Cell, Vol. 82, 937–948, September 22, 1995, Copyright © 1995 by Cell Press

SCA1 Transgenic Mice: A Model for Neurodegeneration Caused by an Expanded CAG Trinucleotide Repeat

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Summary

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant inherited disorder characterized by degeneration of cerebellar Purkinje cells, spinocerebellar tracts, and selective brainstem neurons owing to the expansion of an unstable CAG trinucleotide repeat. To gain insight into the pathogenesis of the *SCA1* mutation and the intergenerational stability of trinucleotide repeats in mice, we have generated transgenic mice expressing the human *SCA1* gene with either a normal or an expanded CAG tract. Both transgenes were stable in parent to offspring transmissions. While all six transgenic lines expressing the unexpanded human *SCA1* allele had normal Purkinje cells, transgenic animals from five of six lines with the expanded *SCA1* allele developed ataxia and Purkinje cell degeneration. These data indicate that expanded CAG repeats expressed in Purkinje cells are sufficient to produce degeneration and ataxia and demonstrate that a mouse model can be established for neurodegeneration caused by CAG repeat expansions.

Introduction

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant inherited neurologic disorder characterized pathologically by loss of Purkinje cells in the cerebellar cortex and neurodegeneration within the brainstem and spinocerebellar tracts (Zoghbi and Orr, 1995). Clinical features of SCA1 include limb and gait ataxia, dysarthria, dysmetria, nystagmus, and variable degrees of muscle wasting and neuropathy. Although several cases of SCA1 in juveniles have been reported (Haines et al., 1984; Zoghbi et al., 1988), SCA1 is typically considered an adult-onset disorder, in which symptoms appear within the third or fourth decade of life and progressively worsen over a period of 10 to 30 years, ultimately resulting in death owing to brainstem dysfunction.

The *SCA1* gene has been localized to 6p22-p23 based on detailed genetic and physical mapping studies (Zoghbi et al., 1991; Ranum et al., 1991; Kwiatkowski et al., 1993; Banfi et al., 1993). Using a positional cloning strategy, the *SCA1* gene was isolated, and the mutational basis of disease was determined to be the expansion of an unstable CAG trinucleotide repeat located within the coding region of the identified gene (Orr et al., 1993; Banfi et al., 1994). Among unaffected chromosomes, the *SCA1* CAG repeat is highly polymorphic, with a heterozygosity rate of 84% (Ranum et al., 1994). Sequence analysis of the repeat from unaffected and affected chromosomes revealed that 98% of the unexpanded alleles had an interrupted repeat configuration with at least one CAT triplet interspersed between two CAG repeat tracts (Chung et al., 1993). The overall size of the trinucleotide repeat on unaffected alleles ranges from 6 to 40 U (Orr et al., 1993; Chung et al., 1993; Matilla et al., 1993; Ranum et al., 1994; Jodice et al., 1994). The remaining 2% of normal alleles that lack the CAT interruption contain fewer than 21 repeats. Expanded *SCA1* alleles, on the other hand, have perfect CAG repeat tracts without a CAT interruption and range in size from 40 to 83 repeat units.

Interrupted normal alleles and uninterrupted expanded alleles display dramatically different stability characteristics. A study of more than 600 germline transmissions of normal alleles containing at least one CAT interruption failed to detect a single instance of repeat instability (Jodice et al., 1994). Conversely, uninterrupted expanded alleles are highly unstable and change in size in approximately 70% of transmissions (Chung et al., 1993; Ranum et al., 1994). Paternal transmissions of expanded alleles often result in further expansions of the CAG repeat and subsequently earlier ages of onset of disease (Chung et al., 1993). In contrast, maternal transmissions of expanded alleles tend to yield contractions in repeat number. Polymerase chain reaction (PCR) analyses performed on either single sperm or small quantities of genomic DNA have suggested that loss of the CAT interruption in the CAG tract is an early step in the process of expansion and the development of repeat instability (Chong et al., 1995).

The wild-type *SCA1* gene encodes ataxin-1, a protein that consists of 792 to 826 amino acids depending on the number of CAG trinucleotides (Servadio et al., 1995). The *SCA1* gene is highly homologous to murine *Sca1* (S. Banfi and H. Y. Z., unpublished data). Interestingly, *Sca1* contains only two CAGs at the site of the CAG tracts in *SCA1*. Data base searches have failed to reveal any significant homologies with previously characterized genes or proteins. Ataxin-1 is expressed in a variety of both neuronal and nonneuronal cell types. Furthermore, immunoblot studies have shown that both the unaffected and affected expanded alleles of *SCA1* are translated and produce protein in both lymphoblastoid cell lines and cerebellar tissue (Servadio et al., 1995). Immunohistochemical studies have revealed that ataxin-1 is cytoplasmic in nonneuronal cell

types and nuclear in neuronal cells in the brain with one notable exception, the Purkinje cells of the cerebellum (Servadio et al., 1995). In Purkinje cells, the cerebellar site of SCA1 pathology, ataxin-1 has both nuclear and cytoplasmic localization.

SCA1 is a member of a family of neurodegenerative disorders caused by CAG repeat expansions. This family currently consists of spinal and bulbar muscular atrophy (SBMA) (La Spada et al., 1991), Huntington's disease (HD) (Huntington's Disease Collaborative Research Group [HDCRG], 1993), SCA1 (Orr et al., 1993), dentatorubral–pallidoluysian atrophy (Koide et al., 1994; Nagafuchi et al., 1994) and the allelic Haw–River syndrome (Burke et al., 1994), and, most recently, Machado–Joseph disease and the allelic type 3 spinocerebellar ataxia (Kawaguchi et al., 1994). The diseases in this group are neurodegenerative in nature, are inherited in an autosomal dominant manner (except X-linked recessive SBMA), and often display anticipation, worsening, and earlier onset in successive generations. Interestingly, the coding regions of the genes associated with each disorder fail to share any homologies to the other members of this group except in the highly polymorphic CAG tract, which is predicted to encode a stretch of glutamines. In each of the genes, the CAG tract on unaffected chromosomes is highly polymorphic and on disease chromosomes is unstable, particularly when paternally transmitted. In addition, in each disorder the length of the CAG reiteration on affected chromosomes correlates inversely with the age of onset and disease severity, such that large repeats are found in juvenile onset cases. The striking similarities in clinical, genetic, and molecular features of these disorders suggest that, as a group, they share a common mechanism of pathogenesis. Thus, insights obtained into any given disorder are likely to have important implications for the other members of this family.

The molecular mechanism by which the expansion of the CAG tract leads to the loss of specific neurons remains unclear. We and others have proposed that the development of disease results from a gain of function in the mutant protein encoded by the expanded CAG allele. Several observations support this hypothesis. First, deletions of the androgen receptor (AR) and HD genes fail to result in SBMA or HD, respectively (Quigley et al., 1992; Gandelman et al., 1992; HDCRG, 1993). A corollary to this point is that expansion of the CAG tract has a limited effect on normal gene function. Illustrative of this point is the observation that SBMA patients display minimal signs of testicular feminization, a hallmark for loss of AR gene function. Furthermore, individuals homozygous for the HD mutation have a disease that is no more severe than individuals having only one mutant HD allele (Wexler et al., 1987), suggesting that HD is caused by a true dominant mutation. Finally, that both SBMA and HD disease phenotypes do not include symptoms associated with the loss of gene function, which are seen in patients with gene deletions, also argues against a classical dominant negative mechanism of pathogenesis, in which the mutant allele disrupts the function of the wild-type allele.

To gain insight into the pathogenesis of SCA1 and to examine intergenerational stability of trinucleotide repeats in mice, we have generated transgenic animals harboring the human *SCA1* gene with either a normal or an expanded CAG tract, containing 30 or 82 repeats, respectively. Expression of the transgenes was directed to cerebellar Purkinje cells, a primary site of SCA1 pathology, using the regulatory region of the Purkinje cell–specific gene *Pcp2* (Vandaele et al., 1991).

Results

SCA1 Transgenes

In an effort to test the gain-of-function hypothesis, the promoter elements of the murine *Pcp2* (L7) gene (Vandaele et al., 1991; Oberdick et al., 1990) were used to direct the expression of the human *SCA1*-coding region containing either a normal interrupted allele with 30 repeats, $(CAG)_{12}CATCAGCAT(CAG)_{15}$, or an expanded uninterrupted allele containing 82 repeats. The resulting constructs were designated PS-30 and PS-82, respectively. We (Vandaele et al., 1991; Feddersen et al., 1992, 1995) and others (Oberdick et al., 1990) have previously shown that the 5′ regulatory sequences of the *Pcp2* gene are capable of directing transgene expression specifically to Purkinje cells, the primary site of SCA1 cerebellar pathology. The simian virus 40 (SV40) polyadenylation signal was also included in these constructs to allow for proper processing of the transgene mRNA (Figure 1).

To assess the translational capabilities of the *SCA1* cDNAs containing either 30 or 82 CAG repeats, the *SCA1*-coding regions from the PS-30 and PS-82 constructs were subcloned into pcDL-SRα296 (Takebe et al., 1988) and

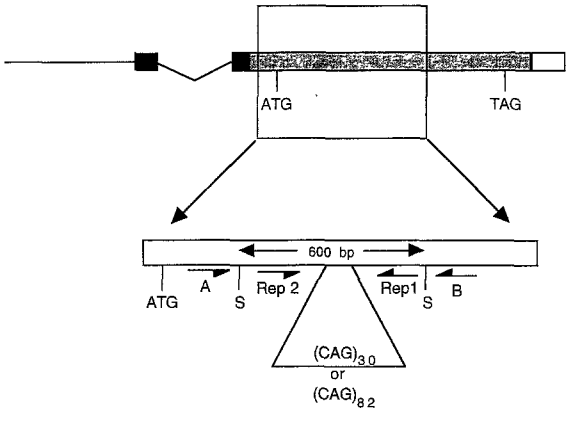

Figure 1. Configuration of the *Pcp2/SCA1* cDNA Transgenes

The thin line depicts the 5′ upstream regulatory region from *Pcp2*. Closed boxes indicate the first two noncoding exons from *Pcp2* separated by the endogenous *Pcp2* intron. The human *SCA1* cDNA is depicted by the stippled box. The open box depicts the poly(A) addition site from SV40. The $(CAG)_n$ configurations are $(CAG)_{12}CATCAGCAT(CAG)_{15}$, designated $(CAG)_{30}$, for the unexpanded allele and $(CAG)_{82}$ for the expanded allele. PCR primer sets A and B and Rep1 and Rep2 are indicated within detailed view of the *SCA1*-coding region. Recognition sites for the restriction enzyme SfiI are indicated by an S.

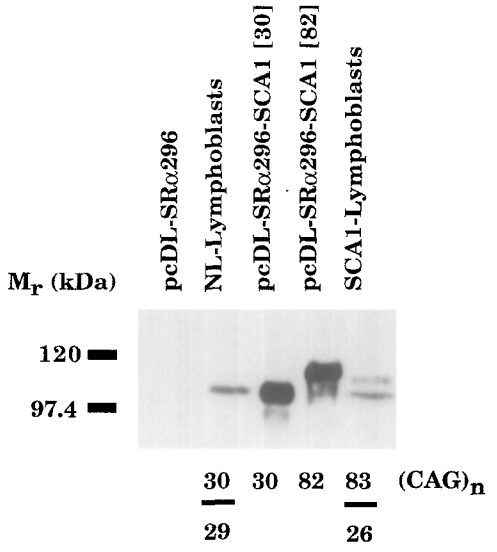

Figure 2. Immunoblot Analysis of Ataxin-1 Expression in Transfected COS7 Cells

COS7 cells transfected with *SCA1* cDNAs containing either the 30 or 82 CAG repeats express ataxin-1 of the expected size based on comparison with extracts from patients harboring similar numbers of repeats. Protein lysates (5 μg) prepared from COS7 cells transfected with vector alone (pcDL-SRα296) or with vector containing a *SCA1* cDNA with either 30 or 82 CAG repeats (pcDL-SRα296-SCA1-30 and pcDL-SRα296-SCA1-82, respectively) were loaded. For comparison, we used 200 μg of lymphoblastoid cell extracts from a normal individual (NL) with 29 and 30 CAG repeats and a SCA1-affected individual with 26 and 83 CAG repeats.

transfected into COS7 cells. Figure 2 shows that COS7 cells transfected with either the pCDL-SRα296-SCA1-30 or pCDL-SRα296-SCA1-82 construct expressed detectable levels of ataxin-1 by Western blot analysis. Moreover, the size of the detected protein was consistent with the number of CAG repeats present within the *SCA1* expression construct. These results demonstrate that both the 30 and 82 CAG repeat–containing *SCA1* cDNAs encode a message capable of producing ataxin-1 of the appropriate size.

The PS-30 and PS-82 transgenes were microinjected into single-cell FVB/N embryos as previously described (Hogan et al., 1986; Taketo et al., 1991). Founder animals were identified using a transgene-specific PCR assay and backcrossed to the parental FVB/N strain to establish independent lines.

A total of 12 founder animals (six for each transgenic construct) were identified. Transgene incorporation was determined by Southern blot analysis. One of the six founder animals containing the PS-30 transgene had two sites of transgene integration, allowing the establishment of seven independent lines containing this construct. Each of the six PS-82 founders had a single transgene integration site, resulting in the establishment of six independent lines. Transgene copy number in each line was determined by densitometric comparison of Southern blot hybridization intensities of N1 tail DNAs with known standards. The transgenic lines obtained (see Table 1) contained between three and 200 copies of the transgene.

Intergenerational Stability of the CAG Repeat within Transgenes

To investigate the stability of the CAG trinucleotide sequences within the *SCA1* transgenes, tail DNA was prepared and subjected to PCR using primers immediately flanking the CAG repeat (Rep1 and Rep2 in Figure 1). PCR products were obtained from 129 progeny of the six founder animals containing the PS-30 construct (A01, n = 7; A02, n = 21; A03, n = 16; A04, n = 17; A05, n = 15;

Table 1. *Pcp2/SCA1* Transgenic Mouse Lines

Transgenic Line	Transgene Copy Number	Analysis of mRNA (weeks)	Analysis of Protein (weeks)	Analysis of Pathology (weeks)	Onset of Ataxia (weeks)
PS-30					
A01	200	− (6,8)	− (7)	ND	− (33)[a]
A02	100	+ (7,8,9)	+ (7,12,24)	− (20,26,48)	− (52)
A03	5	+ (4,7,22)	+ (6,14)	ND	− (43)[a]
A04	5	+ (5,9)	+ (12)	ND	− (43)[a]
A05	20	+ (6,9)	+ (8,16)	ND	− (42)[a]
A06.1	50	+ (6,7,12)	+ (10,13,18)	− (36,48)	− (52)
A06.2	200	+ (12,16)	+ (10,14)	− (20)	− (40)
PS-82					
B01	30	+ (8,14)	− (4,12)	− (7)	− (36)
				+ (26)[b]	+ (24)[b]
B02	10	+ (5,11,18)	− (4,11)	+ (36)	− (36)
				+ (26)[b]	+ (20)[b]
B03	50	− (8,9)	− (12,19)	− (26)	− (20)
B04	3–5	+ (10,14)	− (11,12,17)	+ (17,29)	+ (16)
B05	30	+ (10)	− (5,11,16)	+ (16)	+ (12)
B06	10	+ (9,14)	− (4,9)	+ (26)	+ (26)

Transgenic animals were analyzed for mRNA by Northern blot analysis, protein by Western blot analysis, and pathology by hematoxylin- and eosin-stained cerebellar sections at the ages indicated in parentheses. Age of onset of ataxia or absence of a phenotype in the oldest animal is also indicated. Plus indicates positive; minus indicates negative. ND, not done.

[a] Time at which line was discarded.

[b] These animals were homozygotes; all others were transgene heterozygotes.

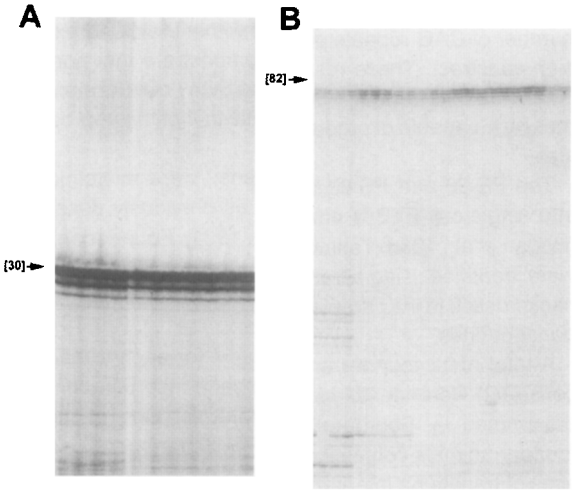

Figure 3. Intergenerational Stability of the *SCA1* $(CAG)_n$ Repeat in Transgenic Mice

Sequencing gel analysis of PCR products amplified from tail DNA of mice containing either the PS-30 (A) or PS-82 transgene (B). The sizes of the 30- and 82-repeat units are indicated. Each lane contains amplification products from DNA of a different animal. The primary PCR product obtained from each animal showed no change in repeat length. There were, however, minor line-specific PCR products observed in several of the PS-82 transgenic lines (B). These products were also stably transmitted.

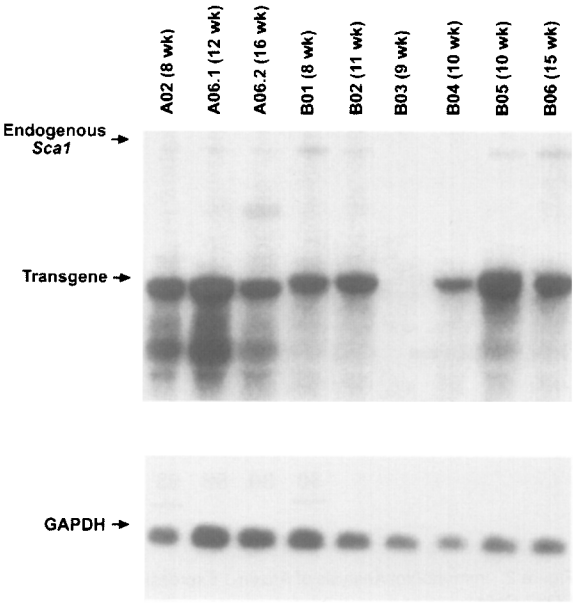

Figure 4. Northern Blot Analysis of Cerebellar RNA from PS-30 and PS-82 Transgenic Mice

Total RNA was prepared from the cerebellum of a single animal from each of the transgenic lines at the age in weeks (wk) indicated. RNA (15 μg) from each animal was fractionated on a glyoxyl–sodium phosphate–agarose (1%) gel and blotted to a nylon membrane. The membrane was sequentially hybridized with human *SCA1* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probes. The positions of the endogenous *Sca1*, transgene, and GAPDH transcripts are indicated. All lines shown, except B03, expressed the transgene at levels 10- to 100-fold greater than *Sca1*. The slightly reduced electrophoretic mobility of the transgene message in the expressing B lines is due to the increased length of the CAG tract in the PS-82 transgenic animals.

A06, n = 61) and from 125 progeny of six founder animals containing the PS-82 construct (B01, n = 29; B02, n = 37; B03, n = 20; B04, n = 19; B05, n = 7; B06, n = 11). The animals examined included groups of progeny that had either paternally or maternally inherited the transgene. In all 254 animals examined, the primary PCR product showed no change in repeat size as compared with the microinjected transgene. Representative results are shown in Figure 3. Four of the six lines containing the PS-82 transgene did have line-specific amplification products, albeit at reduced quantities relative to the 82-repeat product. In each case, the additional PCR product was amplified from founder DNA and was smaller than the full-length 82-repeat tract present in the microinjected construct. Importantly, these products were observed in the amplification products of all the progeny of the line involved, indicating the meiotic stability of the CAG trinucleotide tract over a variety of size ranges. These results indicate that in some copies of the transgene within a tandem array there was rearrangement of the CAG repeat upon integration into the murine genome.

Transgene Expression

Expression of the transgene in each of the lines was assessed by reverse transcriptase–PCR reaction (data not shown) and Northern blot analyses (Figure 4). Of the 13 PS transgenic lines, five lines either failed to express the transgene (A01 and B03) or expressed the transgene at significantly lower levels than murine *Sca1* (A03, A04, and A05; data not shown). Lines A02, A06.1, and A06.2 had comparable levels of transgene expression, approximately 50- to 100-fold greater than endogenous murine *Sca1* (Figure 4). The PS-82-expressing lines had levels of transgene RNA expression approximately 10-fold (B04), 50-fold (B01, B02, and B06), and 100-fold (B05) greater than endogenous levels of *Sca1* RNA (Figure 4).

Immunoblot analysis of protein extracts from cerebellar and total brain tissue using anti-ataxin-1 antisera (Servadio et al., 1995) revealed expression of human ataxin-1 in transgenic lines harboring the PS-30 gene (Figure 5). Surprisingly, human ataxin-1 protein could not be detected in transgenic lines with the PS-82 transgene (Figure 5), despite abundant levels of transgene RNA (see Discussion). The immunoblot analyses were done using tissues obtained at a variety of postnatal timepoints (Table 1).

Neurological Phenotype

The ataxic neurological phenotype provides evidence for disrupted Purkinje cell function. PS-30 transgenic animals expressing the normal human *SCA1* allele, currently more than 1 year of age, display a normal neurological phenotype, indicating intact Purkinje cell function. The apparent wild-type phenotype of these animals suggests that overexpression of normal human ataxin-1, despite the increased length of the polyglutamine tract relative to endogenous murine ataxin-1 (two glutamines; S. Banfi and

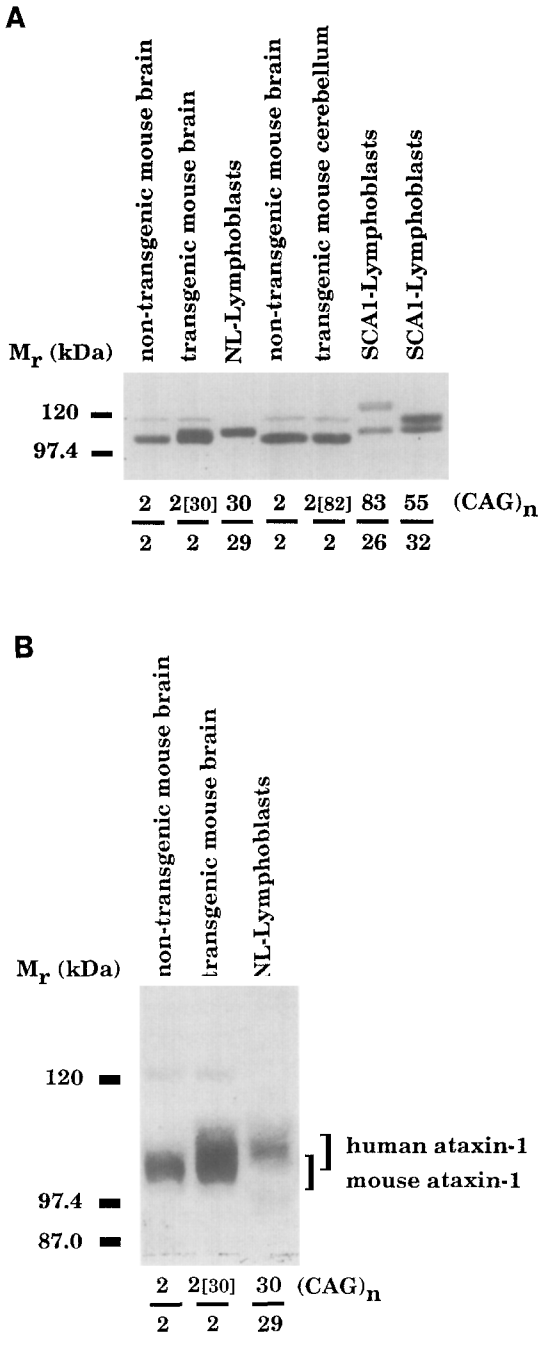

Figure 5. Expression Analysis of Normal and Expanded Human Ataxin-1 Protein in Transgenic Mice

Protein extracts from brain or cerebella of normal and transgenic mice, as well as from lymphoblasts obtained from normal (NL) and SCA1-affected individuals, were analyzed by immunoblotting. Tissue sources are indicated above each lane. The number of CAG repeats within the human *SCA1* and the murine *Sca1* gene for both alleles are shown below each lane. The number of CAG repeats within the *Pcp2/SCA1* transgene cDNA is indicated within the brackets.

(A) Murine ataxin-1 is detected in brain extracts of a nontransgenic mouse and is clearly smaller than wild-type human ataxin-1. In transgenic animals harboring the PS-30 transgene, a denser band is seen, which extends into the size range of wild-type human ataxin-1, indicating that human ataxin-1 is translated from the transgene. Only wild-type murine ataxin-1 is detected in transgenic animals harboring the PS-82 transgene. Analysis of lymphoblast protein extracts from two SCA1 patients with expanded alleles containing 82 and 55 CAG repeats reveals proteins of increased M_r. Analysis of protein expression in isolated cerebella of transgenic animals with the PS-30 cDNA confirmed that the transgene is predominantly and abundantly expressed in the cerebellum (data not shown).

(B) Immunoblot analysis using an extended SDS–polyacrylamide gel demonstrates the presence of both wild-type murine ataxin-1 and a larger protein in transgenic mice with the PS-30 transgene. A cross-reacting band of approximately 120 kDa is seen in brain samples in both panels. This band has the same intensity in all lanes, indicating the equal loading of samples.

H. Y. Z., unpublished data), is not detrimental to murine Purkinje cell differentiation, maintenance, or function.

Conversely, transgenic animals from all five lines expressing the human PS-82 transgene developed ataxia and displayed an abnormal neurological phenotype when compared with nontransgenic littermates. In the first 8–10 weeks of life, the unusual phenotype was subtle but sufficiently distinctive to allow identification of transgenic animals. Phenotypic abnormalities included slightly reduced cage activity, a gentle swaying of the head while walking, and early signs of general incoordination. These abnormalities gradually became more profound over the next 6–20 weeks, until the animals were clearly ataxic when walking (Figure 6) and routinely fell when attempting to stand on their hind legs.

The onset of the neurological abnormalities and discernible ataxia varied in the transgenic lines (Table 1). Heterozygous animals in the highest expressing line, B05, had the earliest onset of ataxia (12 weeks). B04 heterozygous animals developed ataxia at approximately 16 weeks. Although heterozygous B01 and B02 animals express the transgene at levels approximately 50-fold higher than endogenous *Sca1* (see Figure 4) and are currently more than 36 weeks old, they do not display signs of ataxia. In contrast, homozygous animals in these lines become ataxic at 24 and 20 weeks, respectively. Another transgenic line, B06, also expresses transgene mRNA at a level substantially above that seen in transgenic B04 animals (see Figure 4), yet B06 animals display only subtle signs of ataxia at 26 weeks of age. These results suggest that *SCA1* transgene mRNA levels and the onset of ataxia are not strictly correlated.

Pathology

Pathologic examination of several of the PS-30 transgenic lines (Table 1) revealed no gross or microscopic abnormalities of the cerebellum. Similarly, the PS-82 transgenic line, B03, which failed to express the transgene mRNA, had no cerebellar pathology. All of the remaining PS-82 transgenic lines, however, had evidence of cerebellar abnormalities.

The transgenic lines with the earliest onset of ataxia tended to have the most severe neuropathologic changes. Transgenic lines B04 and B05 and lines B01 and B02 bred to homozygosity (Table 1) had evidence of significant loss of the Purkinje cell population, with Bergmann glial proliferation, and shrinkage and gliosis of the molecular layer (Figure 7). In addition, there were numerous ectopic Pur-

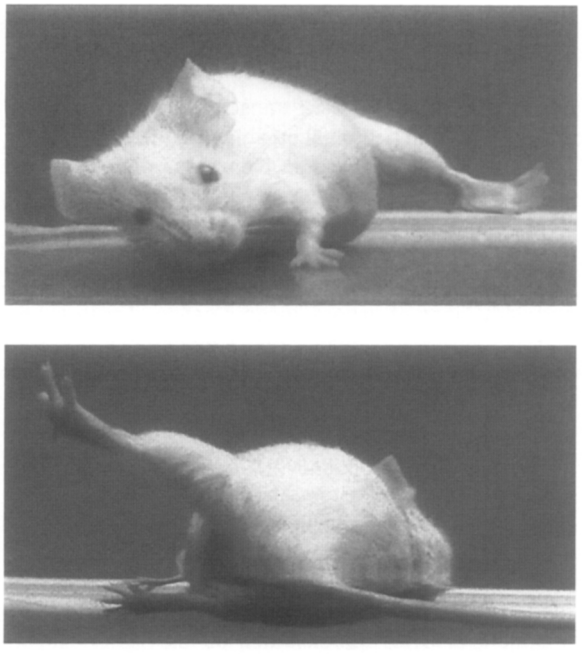

Figure 6. Ataxic Phenotype of a PS-82 Transgenic Animal
A 30-week-old transgenic animal from line B05 displays clear signs of ataxia by falling to one side when walking.

kinje cells present in the molecular layer and occasionally in the granular layer. Using immunostaining for calbindin, the dendritic arrays of the PS-82 transgenic mice also appeared to be abnormal (Figure 8). Occasional Purkinje cells had large clear vacuoles within their cytoplasm. Axonal torpedo bodies could not be identified. The granular layer in these mice was unremarkable.

Heterozygous animals from transgenic lines B01 and B02 (Table 1), although not ataxic, had evidence of mild cerebellar pathology. There were occasional ectopic Purkinje cells in both the molecular and granular layers, but little or no suggestion of loss of Purkinje cells or gliosis. Transgenic line B06, while only mildly ataxic at the time examined (26 weeks), had somewhat more severe pathologic changes, most of which were localized to lobule IX in the posterior cerebellar cortex. In that lobule, the changes resembled those described for the other ataxic transgenic lines. The remaining lobules had only occasional ectopic Purkinje cells without evidence of significant neuronal loss.

Discussion

Targeted expression in transgenic mice of a *SCA1* cDNA transgene carrying an expanded perfect CAG repeat with 82 reiterations induced the loss of Purkinje cells from the cerebellar cortex and the neurological phenotype of ataxia. In contrast, transgenic mice generated using a *SCA1* cDNA carrying an unexpanded normal CAG repeat with 30 reiterations had no cerebellar pathology or ataxia for more than 1 year, well past the age of onset of ataxia and neurodegeneration in transgenic mice with an 82 repeat–containing transgene. These data demonstrate that, even though disorders associated with the expansion of trinucleotide repeats have yet to be observed in any species other than human, neurons within the mouse central nervous system (CNS) are susceptible to degeneration upon the expression of a *SCA1* allele containing an expanded CAG repeat tract.

A previous study reported that transgenic mice carrying a human AR cDNA with an expanded number of CAG repeats (45) failed to develop motor neuron disease similar to that observed in SBMA. This study by Bingham and colleagues (1995) demonstrated that although the AR transgene was transcribed, it was expressed at a level significantly lower than the wild-type endogenous AR gene. Bingham and coworkers suggested that the lack of an appropriate level of expression might account for the absence of a phenotypic effect. In the present study, we found levels of *SCA1* transgene mRNA at least 10-fold greater than the endogenous level of *Sca1*. Therefore, our results are consistent with the notion that expression levels may contribute to the induction of CAG repeat–based neurodegeneration in the mouse. It should, however, be noted that the AR transgenic study used an expanded allele with only 45 CAG repeats. While this number of repeats is clearly within the size range for development of SBMA in humans, it is possible that mouse neurons have a reduced sensitivity to the detrimental effects of CAG repeat expansions. If so, our use of a *SCA1* allele with 82 CAG repeats, almost twice the number of repeats used in the AR transgenic study, may have been another essential factor in the Purkinje cell neurodegeneration of the PS-82 transgenic mice.

In two of the transgenic lines carrying an expanded *SCA1* cDNA, B01 and B02, animals heterozygous for the transgene failed to develop a neurological phenotype, while animals homozygous for the transgene from these lines developed ataxia by 24 and 20 weeks, respectively. Histological examination of the cerebellum of a heterozygous transgenic animal from line B02 at 36 weeks of age did reveal cerebellar pathology. This result demonstrates that considerable neuropathology can occur without the manifestation of a neurological phenotype and is consistent with previous work demonstrating that overt ataxia occurs in mice only after there is loss or dysfunction of a substantial number (50%–75%) of the Purkinje cells (Feddersen et al., 1992, 1995). Thus, we conclude that the absence of an abnormal neurological phenotype in heterozygous animals from lines B01 and B02 reflects that Purkinje cell dysfunction progresses in these transgenic mice at a rate insufficient to cause ataxia over the time course of this study. By breeding the transgene to homozygosity, and thereby increasing the level of its expression, the progression of Purkinje cell dysfunction was increased, and the development of ataxia occurred within the time frame of this study.

In humans, while CAG repeats in the unexpanded range are stable, expanded *SCA1* CAG repeats are subject to intergenerational instability, with a paternal bias for expansions (Chung et al., 1993; Jodice et al., 1994). Unlike the

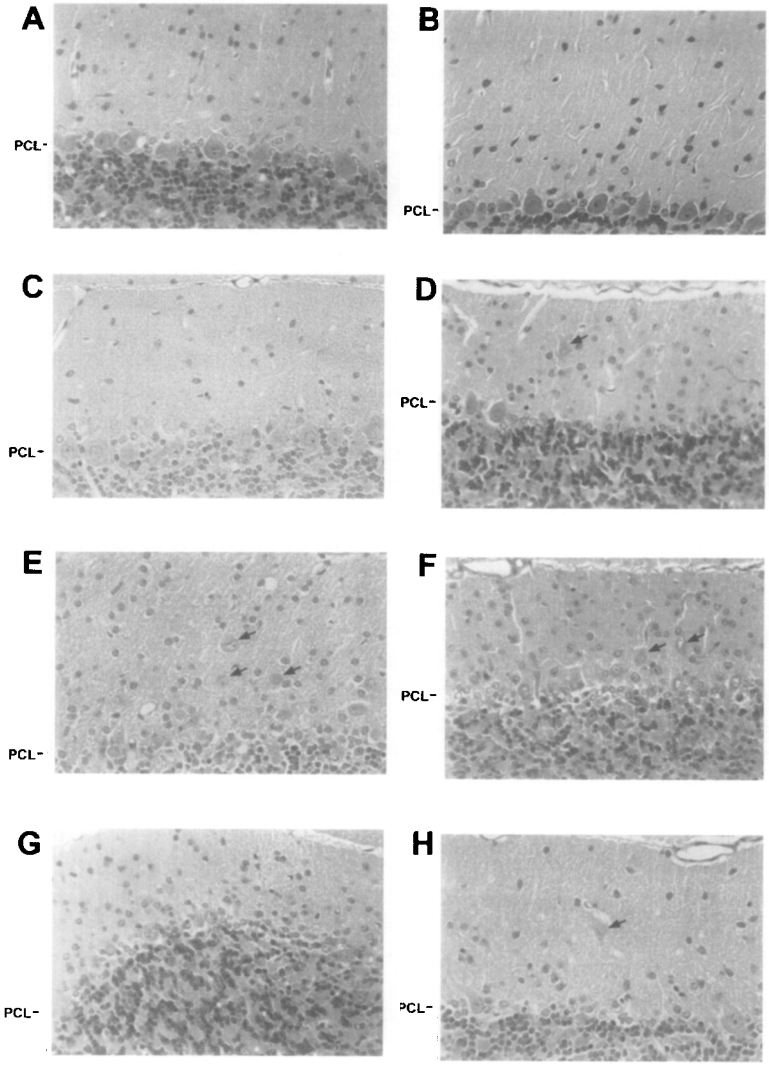

Figure 7. Cerebellar Histology of Normal and Transgenic Mice Using Hematoxylin and Eosin Staining

(A) Nontransgenic littermate control at 29 weeks of age.
(B) A06.2 transgenic mouse at 20 weeks with a normal cerebellar cortex structure.
(C) B03 transgenic mouse at 24 weeks with a normal cerebellar cortex structure.
(D) B01 homozygous transgenic mouse at 26 weeks with an ectopic positioned Purkinje cell (indicated by the arrow), gliosis in the molecular layer, and loss of Purkinje cells and gliosis in the Purkinje cell layer.
(E) B02 homozygous transgenic mouse at 26 weeks with ectopic positioned Purkinje cells, mild gliosis in molecular layer, and some disorganization of the Purkinje cell layer with mild Purkinje cell loss.
(F) B04 transgenic mouse at 29 weeks with ectopic positioned Purkinje cells, gliosis in the molecular layer, and loss of Purkinje cells and gliosis in the Purkinje cell layer.
(G) B05 transgenic mouse at 16 weeks with Purkinje cell loss and gliosis.
(H) B06 transgenic mouse at 26 weeks with an ectopic positioned Purkinje cell, but little evidence of Purkinje cell loss or gliosis. Pathology in B06 was mild and patchy in its distribution.

Magnification, 168 x . PCL denotes Purkinje cell layer.

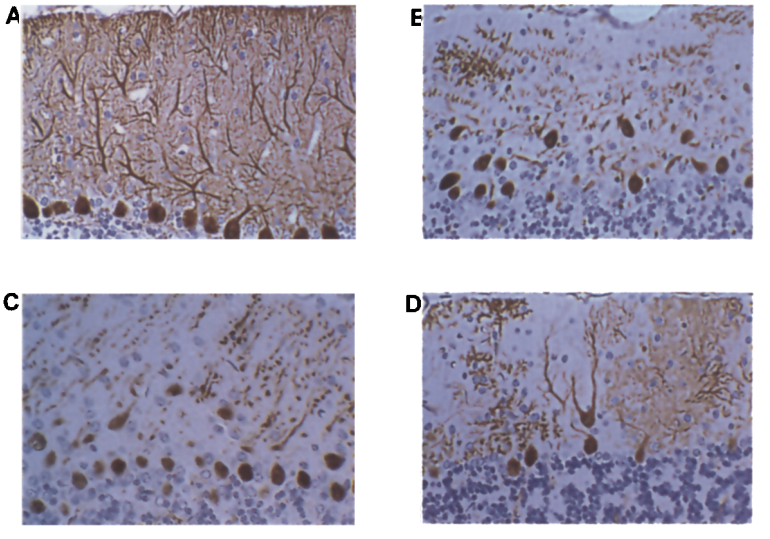

Figure 8. Immunohistochemical Staining for Calbindin to Illustrate Purkinje Cell Morphology

(A) Nontransgenic animal at 17 weeks of age displaying normal appearance of the cerebellar cortex.
(B) B04 transgenic mouse at 17 weeks of age with ectopic Purkinje cells and disorganization of the Purkinje cell layer.
(C) B05 transgenic mouse at 16 weeks of age with changes similar to those described in (B).
(D) B06 transgenic mouse at 26 weeks of age with ectopic Purkinje cells and some Purkinje cell loss seen focally in lobule IX.

(Magnification, 165 x)

observed instability of expanded *SCA1* alleles in humans, the *SCA1* cDNA containing an expanded number of CAG trinucleotide repeats showed no variation in repeat length upon parent to offspring transmission in transgenic mice. Transgenic mice carrying an expanded CAG repeat in an AR cDNA transgene also exhibited intergenerational repeat stability (Bingham et al., 1995). Thus, expanded CAG repeats positioned within cDNA transgenes fail to manifest intergenerational instability. The observed stability may reflect basic differences between mice and humans in their mechanisms of DNA repair, replication, or both. It also suggests that the chromosomal milieu of the CAG repeat may be an important factor in determining repeat instability. Sequences flanking the repeat or the repeat-containing gene, or the structure of the chromatin, may have a critical role in determining whether an expanded repeat is unstable upon transmission from parent to offspring. The fact that a *SCA1* allele of 82 repeats, which in humans had expanded from a paternal allele of 53 repeats and resulted in a juvenile form of disease, is stable in the mouse suggests that repeat number alone in the DNA and parental transmission effects is not sufficient to impart repeat instability.

Recently, it has been demonstrated that the repeat units from the *SCA1* gene, as well as other disease-associated genes with unstable trinucleotide repeats, are capable of forming a DNA hairpin structure in solution (Gacy et al., 1995). The stability of the hairpin was found to be influenced not only by the length of the repeat, but also by the nucleotide sequence of the repeat and its flanking sequences. These investigators suggested that hairpin stability provides a structural basis for repeat instability. Gacy et al. (1995) found that a *SCA1* of 25 repeats plus short stretches of flanking DNA formed a stable hairpin structure in solution. Although both transgene constructs used in this study contained CAG repeats greater than 25 repeats in length, as well as flanking sequences implicated in hairpin formation, neither transgene including the *SCA1* transgene with 82 repeat units was found to be unstable in even a single parent to offspring transmission. If, in fact, a hairpin structure is formed within the *SCA1* transgenes, the lack of intergenerational variability indicates that formation of this structure alone is not sufficient to induce repeat instability. Regardless of the mechanisms important for repeat instability, the ability to induce neurodegeneration in the *SCA1* transgenic mice clearly demonstrates that pathogenesis can occur in the absence of repeat instability.

In terms of SCA1 pathogenesis, the PS-82 transgenic mice are a model of the cerebellar component of the human disease. Each of the transgenic lines expressing the PS-82 transgene was found to have substantial loss of Purkinje cells within the cerebellar cortex (Table 1; Figure 7), a prominent pathological finding in human SCA1 necropsy material (Zoghbi and Ballabio, 1995). Thus, the *SCA1* transgenic mice recapitulate this important pathological feature of the human disease. Since regions of the brainstem that send projections to Purkinje cells are also involved in SCA1 pathology, it is conceivable that loss of Purkinje cells is due to a secondary effect. By directing the expression of the PS-82 transgene selectively to Purkinje cells, the present study demonstrates that the expression of an expanded *SCA1* allele specifically induces the degeneration of these neurons, indicating that loss of Purkinje cells in SCA1, and hence development of ataxia, is due to a direct action of an expanded *SCA1* allele on Purkinje neurons in a cell-autonomous fashion.

Degeneration in SCA1 is not limited to Purkinje cells of the cerebellar cortex, but also includes neurons of the olives and certain lower motor neuron nuclei of the brainstem (Zoghbi and Ballabio, 1995). In fact, it is the neuronal cell loss within the brainstem that is ultimately responsible for the lethal nature of SCA1. The use of other regulatory regions capable of directing transgene expression to broader neuronal cell types within the CNS should provide a means to determine whether expression of an expanded allele of *SCA1* is also able to induce degeneration of other neuronal cell types in addition to cerebellar Purkinje cells.

The presence of ectopically localized Purkinje cells was also a prominent feature of the cerebellar pathology of the PS-82 transgenic mice in this study. In an effort to investigate the possibility of similar pathologic anomalies in human SCA1, we have undertaken an initial pathological examination of cerebellar necropsy material from a juvenile case of human SCA1 resulting from a *SCA1* allele with 83 CAG repeats. The cerebellar findings include severe degeneration of Purkinje cells with Bergmann gliosis and shrinkage and gliosis of the molecular layer, similar to what was observed in the transgenic mice. Torpedo bodies, large dilatations of the proximal axons of Purkinje cells, a common feature of Purkinje cell degeneration in human ataxias (Koeppen and Barron, 1984), were abundant in the human case, but apparently absent in the ataxic mice. Although a small number of Purkinje cells were seen ectopically within the molecular layer, this finding was substantially less striking than that observed in the affected transgenic mouse lines. It should be noted that the *Pcp2* regulatory region can direct transgene expression to embryonic stages, beginning at embryonic day 14 (Smeyne et al., 1991; Feddersen et al., 1995), which is substantially earlier than the transient burst of endogenous *Sca1* expression detectable around postnatal day 14 (S. Banfi and H. Y. Z., unpublished data). Furthermore, murine cerebellar development proceeds rapidly over a few weeks, as compared to many months in humans. These two factors might contribute to greater frequency of ectopic Purkinje cells in PS-82 transgenic mice that are not associated with SCA1 in humans.

The mechanism by which CAG repeat expansions cause neurodegeneration in humans is not understood. The lack of neuropathology in males with a deletion of the AR (Quigley et al., 1992) and the absence of HD-like pathology in individuals with a heterozygous deletion of the HD gene (Gandelman et al., 1992; HDCRG, 1993) argue that the pathology caused by CAG repeat expansion is not simply the result of a loss-of-function mutation. However, certain symptoms associated with SBMA, including gynecomastia and reduced fertility (Arbizu et al., 1983),

have been used to suggest that the wild-type function of the AR gene is partially compromised by the CAG repeat expansion (La Spada et al., 1991). The recent description of behavioral alterations and neuropathological changes in subthalamic nuclei of mice heterozygous for a targeted disruption of the HD gene raises the possibility that some of the symptoms seen in these diseases may be secondary to a partial loss of function (Nasir et al., 1995). The presence of a clinical phenotype in transgenic animals carrying a *SCA1* cDNA with an expanded number of CAG repeats clearly demonstrates that mouse neurons are susceptible to mechanisms by which CAG repeat expansions cause neurodegeneration. Furthermore, data from the PS-82 transgenic mice indicate that development of a phenotype is dependent upon transgene expression. Only those lines expressing the expanded PS-82 transgene developed a phenotype. Although line B03 contained 50 copies of the PS-82 transgene, it failed to express the transgene and did not develop ataxia or show signs of cerebellar pathology. These data argue that a gain of novel properties by the product of an expanded *SCA1* allele is directly involved in the pathogenesis of this disorder. Moreover, if pathogenesis requires the interaction of mutant ataxin-1 with another cellular protein, the fact that expression of an expanded allele of *SCA1* induces neurodegeneration in transgenic mice indicates that mice can be used as a source for proteins that specifically interact with mutant ataxin-1. Whether the symptoms associated with SCA1 are also due to a partial loss of function will be determined by the analysis of mice with targeted disruptions of the *SCA1* gene.

Despite the substantial levels of transgene RNA detected in the cerebella of animals carrying either the PS-30 or PS-82 constructs, human ataxin-1 derived from the transgene was detectable only in extracts of cerebella from mice expressing the PS-30 transgene. Animals displaying PS-82 message levels more than two orders of magnitude greater than murine *Sca1* failed to produce levels of human ataxin-1 detectable by Western blot analysis. These results raise the possibility that the abnormal neurological phenotype may be dependent upon the presence of RNA with an increased stretch of CAGs, rather than the presence of ataxin-1 with an expanded polyglutamine tract. There are, however, several observations contradictory to an RNA-based mechanism of neuronal degeneration. First, COS7 cell transfection studies using the *SCA1* cDNA with 82 trinucleotide repeats resulted in detectable levels of ataxin-1 with an expanded polyglutamine tract, demonstrating that the employed cDNA encodes a translatable mRNA. In addition, cerebella from transgenic animals carrying the PS-30 transgene express *SCA1* RNA at levels 50- to 100-fold in excess of endogenous *Sca1*. Thus, elevated levels of *SCA1* mRNA are not, in themselves, pathogenic. Furthermore, protein extracts prepared from PS-30 transgenic cerebella contain abundant amounts of human ataxin-1. Therefore, wild-type human ataxin-1 can be expressed by murine Purkinje cells, and its expression is not detrimental to Purkinje cell differentiation, morphology, or function. In addition, the levels of PS-82 RNA found in cerebella from transgenic lines of the B0 series did not directly correlate with the onset of ataxia in these animals. For example, transgenic line B06 expressed among the highest levels of PS-82 mRNA and yet had a relatively slow progression of pathology, whereas the B04 line had both the lowest transgene copy number and level of transgene mRNA, but developed early and pronounced pathology, indicating that the neurodegeneration is not simply a consequence of expanded *SCA1* mRNA.

Finally, M. R. Hayden and colleagues (personal communication) have established a series of transgenic mice carrying an expanded allele of the HD gene (*IT15*). These mice expressed substantial levels of transgene mRNA, but failed to express protein from the transgene owing to inclusion of DNA sequence in the 5′ untranslated region, which acts as a repressor and an in-frame stop codon within the *IT15* construct. The *IT15* transgenic mice do not develop an abnormal neurological phenotype or CNS morphological alterations out to 6 months of age. These results further support the notion that pathology due to the expression of an expanded CAG repeat gene is not dependent on RNA.

Detection of *SCA1* mRNA in PS-82 transgenic mice at different ages (4–19 weeks) and with variable degrees of Purkinje cell degeneration (Table 1) argues against the loss of Purkinje cells being the sole reason for lack of detectable levels of mutant ataxin-1. Rather, the results suggest that ataxin-1 with an expanded number of glutamines expressed in Purkinje cells is either rapidly degraded or modified such that it is no longer recognized by the anti-ataxin-1 antibody. If one assumes the mouse is an accurate model of pathogenesis, it might be hypothesized that mutant ataxin-1 is degraded or modified in human Purkinje cells as in mouse. This would not necessarily be in conflict with available human data (Servadio et al., 1995), since the detected expression of mutant ataxin-1 in human cerebellum might have been derived from non-Purkinje cells, and the immunohistochemical staining of Purkinje cells in patients may have represented ataxin-1 produced from the normal allele. Immunohistochemical examination of human SCA1 brain tissue with an antibody specific for mutant ataxin-1 would address this question. It is notable that Purkinje cells, a primary site of SCA1 pathology, are unique in that they are the only cell type that has both cytoplasmic and nuclear localization of ataxin-1 (Servadio et al., 1995). Therefore, it could be hypothesized that ataxin-1 is translocated to the nucleus in disease-related cell types; that mutant ataxin-1 is degraded or modified in disease-related but not in other cell types; and that the degradation or alteration of mutant ataxin-1 is directly related to its gain-of-function property.

The clinical and neuropathologic findings in multiple transgenic mouse lines harboring 82 CAG repeats within the *SCA1* cDNA clearly demonstrate that gain of the expanded CAG tract within the *SCA1* gene leads to Purkinje cell degeneration. The targeted expression to Purkinje cells and the absence of detectable mutant ataxin-1 in these cells will provide insight into the mechanism of

pathogenesis. Furthermore, the inability to detect mutant ataxin-1 in these cells, despite the characteristic pathology and known translatability of the mutant protein, may explain the specificity of neurodegeneration in SCA1. Finally, it is hopeful that a mouse model of neurodegeneration induced by the expression of an expanded CAG repeat can provide an in vivo means for testing promising therapeutic strategies.

Experimental Procedures

Transgene Construction

The *Pcp2/SCA1* transgenes were generated by joining the *Pcp2* 5′ sequences, the human *SCA1*-coding region, and the SV40 polyadenylation signal. In brief, an 835 bp SalI–PvuI fragment of the *Pcp2* genomic sequences containing exon 1, intron 1, and a portion of exon 2, and a 237 bp BamHI–BclI fragment containing the SV40 polyadenylation signal (Lebowitz and Weissman, 1979) were subcloned into pGEM11 as previously described (Feddersen et al., 1992). This plasmid, Purkinje Cell Expression Vector II (PCEVII), also contains a truncated polylinker with SpeI and NotI sites between the *Pcp2* and SV40 sequences. PCEVII was digested with NotI, treated with T4 polymerase to generate blunt ends, and then digested with SpeI in preparation for the *SCA1* insert. The EcoRI insert of cDNA clone 31-5 (containing base pairs 835–3516, including the entire coding region of the human *SCA1* gene with 30 CAG repeats, base pairs 916–3384; Banfi et al., 1994) was initially subcloned into the EcoRI site of pBluescript SK(−). The *SCA1* insert of this plasmid was then released with SpeI (5′ site) and EcoRV (3′ blunt site) and directionally ligated into SpeI–NotI (blunt)–prepared PCEVII. The resulting plasmid contained the PS-30 transgene, flanked by SalI restriction sites. The PS-82 transgene was generated by amplifying the CAG repeat tract from SCA1 patient DNA with primers A (5′-ACCGCCAACCCCGTCACC) and B (5′-GCTCTTCTCCATCTCACCGT), digesting the products with SfiI, and then substituting the SfiI fragment within the PS-30 transgene with the PCR product containing the expanded CAG tract (Figure 1). The described restriction fragment junctions and CAG repeat tracts were sequenced to confirm proper ligation and maintenance of reading frame. The transgene inserts were isolated by SalI digestion, gel purified, extracted with organic solvent, and extensively dialyzed prior to microinjection.

Transgenic animals were identified using a transgene-specific PCR assay or by Southern analysis (or both). Postweaning tail biopsy DNA was generated as previously described (Hanley and Merlie, 1991). We used 0.1–1.0 μg of DNA in a 25 μl PCR reaction. The 5′ primer, 5EX2B (5′-AGGTTCACCGGACCAGGAAGG), located within *Pcp2* exon 2 and an ataxin-1-specific primer, Sau3A-40 (5′-ATGGAGTGGTGGCCCCTG), were used to amplify a 581 bp transgene-specific fragment. Cycle conditions were 94°C (1 min), 57°C (1 min), and 72°C (2 min). Homozygous animals were distinguished from heterozygous littermates by densitometric comparisons of Southern blot hybridization intensities.

Expression of Ataxin-1 in COS7 Cells

SCA1 cDNAs containing either 30 or 82 CAG repeats were cloned into the EcoRI-digested plasmid of pcDL-SRα296 (Takebe et al., 1988). DNA (2 μg) from each construct was individually mixed with 90 μl of Optimem (GIBCO BRL) and subsequently mixed with 90 μl of Optimem and 10 μl of lipofectamine (GIBCO BRL). A control plasmid containing the β-galactosidase gene was used to assess transfection efficiency. The optimal ratio of DNA/lipofectamine/Optimem was evaluated by determining the number of COS7 cells that turned blue when transfected with the pcDL-SRα296-β-galactosidase plasmid. The DNA/lipofectamine mixture was set for 45 min at room temperature and then added to 6×10^4 COS7 cells, which were grown overnight in 3.5 cm tissue culture dishes at 37°C in 10% CO_2 incubators. Lipotransfection was stopped 4 hr later with 1 ml of DMEM medium, and the samples were incubated at 37°C in 10% CO_2.

70 hr later, the cells were washed three times with phosphate-buffered saline, and three times with TEE buffer (50 mM Tris [pH 8], 5 mM EDTA, 5 mM EGTA). Cells were scraped from the dishes with 300 μl of TEE buffer a total of three times. Cells were subsequently pelleted and resuspended in 20 ml of TEE buffer containing 50 mM DTT and 0.3% SDS. The samples were sonicated and spun, and 4 μl of the supernatant was analyzed by 7% SDS–PAGE and immunoblotting using anti-ataxin-1 antiserum (11750 V).

CAG Repeat PCR Analysis

PCR reactions were performed as previously described (Orr et al., 1993). In brief, each 15 μl reaction contained 1 μM Rep2 (5′-CAACATGGGCAGTCTGAG) and [γ-^{32}P]ATP-end-labeled Rep1 (5′-AACTGGAAATGTGGACGTAC) primers, 250 μM dNTPs, 2% formamide, 1.25 mM $MgCl_2$, and 0.75 U of AmpliTaq polymerase. Cycling conditions were as follows: 94°C for 4 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1 min, and final elongation at 72°C for 8 min. A 6 μl aliquot of the PCR was mixed with 4 μl of formamide-containing loading dye. After denaturation, 4–6 μl of the mixture was run on a 4% sequencing gel and visualized by autoradiography.

RNA Isolation and Northern Blot Analysis

RNA was isolated from individual cerebella by the acid guanidinium thiocyanate–phenol/chloroform method (Chomczynski and Sacchi, 1987). RNA samples were glyoxylated, fractionated on 10 mM sodium phosphate–agarose (1%) gels, and blotted onto nylon filters (Micro Separations Incorporated). Sizes were determined by using RNA markers (GIBCO BRL). Blots were stripped with a boiling 10 mM Tris, 1% SDS solution prior to reprobing.

Immunoblot Analysis and Preparation of Tissue Extracts

Freshly collected mouse brains or cerebella were homogenized in 50 mM Tris–HCl (pH 8.0), 1% (v/v) Triton X-100, 0.5% (v/v) NP-40, 10 mM BME, 4% (v/v) glycerol, plus a cocktail of 10 protease inhibitors. Samples were spun at 17,500 × g, and the supernatants were used for analysis of protein expression. Extracts from lymphoblastoid cells were prepared as previously described (Servadio et al., 1995). Protein extracts (160 μg) were separated in a 6% SDS–polyacrylamide gel and transferred to nitrocellulose (Schleicher & Schuell). For the large gel (Figure 5B), 200 μg of protein extracts were used. Blots were preblocked in TBST/NGS (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 0.1% (v/v) Tween 20, 11% (v/v) normal goat serum. Sera were diluted 1:6000 in TBST/NGS. The immune complexes were detected using a horseradish peroxidase–conjugated secondary antibody (Sigma) and enhanced chemiluminescence (Amersham). Anti-ataxin-1 (11750 V) antibodies were preabsorbed on nitrocellulose saturated with protein extracts from Escherichia coli expressing Maltose Binding Protein to reduce the background.

Histological Examination and Immunohistochemistry

Brains of transgenic and nontransgenic littermate mice were immersion fixed in 10% phosphate-buffered formalin for a minimum of 3 days. Tissue was processed by paraffin infiltration in midsagittal orientation. Sections were cut on a rotary microtome at 6–8 μm, and routine histologic examination was done after staining with hematoxylin and eosin.

Paraffin sections (6–8 μm) of the tissues described above were deparaffinized and rehydrated through xylol and graded alcohols. Sections were processed in citrate buffer (pH 6.0) using a microwave oven seven times for 3 min. Endogenous peroxidase was quenched by treatment with 6% hydrogen peroxide in methanol for 10 min. Sections were washed three times in 0.1 M phosphate-buffered saline (pH 7.5) for 5 min, followed by a 20 min incubation with 10% normal horse serum in 0.1 M phosphate buffer (pH 7.5). Immunohistochemical detection of calbindin was performed using mouse monoclonal antibodies to calbindin (Sigma). Detection of primary antibodies was performed using routine avidin–biotin–peroxidase techniques (Vector Labs). The diaminobenzidine reaction was enhanced with ions to form a black precipitate (DAB-Ni). After the reaction, the slides were washed in distilled water, counterstained with eosin, dehydrated in graded alcohols and xylol, and mounted.

Acknowledgments

Correspondence should be addressed to H. T. O. The paper is dedi-

cated to the memory of Dr. Daniel T. Nordquist. The authors thank Dr. Dawna Armstrong for allowing us to review pathology on an 83-repeat case of SCA1 and Dr. Arthur L. Beaudet for critical reading of this manuscript and continued thoughtful discussions. This work was supported by grants from the National Institute of Neurological Disorders and Stroke of the National Institutes of Health to H. T. O and H. B. C. (NS33718), to H. Y. Z. (NS27699), and to E. N. B. (NS09724-01). A. S. was supported by an Italian Telethon Fellowship, and T. M. was supported by the Spanish Ministerio de Educacion y Ciencia (PF 94 969 798).

Received June 21, 1995; revised July 31, 1995.

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