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# Evidence for a recruitment and sequestration mechanism in Huntington's disease

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Polyglutamine (polyQ) extension in the coding sequence of mutant huntingtin causes neuronal degeneration associated with the formation of insoluble polyQ aggregates in Huntington's disease. We constructed an array of CAG/CAA triplet repeats, coding for a range of 25–300 glutamine residues, which was used to generate expression constructs with minimal flanking sequence. Normal-length (25 glutamine residues) polyQ did not aggregate when transfected alone. Remarkably, when co-transfected with extended (100–300 glutamine residues) polyQ tracts, normal-length polyQ-containing peptides were trapped in insoluble detergent-resistant aggregates. Aggregates formed in the cytoplasm but were visible in the nucleus only when a strong nuclear localization signal was present. Intermolecular interactions between polyQ tracts mediated the localization of heterogeneous aggregates into the nucleolus by nucleolin protein. Our results suggest that extended polyQ can interact with cellular polyQ-containing proteins, transport them to ectopic cellular locations, and form heterogeneous polyQ aggregates. We provide evidence for a recruitment mechanism for pathogenesis in the polyQ neurodegenerative disorders. In susceptible cells, extended polyQ tracts in huntingtin might interact with and sequester or deplete certain endogenous polyQ-containing cellular proteins.

**Keywords:** polyglutamine; trinucleotide; triplet repeats; aggregation; neurodegeneration; Huntington's disease

## 1. INTRODUCTION

Eight dominantly inherited neurodegenerative diseases, including Huntington's disease, are caused by the expansion of a CAG trinucleotide repeat within protein coding sequences (Reddy & Housman 1997). If translated, these mutant expanded trinucleotide repeat domains can encode up to 100 glutamine residues embedded within the affected proteins. Each of these polyglutamine (polyQ) expansions occurs in one of a diverse set of widely expressed and seemingly unrelated proteins. Late-onset progressive neurodegeneration is a common feature in these diseases, yet they differ in important respects. Specific regions of the brain are affected in the various diseases, with correspondingly distinct clinical presentations. Thus Huntington's disease is characterized by late-onset degeneration and profound neuronal loss in a specific subset of striatal neurons, whereas the spinocerebellar ataxias manifest themselves as degeneration of the Purkinje cell layer of the cerebellum. The molecular basis for selective vulnerability within neuronal populations in the polyQ diseases is not known, although differences in expression levels of the affected proteins between different neuronal subpopulations might have a role (Kosinski *et al*. 1997).

Perutz and colleagues (Perutz *et al*. 1994) proposed several years ago a 'polar zipper' model of polyQ interaction, in which homopolymeric polyQ domains can interact in a non-covalent manner through the formation of intermolecular hydrogen bonds. It has now been shown that extended polyQ tracts aggregate as amyloid-like protein *in vitro* and form neuronal intranuclear inclusions in mice transgenic for the Huntington's disease mutation (Scherzinger *et al*. 1997; Davies *et al*. 1997). In Huntington's disease, aggregation of extended polyQ tracts causes formation of neuronal intranuclear inclusions as well (DiFiglia *et al*. 1997).

At the cellular level, the precise mechanisms of polyQ toxicity as a cause of neuronal dysfunction and neuron loss in the trinucleotide repeat diseases are unknown. Although it is clear that a toxic 'gain of function' is attributable to the polyQ tracts themselves, the role of large polyQ aggregates in neuronal dysfunction and in neuronal loss in these progressive diseases remains to be elucidated. Moreover, at least in Huntington's disease, neuronal dysfunction as manifested by clinical abnormalities precedes the development of discernible histopathologic changes such as neuronal degeneration and neuronal loss (Vonsattel *et al*. 1985). There might well be distinct polyQ-dependent pathogenetic mechanisms that precede the formation of frank aggregates and that might be associated with neuronal dysfunction at early stages of disease. One such potential mechanism is suggested by the work of Cha *et al*. (1998), who show a decrease in levels of certain neurotransmitter receptors at the transcriptional level in mice transgenic for an expanded CAG repeat. This altered expression of specific subsets of neurotransmitter receptors precedes clinical symptoms and the formation of aggregates in these mice.

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Here we describe certain anomalous intrinsic biochemical properties of polyQ tracts that might contribute to the ability to self-aggregate. We demonstrate that the subcellular localization of expanded polyQ aggregates within cells is determined by the properties of the molecules within which the polyQ stretch is contained. We clearly demonstrate polyQ-dependent interactions between different polyQ-containing molecules in transient transfection experiments. We show not only that expanded polyQ molecules aggregate on their own in cells but that they can recruit polypeptides with short polyQ domains into cytoplasmic and nuclear aggregates. These results provide evidence for a recruitment mechanism for pathogenesis in the polyQ neurodegenerative disorders. In susceptible cells, extended polyQ tracts in mutant proteins might interact with and sequester or deplete certain endogenous polyQ-containing cellular proteins.

## 2. GENERATING polyQ OF LENGTHS RANGING FROM 25 TO 300 GLUTAMINE RESIDUES

To study polyQ aggregation we generated alternating CAG/CAA repeats encoding 25 glutamine residues (normal in Huntington's disease), extended 47 and 104 glutamine residues (pathological in Huntington's disease), and extended 191, 230 and 300 glutamine residues (elongated beyond pathological range). All lengths of alternating CAG/CAA repeats were quite stable in bacteria, in contrast with native extended CAG repeats. A short-N-terminus Huntington's disease complementary DNA (cDNA) fragment including the Kozak box and the first 17 residues was ligated to polyQ repeats of different lengths (25–300 glutamine residues). To monitor the formation of aggregates in cells, we fused polyQ tracts at the C-terminus with either a 28-residue c-Myc tag or a 230-residue enhanced green fluorescent protein (EGFP) tag. PolyQ fusions were subcloned into the pcDNA 3.1 expression vector, which includes the human cytomegalovirus immediate-early promoter–enhancer for high-level expression in mammalian cells.

## 3. ANOMALOUS PHYSICAL PROPERTIES OF polyQ ON SDS–PAGE

Initially we wished to characterize the products of each of our constructs in a translation system *in vitro* that would permit an analysis of the properties of the encoded polypeptides in the absence of cell metabolism. This characterization demonstrated that polypeptides predicted by the sequence of the synthetic open reading frame were indeed produced, but it also gave an insight into an unusual property of polyQ-containing polypeptides. It has previously been observed that polypeptides containing extended polyQ segments show an atypical mobility on SDS–PAGE. The polypeptides produced by our constructs exhibited a mobility in SDS–PAGE that was proportional to predicted molecular mass but demonstrated a systematic relative decrease in electrophoretic mobility consistent with these previous reports (figure 1). The ability to extend the length of polyQ to 300 residues allowed a systematic evaluation of this altered mobility. We found that the observed mobility of a polypeptide composed almost exclusively of polyQ had a mobility 40% of the expected mobility of a polypeptide of identical length with a random amino-acid sequence (figure 2). Atypical mobility of normal and extended polyQ peptides was seen on SDS–PAGE gels at various

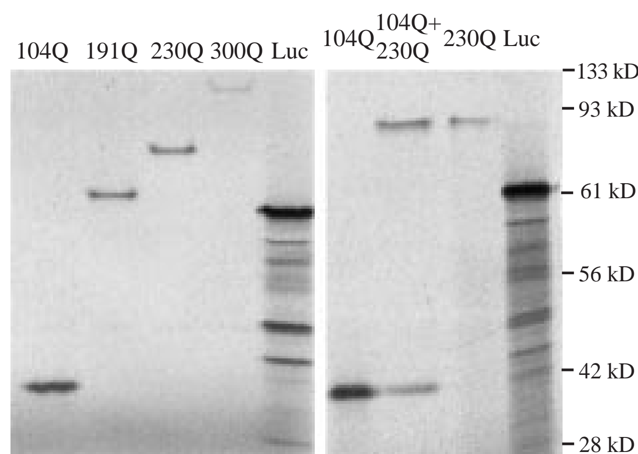


Figure 1. Translation and co-translation *in vitro* of polyQ expression constructs. PolyQ expression constructs were translated *in vitro* using T7 RNA polymerase and rabbit reticulocyte lysates in the presence of [$^{35}$S]methionine. Left, SDS-PAGE analysis of the alternating CAG/CAA repeats translated *in vitro*. Right, no detectable intermediates between co-translated 104Q and 230Q constructs. Luc, 61 kDa luciferase translated *in vitro*.

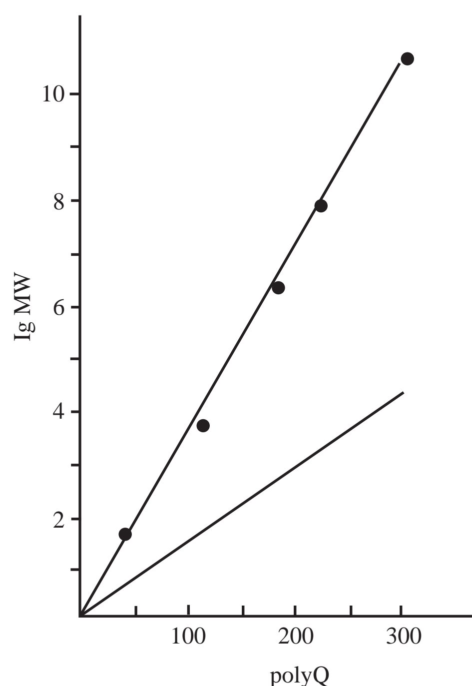


Figure 2. Retarded mobility in SDS–PAGE of polyQ tracts translated *in vitro*. The solid line represents the expected mobility of products translated *in vitro* on the basis of predicted molecular mass. In contrast, filled circles show the observed mobilities of these products in SDS–PAGE. The observed mobility of these products, composed almost exclusively of polyQ, was consistently 40% of that expected across a range of 25–300 glutamine repeats.

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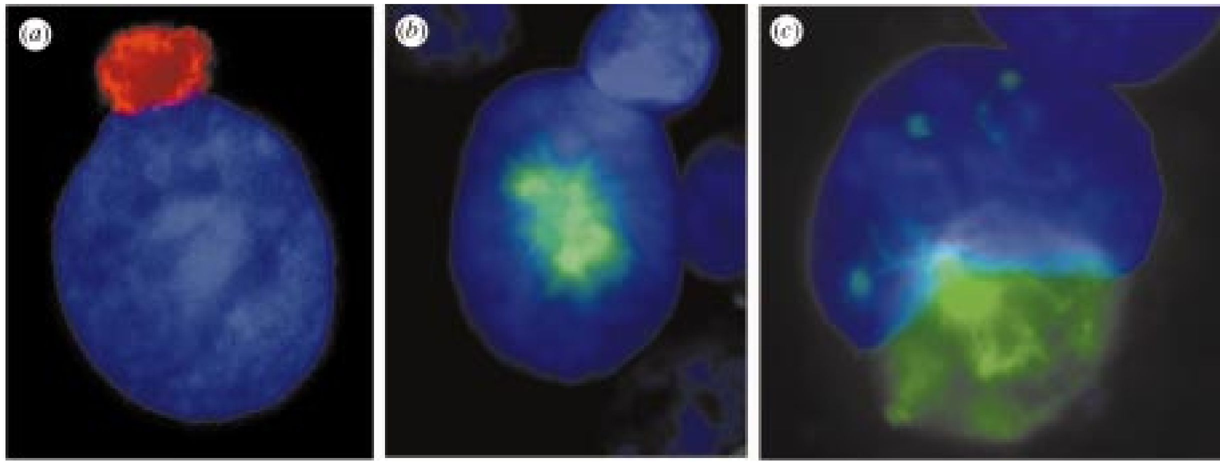


Figure 3. Extended polyQ constructs expressed by transient transfection in Cos-1 cells, 48 h after transfection. (*a*) 104Q–c-Myc fusion construct forms a single dense brilliantly fluorescent spherical structure located in the cytoplasm but closely apposed to the nuclear membrane. Note the poor penetration of the anti-Myc antibody, causing the appearance of a rim around the polyQ aggregate. (*b*) 104Q–nucleolin–EGFP construct. Nucleolar aggregates displacing the nucleoplasm and showing a fibrillary, almost spiculated, appearance. (*c*) 47Q–EGFP construct, showing multiple sites for initiation of aggregation within the cell and a less compact aggregate structure.

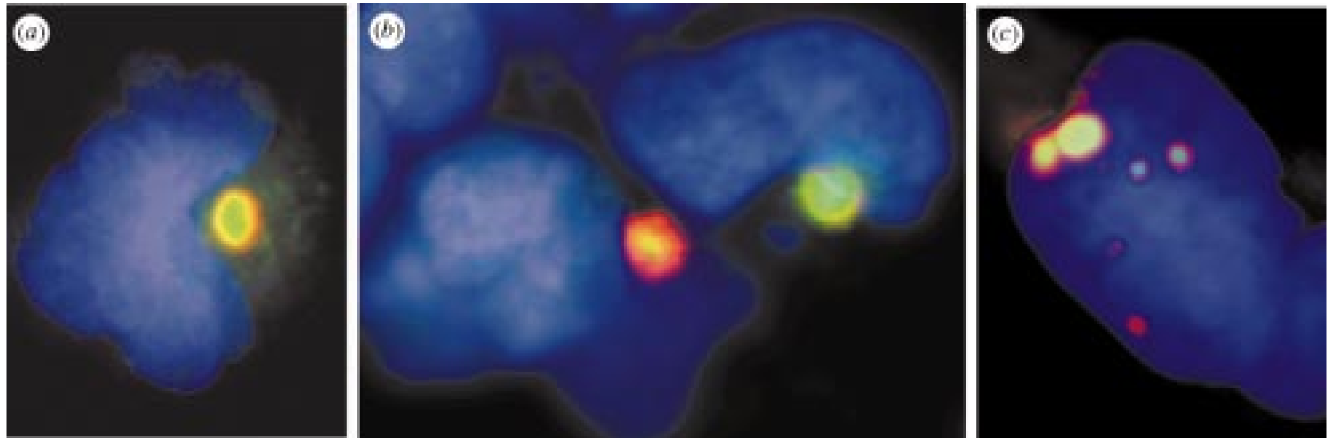


Figure 4. Co-expression and co-aggregation of normal-length and extended polyQ tracts. (*a*) Co-expressed 25Q–EGFP and 104Q–c-Myc detected in a single cytoplasmic perinuclear aggregate. Because the short polyQ (green) is tagged with the intrisically fluorescent EGFP, and the long polyQ (red) is detected by poorly penetrating fluorescent antibody, there is an artefact of the appearance of a red rim around the aggregate. We conclude that both short and long polyQ molecules are present throughout the aggregate, because experiments with reciprocally tagged constructs gave an identical appearance (results not shown). (*b*) Co-expression of 104Q–nucleolin–EGFP (green) and 104Q–c-Myc (red), showing the translocation of 104Q–c-Myc into the nucleus by 104Q–nucleolin. This figure illustrates the heterogeneous composition of the co-aggregates. (*c*) Co-expression of 25Q–nucleolin–EGFP (green) with 104Q–c-Myc (red). Short polyQ fused to nucleolin aggregates in the nucleus when co-expressed with extended polyQ.

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concentrations of acrylamide. Two possible explanations for the anomalous behaviour of polyQ in SDS–PAGE were considered: first, multimers of polyQ might be stable in SDS–PAGE; second, polyQ might form an intrinsic structure with atypical physical properties. To discriminate between these hypotheses we performed an experiment that would allow the association of polyQ tracts of two different lengths after translation *in vitro* by co-translating constructs with 104 and 230 glutamine residues, respectively. The outcome of this experiment is shown in figure 1. Polypeptides migrating with mobilities of 104 and 230 glutamine residues are observed, but not polypeptides with an intermediate mobility. These findings led us to conclude that polyQ translated *in vitro* has an atypical intrinsic structure in SDS–PAGE, because we failed to detect stable polyQ multimers.

## 4. NAKED EXTENDED polyQ PEPTIDES FORM CYTOPLASMIC AND PERINUCLEAR AGGREGATES

We next tested the ability of these naked extended polyQ tracts to form aggregates in cells. In transient transfection experiments in Cos-1 cells, followed by fluorescence microscopy, we found that polypeptides of 25 glutamine residues were expressed diffusely throughout the cytoplasm of transfected cells. When we tested our highly expanded polyQ constructs, we found that 104, 191, 230 and 300 glutamine residues all formed

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aggregates in most transfected cells. Aggregate formation began as early as 16 h after transfection; it appeared typically at this time-point as multiple small crystalline star-like structures distributed throughout the cytoplasm. At this early time-point, a background of diffuse EGFP fluorescence could be seen in these cells as well. By 24 h after transfection, however, aggregate-forming cells had lost this diffuse background fluorescence, which presumably represented expressed polyQ tracts in soluble form. Instead, aggregates within a cell seemed to coalesce into a single, dense, brilliantly fluorescent spherical structure that could be 5 mm or larger (figure 3*a*). These single aggregates were clearly located in the cytoplasm but were often perinuclear or closely apposed to the nuclear membrane. In many instances the nuclear membrane seemed indented or distorted by the presence of the aggregate. Although others have reported a proportional polyQ length-dependent effect on rate of aggregate formation (Li & Li 1998), we found no appreciable difference in the rate of aggregate formation under these conditions among the constructs expressing 104, 191, 230 or 300 glutamine residues. However, we might have been unable to detect such a relationship owing to the very rapid formation of aggregates with any of our long polyQ constructs.

## 5. NUCLEAR LOCALIZATION OF AGGREGATES DEPENDS ON THE FLANKING SEQUENCE OF EXTENDED polyQ

Because neuronal intranuclear inclusions have been reported in the human CAG-repeat diseases as well as in models in the mouse and fly (Paulson *et al.* 1997; Warrick *et al.* 1998; DiFiglia *et al.* 1997) we sought to direct our polyQ peptides to the nucleus by fusing them with a strong nuclear localization signal. To accomplish this we chose the 650-residue nucleolin protein, which has strong nuclear and nucleolar localization signals. We generated expression constructs (polyQ–nucleolin–EGFP) by inserting nucleolin cDNA between polyQ (25 (25Q), 104 (104Q) and 300 glutamine residues) and EGFP sequences. When we expressed normal-length 25Q–nucleolin–EGFP and extended polyQ–nucleolin–EGFP fusion proteins in cells, we found that all polypeptides were located in the nucleus and particularly in the nucleoli. No fluorescent signal was seen in the cytoplasm.

When we transfected cells with extended polyQ–nucleolin–EGFP constructs, we saw nucleolar aggregates formed by extended polyQ tracts (figure 3*b*). These nucleolar aggregates were quite distinct morphologically from the cytoplasmic aggregates generated by the naked polyQ constructs. Cytoplasmic aggregates appeared as dense and compact bodies, whereas nucleolar aggregates showed a fibrillary, almost spiculated, appearance. These results show that the subcellular localization of polyQ aggregates can be determined by polyQ flanking sequences. The basis for the morphological distinction that we observed between cytoplasmic and nucleolar aggregates has not been thoroughly investigated, but it is possible that there are fundamental differences between subcellular compartments in the cellular machinery for processing and degrading these aberrant proteins.

## 6. EXTENDED polyQ CAN TRANSLOCATE polyQ-CONTAINING PROTEINS TO THE NUCLEUS

The naked extended polyQ constructs lack a nuclear localization signal and when transfected alone are found aggregated exclusively in the cytoplasm. To test directly the intermolecular interactions between polyQ-containing molecules, we co-expressed 104Q–c-Myc with 104Q–nucleolin–EGFP. Strikingly, we now found 104Q–c-Myc in heterogeneous aggregates in the nucleus (figure 4*b*). Nuclear localization was strictly dependent on co-aggregation with 104Q–nucleoli–EGFP fusion protein. A nucleolin–EGFP construct without polyQ was unable to pull 104Q–c-Myc into the nucleus, demonstrating the polyQ-dependent nature of the interaction. Despite the presence of the strong nuclear localization signal *in cis*, we found that polyQ–nucleolin–EGFP also aggregated, in some cells, with extended polyQ in the cytoplasm, and thus was excluded from the nucleus. Thus the subcellular localization of aggregation depends in general on the functional characteristics of the protein in which the polyQ is contained. Nonetheless, strong intermolecular interactions mediated by polyQ domains can in some cases be sufficient to override the effects of such intrinsic localization signals.

## 7. EXTENDED polyQ PEPTIDES CAN TRAP NORMAL-LENGTH polyQ PEPTIDES IN AGGREGATES

A number of cellular proteins have been identified with naturally occurring homopolymeric polyQ segments ranging in length from 6 to 38 glutamine residues. We sought to establish in an experimental system whether normal-length polyQ peptides of no more than 40 glutamine residues could interact with and perhaps aggregate with extended polyQ tracts when co-expressed in cells.

To test this hypothesis we co-transfected normal-length 25Q–EGFP and extended 104Q–c-Myc. Normal-length polyQ tracts showed a diffuse pattern of expression when transfected alone. Remarkably, these same normal-length polyQ tracts were recruited into cellular aggregates when they were co-expressed with extended polyQ constructs (figure 4*a*). In contrast, when EGFP lacking a polyQ segment was co-expressed with 104Q–c-Myc, EGFP fluorescence was not detected in aggregates. Co-expression experiments with 25Q–nucleolin–EGFP and 104Q–c-Myc yielded co-aggregates in nucleoli in a small fraction of co-transfected cells (figure 4*c*). Control experiments in which EGFP–nucleolin was co-transfected with 104Q–c-Myc gave cytoplasmic aggregates that had no EGFP signal. These results demonstrate the strict polyQ-dependent nature of the co-aggregation phenomenon.

## 8. DISCUSSION

We synthesized alternating CAG/CAA triplet repeats, ranging from 25 to 300 glutamine residues. All repeats were highly stable in bacteria and were easy to manipulate by using conventional methods of molecular cloning. We generated DNA constructs expressing essentially naked polyQ tracts of different lengths. To direct extended polyQ tracts to the nucleus and to model more closely the neuronal intranuclear aggregates found in

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Table 1. *Some examples of normal cellular proteins containing significant polyQ stretches, as revealed by database searching*

| GenBank accession no. | gene | function | polyQ stretch (single-letter codes) |
|---|---|---|---|
| L37868 | N-Oct 3 | nervous-system specific POU domain transcription factor; homeodomain protein | QQQHQQQQQQQQQQQQQQQQQQQQQ |
| D26155, X72889 | hSNF2a | transcriptional co-activator for glucocorticoid, oestrogen and retinoic acid receptors | QQQQQQQQQQQQQQQQQQQQQQP QQQPPQPQTQQQQQ |
| AF071309 | OPA-containing protein | unknown | QQQQQQQQQQQQQQQQQQQQQQQQ QQYHIRQQQQQQILRQQQQQQQQQQ QQQQQQQQQQQQQQQQHQQQQQQQ AAPPQPQPQSQPQFQRQGLQQTQQQQ Q |
| M55654 | TATA-binding protein | transcriptional activator | QQRQQQQQQQQQQQQQQQQQQQQQ QQQQQQQQQQQQQQQQQ |
| AF012108 | amplified in breast cancer (AIB1) | steroid receptor co-activator | QQQQQQQQQQQQQQQQQQQQQQQQ QQQQQTQ |
| AF016031 | TRAM-1 | thyroid hormone receptor activator | QQQQQQQQQQQQQQQQQQQQQQQQ QQQQQTQ |
| AF010227 | RAC3 | transcriptional co-activator with intrinsic histone acetyl-transferase activity | QQQQQQQQQQQQQQQQQQQQQQQQ QQTQ |
| AF036892 | nuclear receptor co-activator (ACTR) | histone acetyltransferase; transcriptional cofactor | QQQQQQQQQQQQQQQQQQQQQQQQ QQTQ |
| L32832 | ATBF1 | expresses in a neuronal differentiation-dependent manner; zinc-finger homeodomain protein | QQQQQQQQQQQQQQQQQQQAQ |
| U47741 | CREB-binding protein (CBP) | transcriptional adaptor; histone acetyltransferase | QLLQQQQQQQQQQQQQQQQQQ |
| AF010403 | ALR | unknown | QQLQQQQQLQQQQQLQQQQQQQLQ QQQQLQQQQLQQQQQQQQLQQQQQ QQLQQQQQQLQQQQQQQQQQFQQQ QQQQQMGLLNQSRTLLSPQQQQQQQ |

CAG repeat neurodegenerative diseases, we fused alternating CAG/CAA triplet repeats with nucleolin cDNA.

Our results highlight a critical property of polyQ aggregate formation. We show that relatively short, soluble peptides with short polyQ tracts can co-precipitate in cells in the presence of extended polyQ tracts. Once the process is initiated, an extended length of polyQ is not required for joining the aggregate. Moreover, we have demonstrated clearly in our experiments that co-aggregation is strictly dependent on the presence of a polyQ segment in both molecules. Thus, as suggested by Perutz *et al*. (1994), this interaction is mediated directly through the polyQ domains.

We propose that extended mutant polyQ has the potential to interact with any normal polyQ-containing protein in the cell. A possible pathogenic mechanism in the trinucleotide neurodegenerative diseases might involve the depletion or sequestration of normal cellular proteins that contain short homopolymeric polyQ domains. Protein database searches reveal that hundreds of polyQ-containing proteins have been identified so far (see table 1). An interesting class of nuclear proteins that contain glutamine-rich regions and often homopolymeric glutamine stretches are transcription factors and transcriptional co-activators. The expression of many transcriptional activators (or repressors) can and must be exquisitely specific with respect to cell type and developmental stage. Most probably the vulnerability to polyQ expansion of specific neuronal populations in these diseases is governed not simply by the properties of the mutant proteins themselves. In fact, it has been shown that an expanded polyQ repeat inserted into hypoxanthine phosphoribosyltransferase caused a neurological phenotype and intranuclear neuronal inclusions in transgenic mice (Ordway *et al*. 1997). There are certain to be cell-type-specific cofactors for pathogenesis that remain to be elucidated. Among such cell-type-specific cofactors could be normal polyQ-containing molecules that might interact with expanded mutant polyQ tracts within specific cellular compartments or at particular developmental stages. An intriguing candidate for such a role in the neurodegenerative diseases, for example, is the brain-specific Pou-domain-containing homeobox protein N-Oct-3, which contains a nearly homopolymeric polyQ stretch of 27 glutamine residues.

Nuclear localization of mutant polyQ has been shown to be important in the pathogenesis of neurological disease. Klement *et al*. (1998) recently reported that nuclear localization of mutant ataxin-1, but not nuclear aggregation, was necessary for development of ataxia in a

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transgenic mouse model. We have shown that neither nuclear localization nor nuclear aggregation in our transfected cell lines takes place in the absence of a nuclear localization signal. However, we have also shown that interactions between heterogeneous polyQ-containing molecules can mediate the subcellular localization of polyQ aggregates. Thus, although the nuclear localization of mutant extended polyQ might indeed be critical for pathogenesis, a nuclear localization signal *in cis* might not be required for mutant huntingtin, for example, to be found in the nucleus.

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