

Exon 1 of the *HD* Gene with an Expanded CAG Repeat Is Sufficient to Cause a Progressive Neurological Phenotype in Transgenic Mice

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The onset of symptoms is generally in midlife although it can range from early childhood to >70 years. Anticipation is observed, predominantly when the disease is inherited through the male line, with the result that 70% of juvenile cases inherit the disease from their father. The symptoms have an emotional, motor, and cognitive component. A detailed description of all aspects of HD can be found in Harper (1991). Chorea is a characteristic feature of the motor disorder and is defined as excessive spontaneous movement, irregularly timed, randomly distributed, and abrupt. It can vary from being barely perceptible to extremely severe. It involves all parts of the body, can have repetitive and stereotypic elements, and may have a pseudopurposive appearance (Harper, 1991). Other frequently observed motor abnormalities include dystonia (sustained muscle contraction), rigidity, bradykinesia (abnormally slow movements), oculomotor dysfunction, and tremor. Cerebellar dysfunction, upper motoneuron abnormalities, epilepsy, and myoclonus (brief shock-like muscle jerks) are rare except in the juvenile form of the disease, which commonly presents with a "Parkinsonlike rigidity." Voluntary movement disorders include fine motor incoordination, dysarthria (impairment of articulation), and dysphagia (difficulty in swallowing). The emotional disorder is commonly depression and irritability, and the cognitive component comprises a subcortical dementia. The biochemical basis of this disease is not understood, and there is no effective therapy.

The HD mutation results in the expansion of a polyglutamine (polyglu) tract in a large 350 kDa protein of unknown function (Huntington's Disease Collaborative Research Group, 1993). The normal and expanded *HD* allele sizes have been defined as CAG₆₋₃₇ and CAG₃₅₋₁₂₁ repeats, respectively. An inverse correlation between age of onset and repeat length is most pronounced for juvenile HD for which the longest repeats have been observed (Huntington's Disease Collaborative Research Group, 1993; Telenius et al., 1993). Despite the selective cell death, the *HD* transcript is ubiquitously expressed (Strong et al., 1993). The polyglutamines are successfully translated and the huntingtin protein (htt) products arising from expanded alleles have been identified in protein extracts from HD patients (Jou and Myers, 1995; Trottier et al., 1995a).

CAG/gln expansion has been found to be the causative mutation in five neurodegenerative diseases for which the gene has been cloned. In addition to HD, these include spinal and bulbar muscular atrophy (SBMA) (La Spada et al., 1991), spinocerebellar ataxia type 1 (SCA1) (Orr et al., 1993), dentatorubral-pallidolusian atrophy (DRPLA) (Koide et al., 1994), and Machado Joseph disease (MJD or SCA3) (Kawaguchi et al., 1994). Many aspects of the genetics and molecular biology are common to these diseases. They are autosomal dominant (with the exception of X-linked SBMA) and show varying degrees of anticipation on paternal transmission. The size of the normal and expanded CAG repeat ranges are comparable, and available data indicate that age of onset correlations and patterns of repeat stability are

Summary

Huntington's disease (HD) is one of an increasing number of neurodegenerative disorders caused by a CAG/polyglutamine repeat expansion. Mice have been generated that are transgenic for the 5' end of the human *HD* gene carrying (CAG)₁₁₅–(CAG)₁₅₀ repeat expansions. In three lines, the transgene is ubiquitously expressed at both mRNA and protein level. Transgenic mice exhibit a progressive neurological phenotype that exhibits many of the features of HD, including choreiform-like movements, involuntary stereotypic movements, tremor, and epileptic seizures, as well as nonmovement disorder components. This transgenic model will greatly assist in an eventual understanding of the molecular pathology of HD and may open the way to the testing of intervention strategies.

Introduction

Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder (Harper, 1991).

reproduced. A similar ubiquitous expression pattern is also characteristic, and the presence of the expanded forms of ataxin-1 (SCA1 protein) and atrophin-1 (DRPLA protein) in lysates from patient tissues have been observed (Servadio et al., 1995; Yazawa et al., 1995).

Despite the otherwise apparent universality of this mutation, the patterns of cell death differ between these diseases. In HD, the most striking atrophy occurs in the caudate nucleus, which is often reduced to a rim of tissue. The putamen and globus pallidus also undergo atrophy, and there are subtle changes in the cerebral cortex (Vonsattel et al., 1985). SBMA is a form of motor neuron disease with both spinal and bulbar motor neuron involvement (Kennedy et al., 1968). The SCA1 and SCA3 spinocerebellar ataxias are clearly distinguished by major neuropathological features: Purkinje cell, pontine nuclei, and inferior olivary nuclei degeneration in SCA1 (Zoghbi et al., 1993) and pontine nuclei and the molecular layer of the cerebellum in SCA3 (Durr et al., 1996). In DRPLA, neuropathology includes the cerebellar dentate nucleus, globus pallidus, red and subthalamic nuclei, Purkinje cells, brain stem tegmentum, and the lateral corticospinal tract (Takahashi et al., 1988). The proteins containing the polyglu repeats are otherwise unrelated. In SBMA, the repeat lies within the androgen receptor (La Spada et al., 1991), while the others are in novel genes of unknown function. Subcellular localization suggests differing roles for these proteins (DiFiglia et al., 1995; Servadio et al., 1995; Trotter et al., 1995a; Yazawa et al., 1995).

It is essential that transgenic models of these diseases are developed. There have been two previous reports of a neurological phenotype observed in mice transgenic for a protein carrying a polyglu repeat expansion. The first used a SCA1 cDNA construct with (CAG)₈₂ under the control of a Purkinje cell specific promoter (Burright et al., 1995). Three heterozygous lines overexpressing the SCA1 transcript by 10- to 100-fold and two homozygous lines showed a progressive ataxic phenotype between 12 and 26 weeks of age. The mice became clearly ataxic when walking and routinely fell when attempting to stand on their hind legs. Pathologic examination showed significant loss of the Purkinje cell population with Bergmann glial proliferation and shrinkage and gliosis of the molecular layer. More recently, transgenic mice have been reported with a (CAG)₇₉ version of the SCA3 gene and also the (CAG)₇₉ polyglu tract in isolation, both under the control of the Purkinje cell specific promoter (Ikeda et al., 1996). Affected mice transgenic for the isolated polyglu tract were severely ataxic, they exhibit a wide-based hind limb stance, frequently fall when moving, and are unable to rear. Overt Purkinje cell death was observed with secondary effects to the molecular and granular cell layers. No phenotype was observed in the mice transgenic for the entire mutated SCA3 gene. The authors suggested that the polyglu tracts are more toxic in isolation than in the context of a protein, although in the absence of any information concerning transgene copy number, genomic structure of the integration sites, or expression levels, this interpretation should be treated with caution. These reports have shown that Purkinje cell specific overexpression of an expanded polyglu tract, both in the context of the SCA1

gene or in isolation, is toxic to Purkinje cells and causes a corresponding ataxic phenotype.

In our initial attempt to generate a murine model of HD, we have focused on the construction of a mutant yeast artificial chromosome (YAC) for introduction by pronuclear injection. Progress was severely hampered by both instability of YAC intermediates and the severe instability of highly expanded CAG repeats in yeast. Consequently, to address the question of CAG repeat stability in the mouse, transgenic lines were established with a 1.9 kb human genomic fragment containing promoter sequences and exon 1 carrying expansions of approximately (CAG)₁₃₀. Unexpectedly, this fragment has been sufficient to generate a progressive neurological phenotype that displays many of the characteristics of HD. This is the first time that a model of one of these diseases has been generated by a transgene driven from an endogenous promoter. The availability of a mouse model of the disease is extremely informative with regard to the size of the polyglu expansion and level of expression required to produce a phenotype with a given age of onset in the mouse. This work suggests that the polyglu-containing domain of the htt protein may be sufficient to generate a mouse model of HD.

Results and Discussion

Fragment Used for Transgenesis

The microinjection fragment was a 1.9 kb SacI-EcoRI fragment from the 5' end of the human HD gene isolated from a phage genomic clone derived from an HD patient (Figure 1a). It is composed of ~1 kb of 5' UTR sequences, exon 1 carrying expanded CAG repeats of ~130 units and the first 262 bp of intron 1. As the CAG repeats are unstable when propagated in *E. coli*, the DNA preparation used for microinjection contained a heterogeneous set of repeats of varying size but of the order of 130 units. In the event that an unspliced mRNA should be transcribed from this fragment, an "in-frame" stop codon immediately at the beginning of intron 1 would result in a truncated protein corresponding to the first 90 amino acids of the published htt protein (repeat size of (CAG)₂₁).

Genomic Organization of the Integration Events

Transgenic mice were generated by microinjection of single cell CBAx57BL/6 embryos. Of 29 newborn mice, seven died neonatally, and of the remaining 22 pups, one male was transgenic. This founder (R6) was initially backcrossed to both C57BL/6 and to CBAx57BL/6 females. However, a subsequent need to optimize litter size has resulted in the maintenance of the transgene on the CBAx57BL/6 hybrid background. F1 mice were genotyped both by Southern analysis and by PCR to determine the CAG repeat size. Figure 1b shows a Southern blot of BamHI digested DNA from a number of F1 progeny. It was possible to deduce that the microinjection fragment had integrated into five different regions of the founders' genome. The predicted genomic organization of the integration events is illustrated in Figure 1c. In lines R6/1 and R6/0, the fragment has integrated as an intact single copy, and in line R6/T as

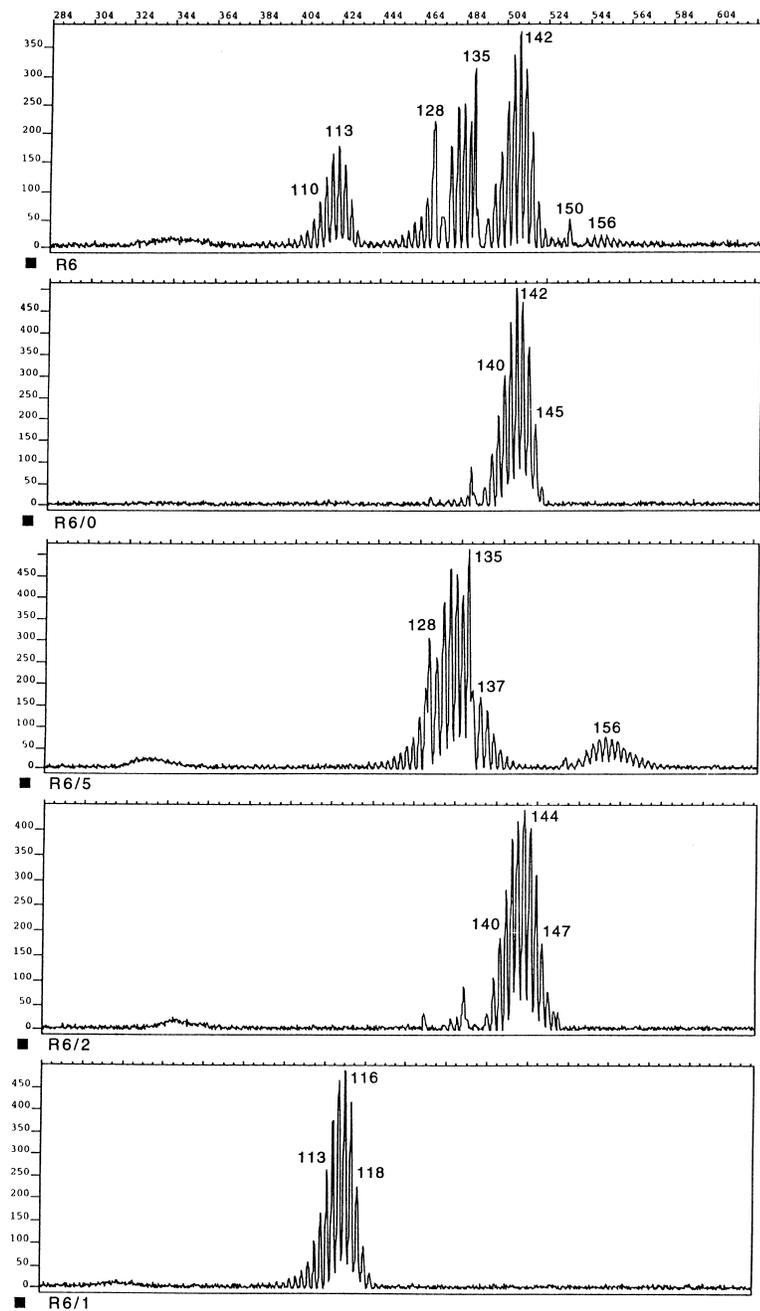


Figure 2. Measurement of the Size of the CAG Expansion in the R6 Transgenic Lines. The CAG repeats were amplified with a FAM-labeled primer as described. The top panel shows the trace specific to the founder (R6) and the four lower panels, the traces obtained in F1 mice with the R6/0, R6/5, R6/2, and R6/1 genotypes.

and 156 repeat units. These repeat sizes are considerably larger than those that have generally been reported to cause the juvenile form of HD in humans. Both gametic and somatic repeat instability have been observed (manuscript submitted).

Segregation of the Integration Events

The specific genotype frequencies found in 321 F1 mice derived from the R6 founder are summarized in Table 1. The integration events appear to segregate independently but are only seen in certain combinations. The founder is therefore a germ line chimera with one set of germ cells containing the R6/0 and R6/T transgenes and

the other containing the R6/1, R6/2 and R6/5 transgenes.

Phenotype Observed in the R6/2 Transgenic Line

The age of onset in line R6/2 has been observed as early as four weeks (one mouse) but most frequently occurs between nine and eleven weeks. Age at death has generally been between 10 and 13 weeks although the mouse with the age of onset at four weeks died at six and a half weeks. The mice display a progressive neurological phenotype. As far as can be ascertained, the mice remain alert, exploratory and inquisitive, and responsive

Table 1. Frequency of Genotypes Arising in 321 F1 Progeny

Genotype	N
R6/0	56
R6/T	61
R6/0 + R6/T	62
Negative ^a	(56/71)
R6/1	8
R6/2	16
R6/5	15
R6/1 + R6/2	9
R6/1 + R6/5	8
R6/2 + R6/5	11
R6/1 + R6/2 + R6/5	4
Negative ^a	(15/71)

^a The total number of nontransgenic mice are divided between the two genotype clusters in a proportion consistent with the genotype frequencies.

to sensory stimuli. The phenotype is complex. There are a number of components to the motor disorder including a resting tremor, movements described as resembling chorea, stereotypic involuntary movements, and in some cases a mild ataxia manifesting as dysmetria. One of the first symptoms is a dyskinesia of the limbs when held by the tail. This progresses to an alternating clasping together and releasing of the feet until the mice clasp their feet together immediately after they are picked up, (Figure 3a), and can no longer release this posture. The mice develop a constant tremor that becomes progressively worse. The tremor tends to be less noticeable when they are quiet or asleep, but worsens under stress (for example, the removal of the cage lid) or if they reach for food or to climb out of the cage. As the disorder progresses, stereotypic involuntary movements are apparent, which include repetitive stroking of the nose and face, and a hind limb kicking/scratching motion. Sudden movements that involve the whole body and may resemble chorea are observed. These are rapid, abrupt, irregular, and manifest as a shaking/shudder of the trunk. The mice do not develop a wide-based gait, can stand on their hind limbs and climb out of the cage without falling. They only consistently lose balance when sitting on their hind limbs, turning, and reaching round to groom their backs, which results in a somersault. The mice exhibit severe handling-induced epileptic seizures that can last for several minutes.

At weaning, the R6/2 transgenes are indistinguishable from their normal litter mates. Coincident with the onset of motor symptoms, their weight plateaus and then progressively decreases. In the end stages, mice have been observed to weigh as little as 60%–70% of their normal sibs. As the disease becomes more severe, they are very frequently observed to be eating but do not gain weight. It appears that the mice are eating rather than just breaking off food. Their food comprises an expanded chow, which does not crumble easily, and excess food crumbs are not observed in the bedding. On autopsy, the mice are often emaciated with an overall loss of muscle bulk although food is observed in the stomach and fecal pellets in the gut. Histological analysis of muscle samples showed no evidence of a myopathy.

Characteristic vocalizations have been observed. These include a sound similar to that made by a new born litter, which resembles teeth chattering from cold, but is likely to have a respiratory basis (since it occurs before the young mice have teeth). A second sound, a type of chirping noise, is more reminiscent of a bird than of a mouse. The mice are more likely to make these sounds when they are under stress (for example, away from the home cage).

The mice appear to urinate more frequently. The bedding at the front of the cage becomes excessively wet as compared to that in cages housing normal mice. They are unlikely to be suffering from spastic bladders as the wetting of the bedding is not uniform. Urine tests in 18 transgenic mice (11 male and 7 female) showed no abnormality in glucose or protein levels. Similarly, blood tests in two mice showed glucose and protein levels to be within the normal range.

R6/2 females are sterile and, of ten R6/2 males that have been placed with females from a time just prior to expected sexual maturity, five have mated. Of these, one mouse produced one litter, two mice produced two litters and two produced four litters. On autopsy, the reproductive organs consistently appear vestigial or atrophied. Females often have miniscule ovaries and a hair-like uterus. Males have small testes, seminal ducts, and coagulation glands. On histology, one male that had failed to mate was found to have testicular atrophy with an absence of spermatazoa, an atrophy of the epididymus with aspermia, and no secretion present in the coagulation gland.

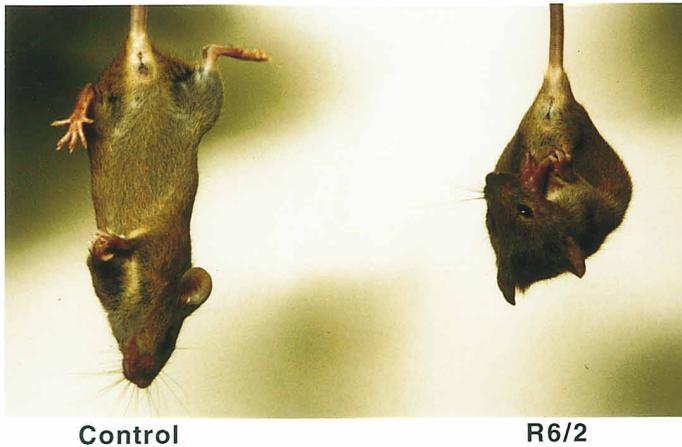
The mice die suddenly and the cause of death is generally unknown although one mouse was observed to die during an epileptic seizure.

Dosage Effect on Age of Onset and Phenotype Severity in Complex Genotypes

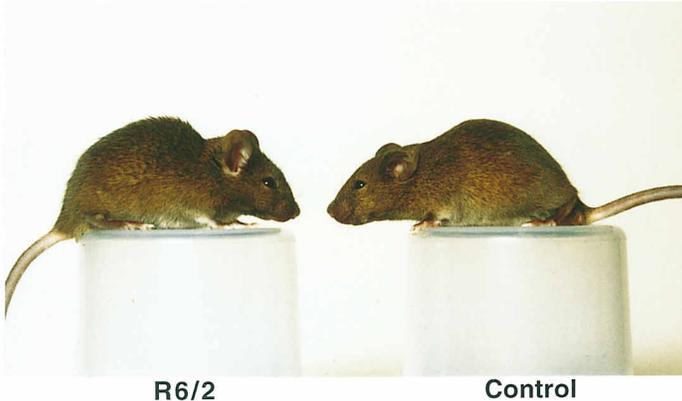
Lines R6/1, R6/2, and R6/5 have been established from the founder. In the F1 generation, mice with all possible combinations of these transgenes were identified. Each aspect of the phenotype, as described for line R6/2, has been observed for the genotypes listed in Table 2. In the end stages of the disease, the transgenes are always considerably smaller than their normal littermates. The age of onset varies from <3 weeks (R6/1+R6/2+R6/5 genotype) to ~4 or 5 months (R6/1 line).

The (R6/1+R6/2+R6/5) genotype is the most severe. Only four such mice were recovered in the F1 generation. The overall genotype frequency (Table 1) would have predicted more than this, and it is possible that some mice with this genotype died neonatally or in utero. All aspects of the phenotype are more severe and have a more rapid progression. The (R6/1+R6/2+R6/5) mice are considerably smaller than their litter mates at weaning. For example, one weighed 5.2 g at 23 days of age as compared to a mean of 9.2 g for her female sibs. She reached a maximum weight of 7.5 g but was only 6.0 g at death at 51 days as compared to a mean of 16.3 g for her sibs. In contrast, line R6/1 has the latest age of onset and the slowest progression. The mice begin to exhibit the feet-clasping posture when suspended by the tail at ~4–5 months. At between 6 and 7 months,

a)



b)



c)

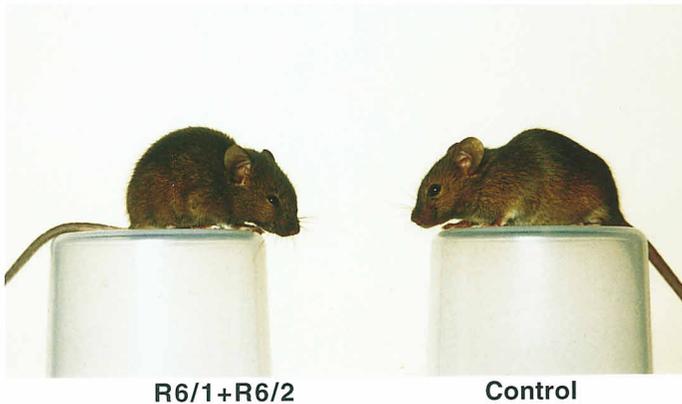


Figure 3. Comparison of R6 Transgenic Mice and Littermate Controls

(a) An R6/2 transgenic mouse demonstrating the feet-clasping posture adopted when suspended by the tail. The normal mouse holds its hind limbs outward in order to steady itself. (b) The R6/2 mouse (17.7 g) and normal littermate (21.3 g) at 12 weeks of age. The transgenic mouse is thinner. (c) An R6/1 + R6/2 (10.1 g) transgenic mouse and normal littermate (19.6 g) at seven weeks, three days. There is a considerable size difference.

some show a mild tremor and intermittently exhibit all aspects of the involuntary movement disorder as described for the R6/2 line. Epileptic seizures have also been observed. The effect of transgene dosage on the size of the mice is illustrated in Figure 3.

On autopsy, atrophy or gross atrophy of the primary and secondary reproductive organs is routinely observed. Otherwise, hepatic changes in the form of polyploid hepatic nuclei and a loss of cytoplasmic mass with

no obvious cell death was the only consistent observation resulting from a routine histopathological examination (two R6/2 and six R6/1 + R6/5 mice in the end stages of the disease and displaying all aspects of the phenotype). Thymic atrophy is sometimes present, more frequently in the more severely affected lines, but this does not correlate with the presence or absence of phenotypic features. In a few mice there is a slight deformation to the cranial vault resulting in a bony ridge over the

Table 2. Comparison of the Onset and Duration of the Phenotype Associated with the R6 Genotypes

	Age of Onset	Age at Last Litter	Age at Death
R6/1 + R6/2 + R6/5	< 3 weeks	N/A	4–7 weeks
R6/1 + R6/2	3–4 weeks	N/A	6–8 weeks
R6/2 + R6/5	6–7 weeks	N/A	8–12 weeks
R6/2	9–11 weeks	6–9 weeks ^a (5 males)	10–13 weeks
R6/1 + R6/5	12–16 weeks	12 weeks ^a (1 male)	24–36 weeks
R6/1	15–21 weeks	14 weeks ^b (1 male)	32–40 weeks ^c

^a Mice bred continuously.

^b Mouse failed to breed when cross set up at 19 weeks.

^c Oldest R6/1 mouse is alive at 40 weeks.

cerebellum. This has been seen more frequently in the lines with the more severe phenotype but has also been observed in line R6/1+R6/5.

A phenotype has not been observed in the heterozygous (R6/5)/+ or (R6/0)/+ lines, the oldest mice now being ~14 months. R6/5 homozygotes are developing symptoms at ~9 months, and the R6/5 transgene clearly contributes to the onset and progression of the disorder when in combination with R6/1 or R6/2 transgenes.

Expression of the Transgene

PCR primers specific to exon 1 of the human *HD* gene were used to examine the expression and tissue distribution of the transgenes. RT-PCR showed the transgene to be expressed in every tissue examined for lines R6/2 (Figure 4a), R6/1, and R6/5, but was not expressed in line R6/0. This ubiquitous pattern of expression for three of the lines suggests that the transgene is most likely expressed from promoter sequences present on the microinjection fragment. The absence of expression in line R6/0 is probably due to a position effect as Southern analysis of this line predicts that the R6/0 transgene has integrated adjacent to a genomic region of unusual structure. Northern analysis revealed transcripts of 2.5 and 2.3 kb in lines R6/1 and R6/2, respectively (Figure 4b) and the suggestion of a larger R6/5 transcript. The 4G6PE0.2 probe is derived from intron 1 of the human gene, and the presence of this sequence in the transcripts indicates that the human exon 1 has not spliced to mouse exonic sequences potentially occurring close to the integration sites.

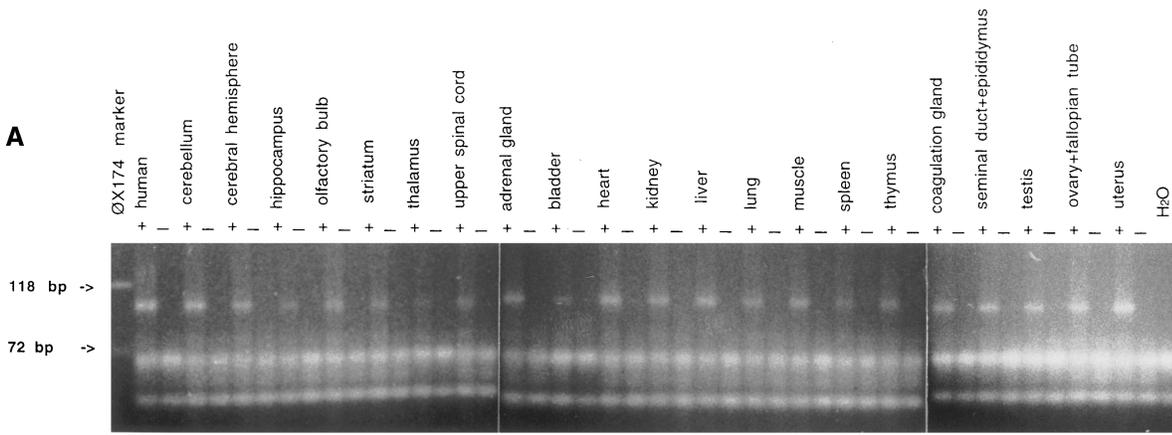
The level of expression of the transgene with respect to the endogenous mouse *hd* gene was assessed in total RNA from six tissues for each of the lines R6/1, R6/2, R6/5, and R6/0. The PCR primers had identical recognition sequences in exon 1 of both the mouse and human genes and amplified mouse and human products of 121 and 114 bp, respectively. No expression was detected in the R6/0 transgene. While the comparative expression level varies between tissues, the average expression of the R6/2, R6/1, and R6/5 transgenes was 75%, 31%, and 77% of the endogenous level (data not shown). The tissue variability made absolute quantitation difficult, but this analysis nevertheless places the level of expression of the transgene within the range of the murine gene.

The monoclonal antibody, 1C2, binds specifically and in a size-dependent manner to pathogenic polyglu

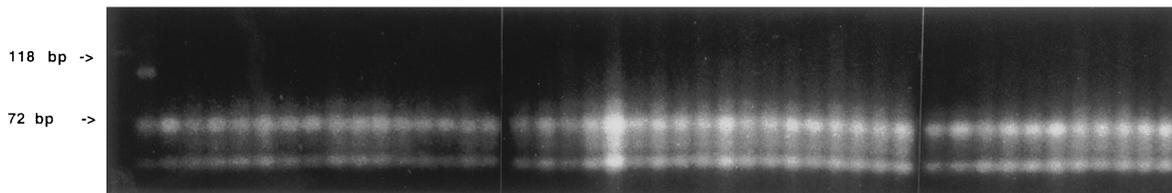
expansions (Trottier et al., 1995b). This antibody was used to immunoprobe Western blots of cell lysates derived from a complete set of tissues from lines R6/1, R6/2, and R6/5. A transgene-specific product was detected in lines R6/2 and R6/5 in all tissues tested. Figure 5 shows the Western blots obtained for a subset of tissues from lines R6/2 and R6/5. The predicted size of the R6/2 protein would be ~23 kDa. The migration of the R6/2 and R6/5 products, at a size larger than this with respect to the markers, is consistent with the aberrant migration observed for the expanded polyglu containing htt, ataxin-1, and atrophin-1 products when compared to their normal counterparts. A constant band detected in all transgene and control tissues was found to be due to cross-reactivity of the antimouse secondary antibody. Comparison of the intensity of the the constant band between the R6/2 and R6/5 tissues suggests that the transgene protein is present at similar levels in these lines. A protein product has not been detected in line R6/1 despite testing ranges of polyacrylamide concentration and antibody dilution. It would be extremely unlikely that a protein product were not present in this line. One possible explanation is that the length of polyglu tract in the R6/1 protein does not present an epitope to the 1C2 antibody. It is not clear from expression analysis why the R6/5 phenotype should be so much milder than that observed in lines R6/2 and R6/1.

Neuropathology

Nine R6/2 transgenic mice, exhibiting a broad spectrum of severe symptoms of 2–3 weeks duration, and nine nontransgenic littermates were used for neuropathological investigation. Brains from the transgenic animals were consistently smaller than controls (controls 490 ± 9.8 mg, transgenes 395 ± 8.0 mg). Serial 40 μm sections in either the coronal (12 mice) or horizontal (6 mice) planes were processed for either Nissl staining (Figures 6 and 7) or the immunocytochemical localization of glial fibrillary acidic protein (GFAP) or the mouse macrophage and microglial marker F4/80. The morphology of the central nervous system (CNS) in the transgenic mice appeared normal with no focal areas of malformation or neurodegeneration; however, sections of the brains of these animals were consistently smaller than those of their litter mates (19% ± 1.6%). This reduction in size appeared to be uniform throughout all CNS structures. Analysis of thionin-stained sections showed no evidence of neuronal cell loss, oligodendrocyte loss, reac-



Transgenic line R6/2.



Litter mate control.

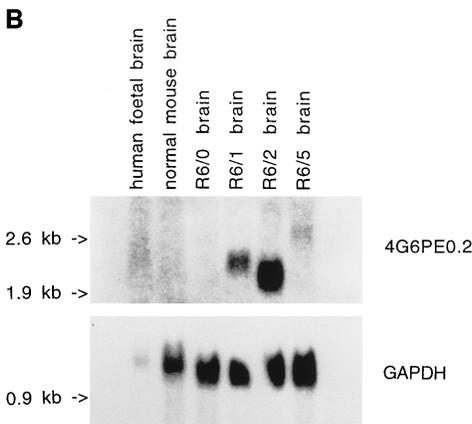


Figure 4. mRNA Size and Expression Pattern of the R6 Transgenes
(a) RT-PCR analysis of the expression of the transgene in the R6/2 line. RNA for PCR had (+) or had not (-) been treated with RT. The expression pattern in RNA extracted from an R6/2 transgenic mouse (top panel) and a littermate control (bottom panel) are shown. In each case, the first track contains RNA from human fetal brain as a positive control.

(b) Northern analysis of the transgene expression in the R6 lines. All lanes contained 20 µg total brain RNA and the blot was hybridized with the human 4G6PE0.2 intronic probe. As expected, a signal was not detected in the human RNA lane. Products of 2.5 and 2.3 kb are present in the R6/1 and R6/2 lanes, respectively, and a larger band can be seen in the R6/5 lane. Lower panel: hybridization with the mouse GAPDH probe. The reduction in intensity in the human RNA track is due to cross species hybridization and not unequal loading of RNA.

tive gliosis, or inflammatory change. These latter two observations were corroborated by the GFAP and F4/80 stained sections, where the normal distribution of astrocytes and ramified microglia cells was observed in the absence of any indication of increased reactivity of astrocyte staining or the presence of rounded microglia or infiltrating macrophages.

Cerebral Cortex and Hippocampus

The cytoarchitectonic structure of the cerebral cortex was maintained in the frontal, temporal, occipital and parietal lobes, although all regions were noticeably thinner when measured between the pia and subcortical white matter. The large pyramidal cells of the motor regions of the frontal cortex were present in normal number and morphological appearance. Similarly the pyramidal cells of hippocampus, subiculum and para-

hippocampal gyrus, the stellate cells of layer II of the entorhinal cortex, and the granule cells of the dentate gyrus were of normal size and distribution.

Basal Ganglia

A detailed analysis of the striatum, nucleus accumbens, globus pallidus, entopenduncular nucleus, subthalamic nucleus, and substantia nigra demonstrated normal neuronal density and patterns of morphological diversity. The striatum is composed of a normal complement of medium-sized striatal neurons interspersed with fewer large and small neurons, together with satellite glia. The white matter of the corpus callosum and the fascicles of fibers forming the internal capsule contain as many oligodendrocytes as similar sections from control mice. The striatum is again consistently smaller in the transgenic animals.

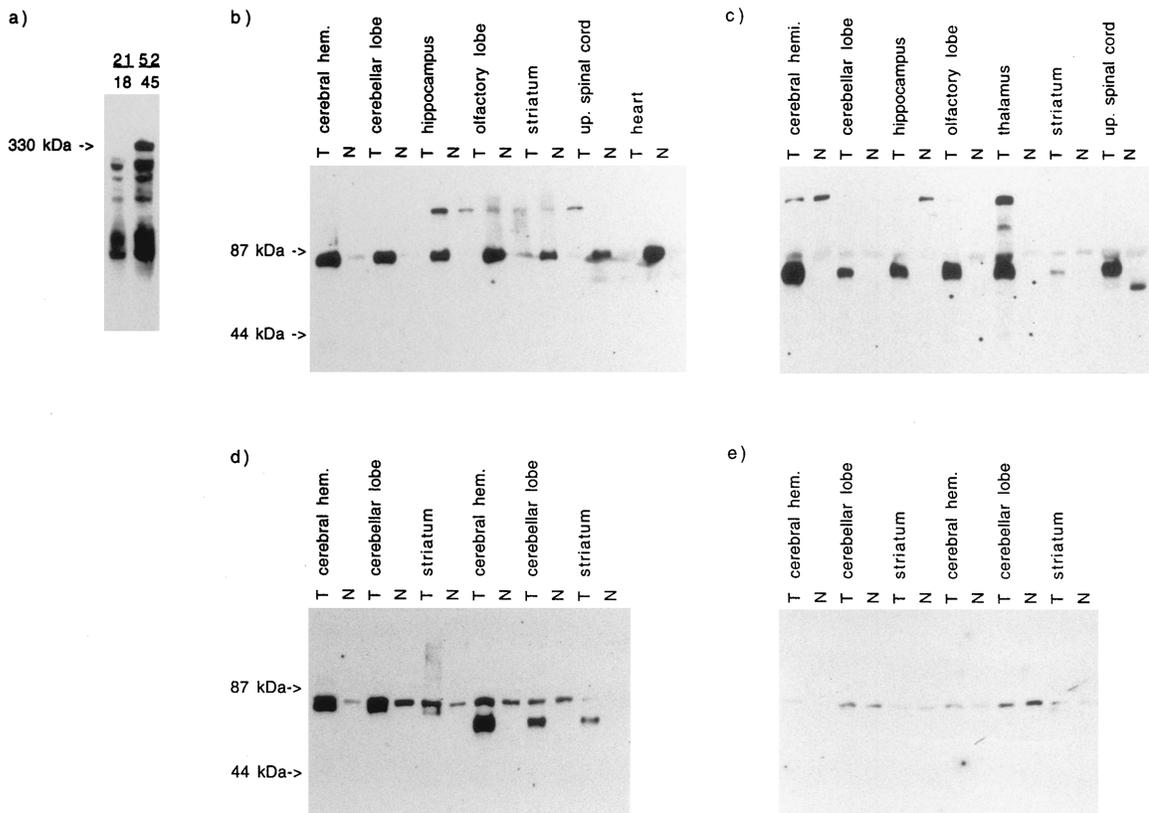


Figure 5. Expression Profile of the Transgene Protein Products

Identification of the transgene protein product in the R6/2 and R6/5 lines using a monoclonal antibody (1C2) that specifically detects polyglin expansions.

(a) Identification of htt in lysates prepared from lymphoblastoid cell lines from a normal individual and an HD homozygote and fractionated on a 6% SDS-PAGE gel. The size of the respective CAG expansions are indicated above the tracts. The position at which the fibrinogen marker (330 kDa) migrates is indicated.

(b) Lysates from an R6/2 transgene (T) and littermate control (N) were fractionated on a 10% SDS-PAGE gel.

(c) Lysates from an R6/5 transgene (T) and a littermate control were fractionated on a 10% gel.

(d) Lysates from the R6/2 and R6/5 lines fractionated on a 10% SDS-PAGE gel.

(e) The filter in (d) stripped and reprobbed with the secondary antimouse antibody, which detects the constant band seen in (b)-(d).

Cerebellum and Spinal Cord

The granule cells, Purkinje cells, and the neurons of the molecular layer of the cerebellum show no differences from the control mice. Similarly, the large motor neurons of the anterior horn of the cervical and lumbar enlargements of the spinal cord and the dorsal horns are again of normal appearance.

Examination of all other areas of the CNS revealed no gross or microscopic abnormalities.

Discussion

Transgenic mice that develop a progressive neurological phenotype have been generated by the introduction of a genomic fragment containing exon 1 of the human *HD* gene. Four lines have been established, with CAG repeat expansions ranging from ~115 to 150 repeat units. In the three lines that exhibit a phenotype, R6/1, R6/2, and R6/5, the transgene has a ubiquitous mRNA and protein expression pattern. The transgene mRNA is most likely transcribed from human promoter elements and extends into the flanking mouse sequences.

The presence of human intron 1 sequences in the mRNA rules out the possibility that the human exon splices to mouse exonic sequences and therefore predicts that the corresponding transgene protein products contain 69 amino acids in addition to the number of polyglin residues encoded by the repeat expansion.

The polyglin expansions in the R6 transgenic mice are of a size considerably greater than is generally associated with the juvenile form of HD. Even so, it is not possible to predict the phenotypic expression of such a mutation in the mouse. In HD, the major focus of neuropathological change is in the striatum (part of the basal ganglia) and the cerebral cortex. The motor disorder observed in the R6 lines is strongly suggestive of a basal ganglia lesion. The mice exhibit involuntary jerky shudders that have been described as resembling chorea and likened to the choreic movements observed in the neurological disease arising from canine distemper (Lauder et al., 1954). As far as we can ascertain, chorea has not previously been described in mice (Lyon and Searle, 1990). The neuropathological correlate of chorea is accepted as a basal ganglia lesion. The pronounced

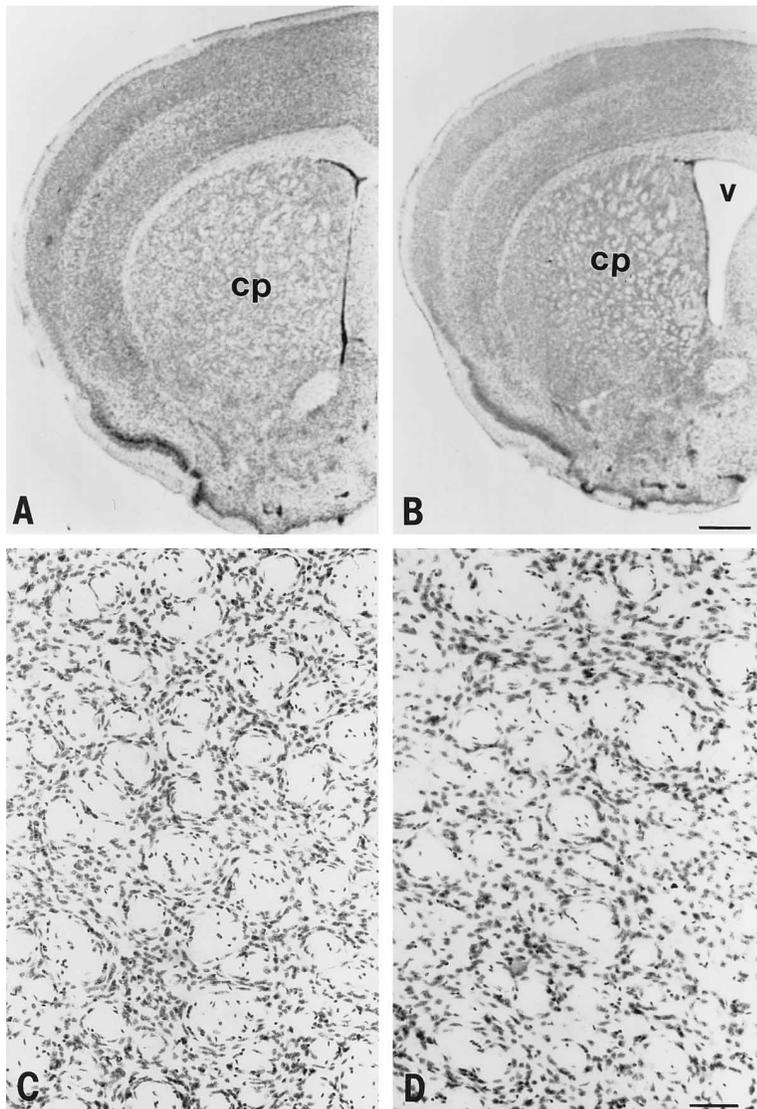


Figure 6. Nissl Sections Through the Mouse Forebrain

Frontal section through the caudate/putamen (cp) at the level of the lateral ventricle (v) of a normal littermate control (A) and R6/2 transgenic mouse (B). The caudate putamen is shown in higher power in C (control) and D (R6/2 transgene). Scale bars, 500 μm in (A) and (B), 80 μm in (C) and (D).

progressive resting tremor that occurs in all limbs, trunk, and head of affected mice also points to a basal ganglia abnormality. The observation of epileptic seizures is compatible with juvenile HD; however, while seizures have a cerebral focus, they could result from many imbalances that are both intracranial or extracranial.

The R6 mice also suffer from a progressive decrease in body weight and an overall loss of muscle bulk. Similarly, loss of body weight and a generalized lack of muscle bulk is a progressive and characteristic symptom of HD, despite increased calorific intake (Sanberg et al., 1981). The weight loss appears to be independent of the hyperkinesia and its molecular basis is not understood (Harper, 1991). In addition, the R6 mice appear to urinate more frequently as judged by wetting of the bedding. Urinary incontinence has also been noted in HD with symptoms including frequency, urgency, nocturia, and incontinence (Wheeler et al., 1985). Finally, chorea affecting face, jaw, and pharyngeal muscles affects both speech and swallowing and can also cause grunting and clicking sounds that may reflect respiratory movements

(Harper, 1991). It is possible that the unusual vocalizations made by the R6 transgenes arise by a similar mechanism.

A landmark study of the neuropathology of HD has classified the neuropathological changes into five grades that progress from grade 0, in which HD brains show no gross or microscopic abnormalities consistent with HD despite premortem symptomatology and positive family history, to grade 4, in which the most extreme atrophy is observed (Vonsattel et al., 1985). The brains from the R6/2 transgenic mice were found to be on average 19% smaller than those of their normal littermates, a reduction in size that was maintained through all CNS structures. This finding is consistent with neuropathological changes occurring in HD in which it has been noted that a 30% reduction in brain weight in HD is associated with 20%–30% areal reductions in cerebral cortex, white matter, hippocampus, amygdala, and thalamus (de la Monte et al., 1988). This atrophy was similar for all grades of HD, suggesting that the shrinkage of these structures occurs early in the

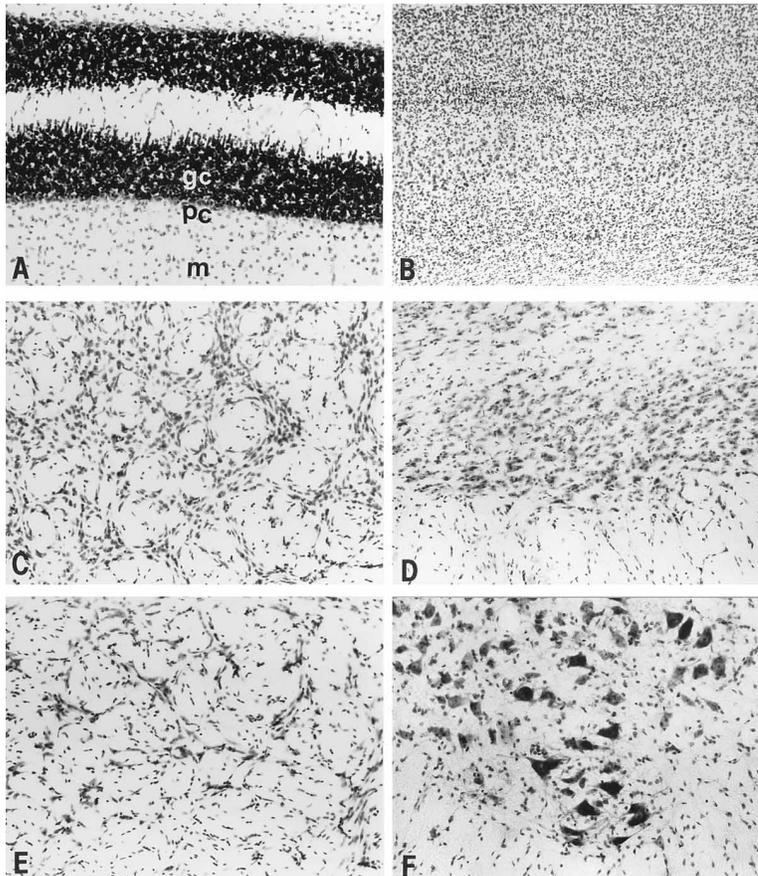


Figure 7. Nissl Sections throughout the CNS. Coronal sections through the cerebellum (A), cerebral cortex (B), globus pallidus (C), subthalamic nucleus (D), entopeduncular nucleus (E) and ventral horn of the lumbar spinal cord (F) of an R6/2 transgenic mouse. Within the cerebellum, note the normal density of the granule cell layer (gc), the monocellular layer of pyramidal cells (pc) and the normal structure of the molecular layer (m).

disease process, is not progressive, and reflects cell loss of both neurons as well as fibers. Interestingly, gliosis was not readily apparent in these structures, and the neuronal density was assessed to be normal (de la Monte et al., 1988). In contrast, a 60% reduction in the cross-sectional area of the caudate, putamen, and globus pallidus increases with the higher grades of HD brains, indicating that these structures progressively degenerate with prolonged survival. It is this specific progressive atrophy, associated with reactive astrocytosis, that was not apparent in the R6/2 transgenes and is also absent from grade 0 HD brains (Myers et al., 1991; Vonsattel et al., 1985). The grade 0 brains came from patients that had had HD symptomatology for between 2 and 13 years (Vonsattel et al., 1985; Myers et al., 1988; Hedreen and Folstein et al., 1995), thereby providing no pathological correlate for chorea and other early signs (Hedreen and Folstein et al., 1995). It seems likely that the brains of the R6/2 transgenes have neuropathology consistent with that found in the early stages of HD and that the progression of the phenotype in these mice is so rapid that there is insufficient time for the progressive atrophy to take place. A detailed morphometric analysis did uncover a neuronal loss in the caudate of grade 0 brains (Myers et al., 1991), and the absence of reactive astrocytosis was taken as evidence that the neuronal cell loss was not a recent event and may support the hypothesis that the HD striatum is compromised from early in development (Myers et al., 1991). A detailed

morphometric analysis of the R6/2 transgene brains is merited. The neuropathological analysis of the transgenes was also focused on the additional regions that undergo neurodegeneration in the polyglu expansion diseases as a whole, and no evidence of localized neurodegeneration was identified.

To date, five neurodegenerative diseases have been described that are caused by polyglu expansions in ubiquitously expressed unrelated proteins. It is most probable that in each case the polyglu expansion confers a gain of function to the proteins and that this may operate by a common molecular mechanism. It has been proposed that the specific selective cell death is directed by the remainder of the respective proteins. The R6 transgene protein products contain polyglu tracts in a domain consisting of only 69 other amino acids amounting to ~3% of the htt protein. Therefore, the R6 transgenic mice might be expected to represent a generic CAG/glu disease model rather than a specific model of HD. However, the R6 mice do not develop a pronounced ataxia as described by Burright et al. (1995) and Ikeda et al. (1996). They do not develop a wide-based gait or fall while moving, are able to rear, and do not lose their righting response when turned onto their backs. This would suggest that there is no major cerebellar lesion and that the R6 lines do not display the major movement disorder of SCA1, SCA3, and late onset DRPLA. Similarly, they do not show a pronounced motor neuron disease, although the SBMA symptoms in

humans are mild with a very slow progression and it would probably be difficult to identify this component as part of the complex R6 phenotype. The diagnosis of HD and DRPLA was not infrequently confused before the advent of mutation analysis afforded an unequivocal test. Both disorders present with complex and variable symptoms that can include chorea, myoclonus, dystonia, dysarthria, and seizures. Some features are more or less associated with the juvenile or adult forms but the boundaries are not absolute. It would therefore be difficult to express any strong claims as to the specificity of a mouse model with respect to these two diseases.

The R6 mice are the first transgenic model of a polyglu expansion disease in which the transgenes are ubiquitously expressed (as are the mutant human genes). The two previous reports of a neurological phenotype observed in mice transgenic for a protein carrying a polyglu repeat expansion used a Purkinje cell specific promoter to drive either a *SCA1* cDNA construct with (CAG)₈₂ (Burrigh et al., 1995) or a (CAG)₇₉ polyglu tract in isolation (Ikeda et al., 1996). Purkinje cell death was identified with a corresponding ataxic phenotype. It is possible that comparable overexpression of these constructs in any other cell would also demonstrate toxicity. The dramatic dosage effect on the phenotype observed with the R6 transgenes expressing at less than endogenous levels suggest that ubiquitous overexpression of the R6 transgene could be lethal.

The apparent absence of specific neurodegeneration in the R6 mice supports the possibility that localized atrophy may be secondary to a primary imbalance that is directly responsible for the clinical symptoms that arise in HD. Indeed, replication of the patterns of cell death observed in HD by intrastriatal injections of quinolinic acid does not cause chorea in rats (Harper, 1991). It remains remarkable that the introduction of the expanded version of the polyglu-containing domain of htt protein into transgenic mice has succeeded in reproducing not only features of the movement disorder, but also other aspects of the complex HD phenotype.

Two further lines of transgenic mice are required to determine the extent to which the R6 mice represent a model of HD. First, mice transgenic for the entire *HD* gene carrying repeat expansions of a comparable size must be generated. The large size of the *HD* gene necessitates that the construct be introduced in the form of a YAC clone (experiments in progress). An identical phenotype would indicate that the remainder of the htt protein is superfluous to the course of the disease, and any differences would aid in the dissection of the protein into functional domains. Second, mice transgenic for the nonexpanded CAG repeat version of the R6 lines have not been described in this paper. The original purpose of the R6 transgenes was to study repeat stability and, consequently, the nonexpanded controls were not generated in parallel. However, it is important to characterize such mice, to rule out the unlikely scenario that the phenotype observed is the result of a novel peptide. Three founders have now been established that contain the *SacI*-*EcoRI* fragment with a (CAG)₁₈ tract: Hdex/6, Hdex/27, and Hdex/28. F1 mice derived from the Hdex/6 founder are currently 20 weeks, and the mice show no signs of a neurological phenotype or weight loss. These

mice are twice as old as the R6/2 mice at the onset of the phenotype. Quantitative RNA analysis shows the Hdex/6 transgene to be expressed at levels comparable to that in the R6/2 and R6/5 lines; however, it is not possible to use the 1C2 antibody to detect the Hdex/6 transgene protein as this is specific to polyglutamine expansions. The Hdex lines will be bred to homozygosity and the mice observed over the course of at least one year.

This work raises the intriguing possibility that exon 1 of the *HD* gene carrying highly expanded repeats is sufficient to generate a transgenic model of HD. The mutation is predicted to operate by conferring a gain of function to the mutated protein to which some cells are particularly sensitive. The cell-selective toxicity may be afforded by differing compartmentalization of the polyglu-carrying proteins or by the specificity of their intermolecular interactions. In order that the small R6 transgene could initiate a chain of molecular events comparable to those involving the entire htt protein, it would be necessary to predict that the transgene occupies the same subcellular localization. It has not been possible to make this comparison as our attempts at immunohistochemistry with the 1C2 antibody have been consistently unsuccessful, and in addition, the subcellular localization of htt remains to some extent controversial. If the selectivity of the cell death arises through the interacting proteins, the polyglu-containing domain of the htt protein must be sufficient to convey this specificity. There may be some evidence to suggest that this could be the case, arising from the isolation of HAP1 (huntingtin associated protein 1) (Li et al., 1995). HAP1 binds to htt containing a polyglu of 21 residues, and the association is enhanced by increasing lengths of the gln repeat. There was no binding to atrophin-1 (the mutant protein in DRPLA) also containing 21 glutamines.

It is impossible to predict the accuracy with which transgenic mouse lines will model a corresponding human disease. The R6 transgenes display many characteristics of HD, and had this phenotype arisen in mice transgenic for the entire mutant protein, the model would have needed little justification. It is clearly possible that the polyglu-containing domain may be the only part of the htt protein involved in the disease process. The R6 transgenic mice already provide a valuable resource for uncovering the molecular pathology of HD and may present a target for the testing of potential therapeutic interventions.

Experimental Procedures

Genotyping

DNA was prepared from tail biopsy and Southern blots and hybridizations were as described (Monaco et al., 1985). CAG repeats were sized by PCR using FAM-labeled primer 31329 (ATGAAGGCCTTC GAGTCCCTCAAGTCCTTC) and primer 33934 (GGCGCTGAG GAAGCTGAGGA) in AM buffer (67 mM Tris-HCl [pH 8.8], 16.6 mM NH₄SO₄, 2.0 mM MgCl₂ 0.17 mg/ml BSA, 10 mM 2-mercaptoethanol), 10% DMSO, 200 μM dNTPs, 8 ng/μl primers with 0.5 U/μl Taq polymerase (Cetus). Cycling conditions were 90" @ 94°C, 25 × (30" @ 94°C, 30" @ 65°C, 90" @ 72°C), 10" @ 72°C. PCR products were sized using an ABI sequencer and the Genescan and Genotyper software packages. The size of the CAG repeat was 85 bp less than the size of the PCR product.

RNA Analysis

Northern blots were prepared by standard methods and hybridized as described (Monaco et al., 1985). RNA was reverse transcribed (14 U/ μ l MMTV RTase, BRL) in 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 6.5 mM MgCl₂, 10 mM DTT, 1 mM dNTPs, 10 ng/ μ l random hexamers with 0.35 U/ μ l RNasin (Promega) at 10' @ 23°C and then 40' @ 37°C. Primers for specific transgene RNA detection were 33935 (CGGCTGAGGCAGCAGCGGCTGT) and 35093 (GCAGCAGCAGCAGCAACAGCCGCCACCGCC). PCR was in AM buffer, 10% DMSO, 200 μ M dNTPs, 10 ng/ μ l primer with 0.5 U/ μ l Taq polymerase (Cetus). Cycling conditions were 90" @ 94°C, 34 \times (30" @ 94°C, 30" @ 68°C, 90" @ 72°C), 10' @ 72°C.

Protein Analysis

Frozen tissue was homogenized in 50–100 μ l 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, and 1 mM 2-mercaptoethanol with 1 mM PMSF, 0.5 mM DTT, 25 mM benzamide and leupeptin, pepstatin and chymostatin each at 200 ng/ml. Homogenates were sonicated on ice 10–20 s, spun at high speed at 4°C, and the supernatant transferred to a fresh tube. Protein was quantified by the Bradford assay when in sufficient quantity. Approximately 50 μ g of protein was loaded per track onto 6% or 10% SDS-PAGE gels. Kaleidoscope prestained standards were used as size markers (Biorad). Fibrinogen (Sigma) was added as a size marker of 330 kDa (Jou and Myers, 1995). Proteins were transferred to PVDF membranes (Biorad) that were blocked at 4°C overnight in PBS with 5% nonfat dry milk and 2% fetal calf serum. Immunoprobings with antibody 1C2 was at a 1:2000 dilution in PBS with 0.5% nonfat dry milk for 1 hr at RT. Washes were in PBS containing 1% NP-40 and 1% fetal calf serum. Secondary antibody probing and detection was by use of the ECL kit (Amersham).

Histopathology

Brains from nine R6/2 transgenes and nine nontransgenic littermates were analyzed for neuropathological change. A 1:3 series of sections was stained for Nissl substance with thionin, or processed free floating for the immunocytochemical localization of the glial marker, glial fibrillary acidic protein (GFAP), or the macrophage/microglial marker F4/80. Nuclear cells groups within the mouse brain were verified by reference to Sidman, Angevine, and Taber-Pierce (Sidman et al., 1971).

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