HA-tagged MJD(27) (not shown) and HA-MJD(78) (*bottom*). (*b*) FLAG-tagged expanded repeat ataxin-1, SCA1(82), forms larger NI (*top*) which also recruit HA-MJD(27) (not shown) and HA-MJD(78) (*bottom*).

and thus it is perhaps not surprising that mammalian EYA proteins do not localize to NI in SCA3/MJD brain (not shown).

The results with EYA provide in vivo evidence that an endogenous polyglutamine-containing protein is recruited into NI. They support a model of disease in which polyglutamine aggregates may cause neuronal dysfunction by sequestering normal polyglutamine-containing proteins. In humans, several nuclear proteins contain polyglutamine tracts or glutamine-rich domains, including several transcription factors. One or more of these proteins might be recruited into NI in a glutamine-repeat–dependent manner, thereby reducing total cellular activity or altering properties of the recruited proteins. TBP, or TFIID, is an intriguing candidate protein because of its relatively long polyglutamine tract of 38 residues and its critical role in transcription. Therefore, we performed immunohistochemistry with anti-TBP antibodies against control and SCA3/MJD brain tissue. These studies showed that TBP colocalizes to NI in SCA3/MJD disease brain (Fig. 9). Immunostaining of NI was seen with two antibodies against TBP, one raised against the full protein (Fig. 9 *b*) and a second against an NH_2-terminal peptide of TBP that does not contain the glutamine domain (Fig. 9 *d*). Western blot of disease brain tissue (Fig. 9 *f*) and immunofluorescence analysis of transfected cells showed that neither antibody cross-reacts with the normal or expanded glutamine domains in ataxin-3 (not shown). Comparative immunostaining of adjacent sections with ataxin-3 antibody indicated that approximately one-fourth of NI in disease tissue contains TBP; coimmunofluorescence staining of brain tissue with ubiquitin and TBP antisera confirmed that only a fraction of NI contains TBP. This finding that not all NI immunostain for TBP is consistent with results from our laboratory and others showing that NI are heterogeneous with respect to size, shape, and molecular composition (Cummings et al., 1998; Paulson, H.L., unpublished observations). These results with EYA protein and TBP are the first to show that recruitment of specific polyglutamine-containing proteins occurs in vivo.

Nuclear Localization Promotes Aggregate Formation

An intriguing feature of glutamine-repeat diseases is that despite a primarily cytoplasmic localization for several disease proteins, the nucleus seems to be the major site of aggregation. Therefore, we sought to determine whether the nuclear environment per se promotes aggregation. To do this, we targeted a nonpathologic glutamine repeat to the nucleus and assessed whether this led to the formation of

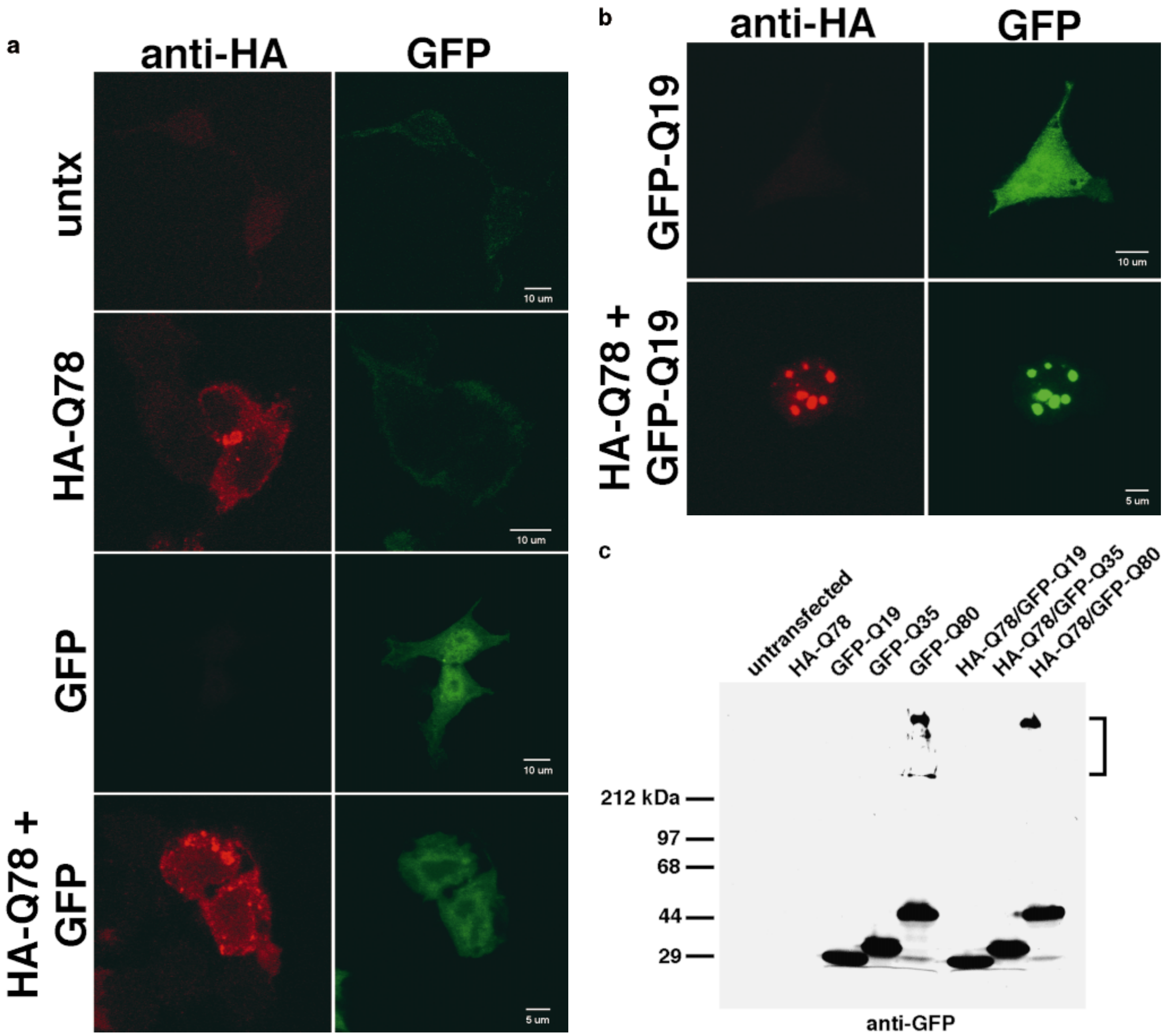

Figure 7. A glutamine stretch on the control protein, GFP, is sufficient for recruitment into polyglutamine inclusions but not SDS-insoluble protein complexes. Untransfected 293T cells (*untx*) do not label for anti-HA or GFP (*a*, *top*). Expression of GFP (*a*, *third row*) or GFP-Q19 (*b*, *top*) alone shows a diffuse staining throughout the cell. However, when coexpressed with HA-Q78, GFP-Q19 becomes redistributed into inclusions (*b*, *bottom*) whereas GFP itself does not (*a*, *bottom*). (*c*) GFP-Q80 forms a high molecular weight SDS-insoluble protein complex (*bracket*) when expressed alone, whereas GFP-Q19 and GFP-Q35 do not. When coexpressed with HA-Q78, neither GFP-Q19 nor GFP-Q35 becomes recruited into the HA-Q78 SDS-insoluble protein complex, showing that recruitment into this complex is dependent on glutamine repeat length.

NI. Typically, a COOH-terminal fragment of ataxin-3 containing a repeat of 27 glutamine residues is expressed diffusely in the cell (Paulson et al., 1997*b*). Adding a NLS to this ataxin-3 fragment (NLS-Q27-myc) led to intranuclear localization and formation of NI (Fig. 10 *a* and Table I). By immunofluorescence, NLS-Q27-myc inclusions resembled those formed by NLS-Q78-myc. However, two lines of evidence suggest that they differ in important respects. First, immunofluorescence analysis showed that NI formed by NLS-Q27-myc were less efficient at recruiting full-length ataxin-3 (Fig. 4 *b*). Second, by Western blot analysis NLS-Q27-myc did not form the high molecular weight SDS-insoluble complex regularly observed with NLS-Q78-myc (Fig. 10 *b*). The simplest explanation for these differences is that the intermolecular polyglutamine interactions in NLS-Q27-myc inclusions differ from those in NLS-Q78-myc inclusions.

Discussion

Misfolding and aggregation of the disease protein appears to be central to the pathogenesis of the glutamine-repeat diseases. In most if not all glutamine-repeat diseases, aggregation is manifested by the formation of NI in suscepti-

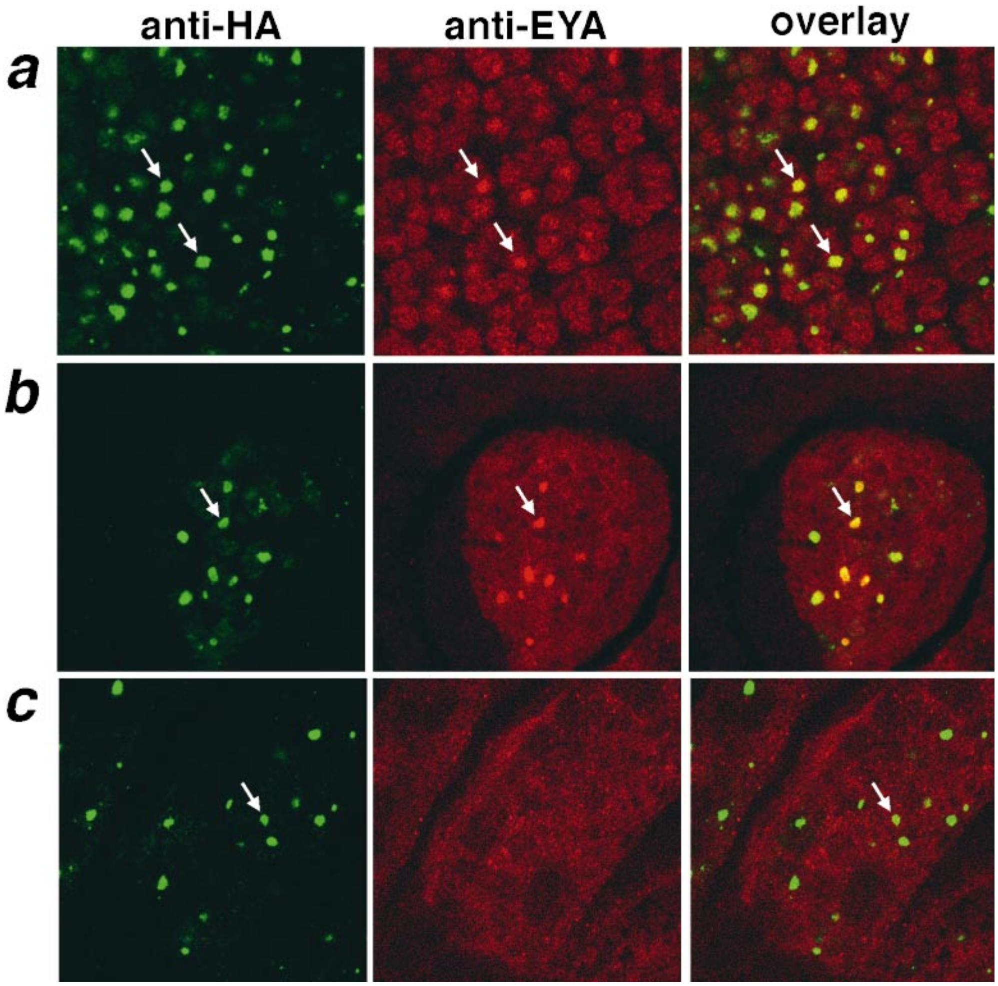

Figure 8. In vivo recruitment of EYA in a *Drosophila* model of polyglutamine disease is dependent on the presence of its glutamine domain. The left column represents sections labeled with anti-HA, detecting the HA-Q78 protein. The middle column is labeled with 10H6, recognizing a conserved peptide domain present in the expressed EYA proteins. The first two columns are merged into the right column. (*a*) NI (*arrows*) formed by truncated ataxin-3 (HA-Q78) are visualized with anti-HA labeling. Anti-Eya label demonstrates that in the transgenic fly expressing an expanded glutamine domain, the endogenous EYA protein is primarily localized throughout the nucleus and in many cells has a distinct punctate appearance. The overlay indicates that the EYA protein colocalizes with polyglutamine inclusions. (*b*) Ectopic expression of the NH_2-terminal half of the EYA protein, which contains the polyglutamine repeat. Coexpression of the HA-Q78 protein with the EYA NH_2 terminus shows that EYA containing the glutamine domain is concentrated in the NI (*arrow*). (*c*) Ectopic expression of the COOH-terminal half of the EYA protein, which contains the highly conserved Eya domain, but not the glutamine repeat. Coexpression of the HA-Q78 protein (*arrow*) with the EYA COOH-terminal domain shows that the COOH-terminal domain lacking the glutamine repeat does not become recruited into NI. Photographs in *a* are from the developing eye field of an eye-antennal imaginal disc from a third-instar larva expressing HA-Q78 with *gmr-GAL4*; those in *b* and *c* are from the antennal field of developing eye-antennal imaginal discs of larvae expressing HA-Q78 and the *eya* constructs with *dpp-GAL4*.

ble neurons (Davies et al., 1998). The molecular events driving polyglutamine aggregate formation are still poorly understood, but a commonly held view is that limited proteolysis may generate aggregation-prone fragments of the mutant protein in at least some glutamine-repeat diseases (Goldberg et al., 1996; Ikeda et al., 1996; DiFiglia et al., 1997; Merry et al., 1998; Wellington et al., 1998). Such fragments might serve to initiate the formation of aggregates that, once begun, could recruit full-length disease protein and other cellular proteins containing a glutamine repeat. In this paper we have provided in vitro and in vivo evidence supporting such a recruitment model. We have demonstrated that full-length disease protein is efficiently recruited into NI seeded by a polyglutamine-containing fragment, and that certain other glutamine-repeat proteins localize to NI in human disease tissue and in a transgenic model. Our results support the general view that polyglutamine is a critical domain in the recruitment process, although in the particular case of ataxin-3, other domains within the protein also likely contribute to recruitment.

Several lines of evidence presented here suggest that interactions between glutamine repeats play an important role in recruitment of proteins into NI. First, adding a glutamine domain to GFP is sufficient to cause recruitment of this otherwise irrelevant protein. This finding argues strongly for direct polyglutamine–polyglutamine interactions as one important molecular mechanism underlying recruitment into aggregates. Second, recruitment of EYA in the fly is dependent upon the presence of the glutamine repeat. Third, recruitment into SDS-insoluble complexes is dependent upon an expanded glutamine-repeat within the recruited protein. Finally, the recruitment of TBP in SCA3/MJD brain is consistent with an important contributory role for the glutamine repeat within the recruited protein. However, we stress that the ability of polyglutamine to confer recruitment on a given protein

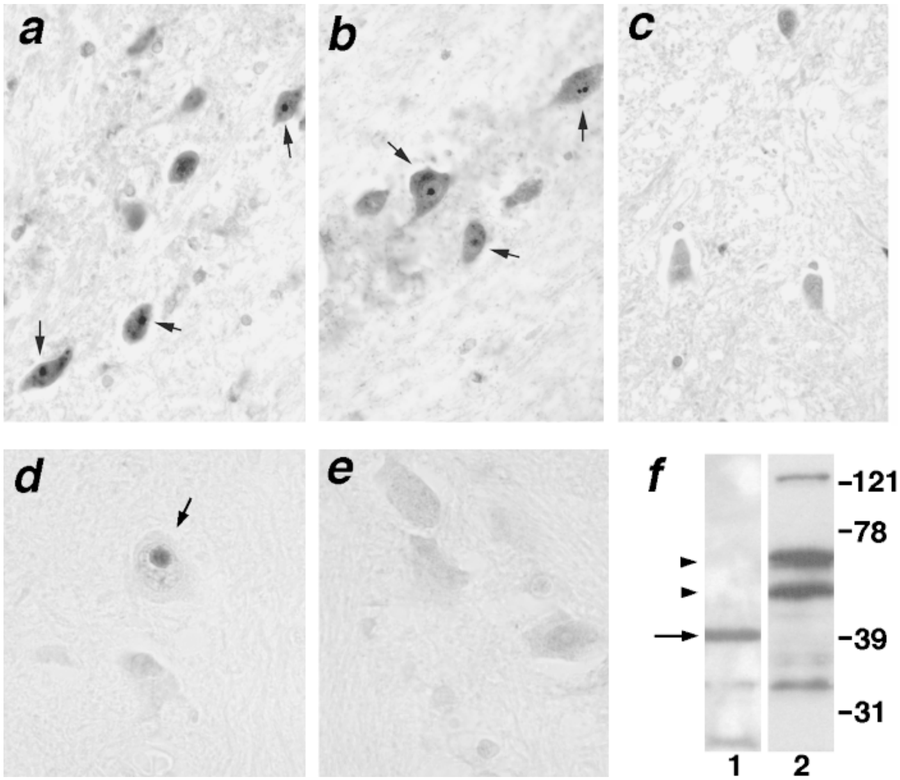

Figure 9. TBP immunoreactivity in NI in SCA3/MJD disease brain. Sections from control and disease pons were immunohistochemically stained with two different anti-TBP antisera or anti–ataxin-3 antiserum. (*a*) Anti–ataxin-3 immunostains NI within neurons of SCA3/MJD brain. (*b*) An adjacent section stained with the anti-TBP antibody SI-1 demonstrates TBP present within a subset of NI. (*c*) Control brain immunolabeled with SI-1. (*d*) Higher power magnification of SCA3/MJD pons showing immunolabeling of a NI with a second anti-TBP antibody, N-12. (*e*) Control brain immunolabeled with N-12. (*f*) Western blot of lysate from SCA3/MJD pons probed with the anti-TBP antibody N-12 (lane *1*) or ataxin-3 antisera (lane *2*) confirms the specificity of the TBP antibody. Arrows in *a*, *b*, and *d* indicate neurons that have NI. Original magnification, ×630 (*a–c*) and ×1,260 (*d* and *e*). The arrow indicates TBP in lane *1* and arrowheads indicate ataxin-3 protein from normal and expanded alleles in lane *2*.

likely varies depending upon its particular protein and cellular context. The results with TBP illustrate this point: TBP recruitment is seen in only a fraction of NI in SCA3/MJD brain, suggesting that the presence of a glutamine repeat is not always sufficient to confer recruitment. Based on our results, we anticipate that some but not all glutamine-repeat proteins will be recruited into NI; it will be important to determine if additional polyglutamine proteins are recruited into aggregates both in vivo and in vitro. It is also important to stress that clearly not all proteins colocalizing to NI are recruited by virtue of their having a glutamine repeat. For example, the colocalization of proteasome and chaperone proteins to NI instead likely reflects the inherent functions of these proteins (Cummings et al., 1998; Paulson, H.L., unpublished observation).

Our results with EYA and TBP represent the first in vivo evidence that nondisease glutamine repeat proteins are sequestered within NI, raising the possibility that the neuronal toxicity of glutamine-repeat diseases may in part result from sequestration of other polyglutamine-containing proteins within aggregates. Given that only a subset of inclusion-positive neurons show TBP recruitment, our data suggest that the presence of a polyglutamine tract favors but is not itself sufficient to cause recruitment into NI. The sequestering of polyglutamine-containing transcription factors might perturb transcriptional events with potentially adverse effects for the neuron (Gerber et al., 1994). It will be important to determine if recruitment of other polyglutamine-containing nuclear factors occurs and to extend our results to other glutamine-repeat diseases. A complete account of all the proteins comprising NI awaits the purification and dissociation of NI into constituent proteins, a challenging task now being pursued by several laboratories.

A controversial and unresolved issue is whether aggregate formation is initiated by the full-length protein or a polyglutamine-containing fragment of the disease protein. The answer, in fact, may differ depending upon the disease. In SCA1, for example, evidence suggests that full-length ataxin-1 is responsible for aggregate formation (Burright et al., 1995; Skinner et al., 1997), whereas in Huntington's disease evidence is consistent with proteolysis playing a role in aggregate formation (Goldberg et at., 1996; DiFiglia et al., 1997; Davies et al., 1998). Regardless of which is the case for SCA3/MJD, our results indicate that once aggregation is initiated, the full-length protein can be recruited efficiently into aggregates. In the human disease state, similar sequestering of the disease protein could contribute to pathogenesis by reducing cellular levels of functional ataxin-3. If this occurs, then the disease process would reflect not only the well-accepted dominant toxic gain-of-function, but also a partial loss-of-function of the disease protein. Since the function of ataxin-3 is unknown, it is not currently possible to test this hypothesis in SCA3/MJD. However, clinical features and in vitro studies in another glutamine-repeat disease, spinobulbar muscular atrophy, are consistent with a partial loss-of-function of the disease protein, the androgen receptor (reviewed in Merry and Fischbeck, 1998).

Our finding that a fragment of normal ataxin-3 with 27 glutamines forms inclusions only when targeted to the nucleus strongly supports a proaggregatory role for the nucleus. Recent studies of SCA1 transgenic mice further sug-

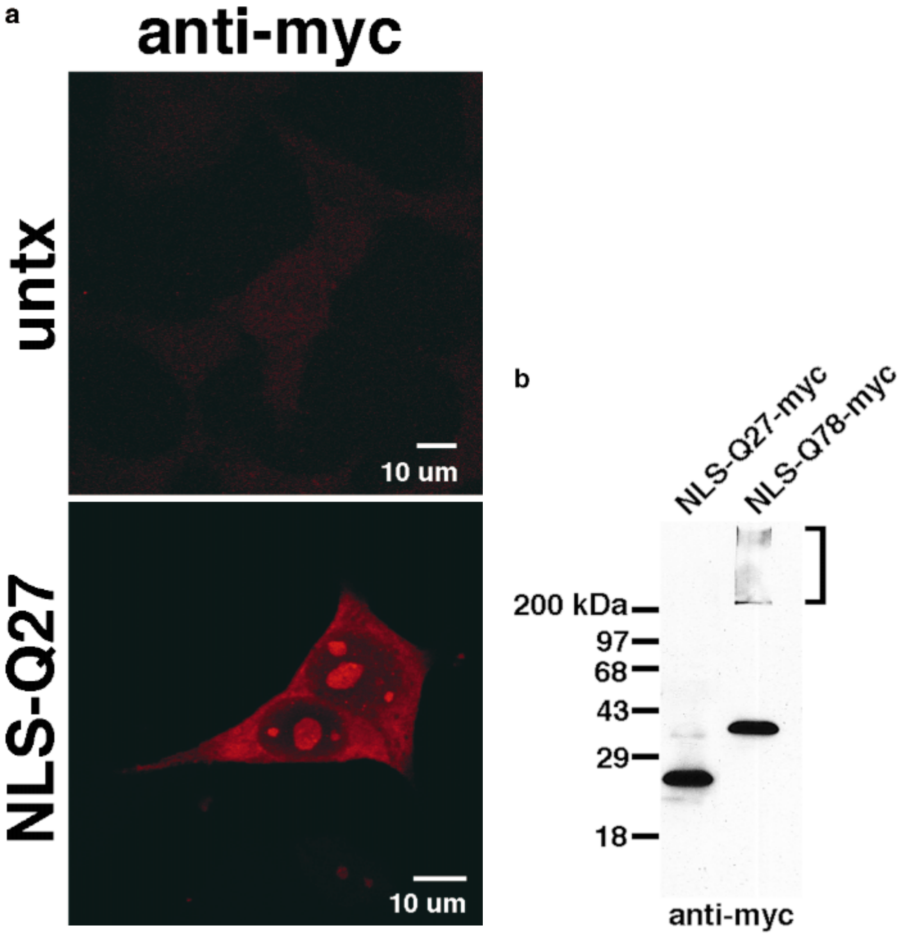

Figure 10. Expression of a nonpathologic glutamine stretch in the nucleus leads to formation of nuclear inclusions but not SDS-insoluble complexes in 293T cells. (*a*) Nuclear localization of a COOH-terminal fragment of ataxin-3 containing 27 glutamines (*NLS-Q27-myc*) leads to the formation of NI detected by anti-myc epitope tag. Untransfected cells (*untx*) are not labeled with anti-myc. (*b*) Western blot of 293T cells transfected with a COOH-terminal fragment of ataxin-3 containing 27 glutamines (*NLS-Q27-myc*) or 78 glutamines (*NLS-Q78-myc*) targeted to the nucleus. Anti-myc labeling shows that NLS-Q27-myc forms a major band at 25 kD, whereas NLS-Q78-myc forms a 40-kD band as well as a high molecular weight, SDS-insoluble complex which remains in the stacking gel (*bracket*).

gest this is true in vivo as well: mutant ataxin-1 that has been modified to stay in the cytoplasm no longer forms aggregates (Klement et al., 1998). Aggregation may occur preferentially in the nucleus for one or more reasons. The nuclear and cytoplasmic compartments may differ fundamentally in their capacity to handle misfolded polypeptides perhaps in part due to differences in their complement of chaperonins and proteasomes (Michels et al., 1997). Alternatively, the cytoarchitecture of the nucleus could lead to higher focal concentrations of disease protein that would accelerate aggregate formation. Subnuclear domains exist that may serve as sites for expanded glutamine disease protein to accumulate and aggregate (Skinner et al., 1997; Lamond and Earnshaw, 1998). Lastly, since the protein composition differs between the nucleus and the cytoplasm, interactions with selective nuclear proteins may render polyglutamine prone to aggregation.

If, as we have shown, an ataxin-3 fragment with a nonpathologic glutamine repeat will aggregate when targeted to the nucleus, what prevents NI from forming in the absence of glutamine-repeat expansion? The simplest explanation for this result is that even a normal glutamine repeat of 27 residues has a low probability of misfolding and aggregating, and that by freeing this repeat from its normal protein context, forcing it into the nucleus and overexpressing it in transfected cells, we are effectively driving this misfolding and aggregation. It is clear that the intermolecular interactions within NLS-Q27 inclusions differ from those within NLS-Q78 inclusions, since NLS-Q27 inclusions do not efficiently recruit full-length ataxin-3 and do not form the high molecular weight SDS-insoluble complexes seen with the expanded glutamine domain. Although NI can form from nonpathological glutamine domains when overexpressed in vitro, in vivo there may be protective mechanisms which can dissociate these weaker aggregates if they are formed (Glover and Lindquist, 1998). This cellular paradigm provides a model to further characterize the nature of nuclear aggregation and to identify specific proaggregatory nuclear factors.

Two recent studies further support a role for the nucleus in the pathogenesis of glutamine-repeat diseases. In SCA1 transgenic mice, mutant ataxin-1 protein lacking a NLS remains in the cytoplasm, fails to aggregate, and does not cause neurodegeneration (Klement et al., 1998). In an in vitro model of Huntington's disease, mutant huntingtin modified with a nuclear export signal no longer forms aggregates or induces apoptotic cell death (Saudou et al., 1998). These same studies also demonstrate that observable NI are not necessary for the onset of pathogenesis in mice or for the induction of apoptosis in cultured neurons. These studies thus raise the question, What role if any do NI play in the glutamine-repeat disease process? We favor the view that in this group of chronic neurodegenerative disorders, disease progression is complex, consisting of at least two stages: an early period of neuronal dysfunction and a later period of exacerbated dysfunction and neuronal demise. Although early neuronal dysfunction probably is a direct consequence of misfolding of the disease protein, observable aggregates may not be necessary. At early points in pathogenesis, one possibility is that expanded polyglutamine alters protein–protein interactions subtly in a disease- and neuron-specific manner, resulting in abnormal neuronal function. In later stages of disease, however, when NI are clearly detectable in pathologic studies, we propose that NI contribute to exacerbated dysfunction and neuronal demise. The findings described in this report do not implicate NI in neuronal demise directly, but suggest ways that these structures might perturb normal nuclear events, including the sequestering of nuclear proteins that contain glutamine repeats or glutamine-rich domains. Clearly, further studies of cellular and animal models are necessary to determine the precise role of NI in later stages of disease.

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