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# Length-dependent gametic CAG repeat instability in the Huntington's disease knock-in mouse

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**The CAG repeats in the human Huntington's disease (HD) gene exhibit striking length-dependent intergenerational instability, typically small size increases or decreases of one to a few CAGs, but little variation in somatic tissues. In a subset of male transmissions, larger size increases occur to produce extreme HD alleles that display somatic instability and cause juvenile onset of the disorder. Initial efforts to reproduce these features in a mouse model transgenic for *HD* exon 1 with 48 CAG repeats revealed only mild intergenerational instability (~2% of meioses). A similar pattern was obtained when this repeat was inserted into exon 1 of the mouse *Hdh* gene. However, lengthening the repeats in *Hdh* to 90 and 109 units produced a graded increase in the mutation frequency to >70%, with instability being more evident in female transmissions. No large jumps in CAG length were detected in either male or female transmissions. Instead, size changes were modest increases and decreases, with expansions typically emanating from males and contractions from females. Limited CAG variation in the somatic tissues gave way to marked mosaicism in liver and striatum for the longest repeats in older mice. These results indicate that gametogenesis is the primary source of inherited instability in the *Hdh* knock-in mouse, as it is in man, but that the underlying repeat length-dependent mechanism, which may or may not be related in the two species, operates at higher CAG numbers. Moreover, the large CAG repeat increases seen in a subset of male HD transmissions are not reproduced in the mouse, suggesting that these arise by a different fundamental mechanism than the small size fluctuations that are frequent during gametogenesis in both species.**

## INTRODUCTION

Huntington's disease (HD), with its hallmark choreiform movements and graded pattern of neuronal cell loss in the basal ganglia (1,2), is caused by a CAG tract in one copy of a novel 4p16.3 gene (*HD*) that is expanded (~35 to >130 units) beyond the normal polymorphic range (6–34 units) (3). The mutation acts at the level of the protein by extending a glutamine segment in huntingtin, triggering a pathogenic mechanism that remains to be elucidated (4,5). Disease onset varies inversely with the length of the expanded glutamine tract (3,6–24). Thus, most HD cases (40–50 CAG units) manifest in mid-life but symptoms may not begin until old age due to tracts of ~35–39 CAGs that exhibit reduced penetrance (19,20). Occasionally, the defect claims its victims in childhood with repeats of ~55 or more units, which are typically inherited from fathers (3,21–24).

The expanded *HD* repeat exhibits striking intergenerational instability that, in conjunction with the CAG length-dependent pathogenic mechanism, explains puzzling genetic aspects of the disorder. In the vast majority of transmissions from either HD mothers or HD fathers (>80%), the expanded repeat (~40–50 units) is subtly altered, increasing or decreasing by one or a few CAG units (6–24). However, on occasion, paternal HD transmissions produce large expansions that cause juvenile onset disease (6,21–24). Similarly, paternal transmission of chromosomes with 34–39 CAGs serves as a source of sporadic HD by producing rare jumps into the overt disease-causing range (25–30). This pattern reflects gametic mosaicism that has been demonstrated in sperm DNA of some HD males (6,31–35).

In contrast to the intergenerational instability of the expanded HD repeats, limited length variation is exhibited in somatic tissues of most HD patients (3,6,31,35–38). Only the largest arrays (>~65 repeats) show dramatic mosaicism in brain and

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other organs of some juvenile onset HD patients (32,37). Intriguingly, in a proportion of the latter cases the cerebellum possesses a single dramatically reduced allele rather than a combination of different repeat sizes (32). Notably, the predominant mitotic stability of *HD* CAG repeats argues that specific somatic mosaicism in the target tissues, the basal ganglia and cerebral cortex, does not explain the selective pathogenesis of the disorder (31,37).

The fundamental processes that participate in HD CAG repeat instability are unknown, but comparison of the stable inheritance of alleles in the normal range with the unstable behavior of expanded CAG repeat tracts demonstrates that instability is length-dependent in both male and female transmissions. The observation of mosaicism in sperm, but not somatic tissue, of typical HD patients and the finding that monozygotic twins have an identical HD repeat size both point to gametogenesis as the primary source of instability (31). In HD males, gametic mosaicism in sperm tends to increase with the length of the constitutional CAG repeat (31,33). Gametic mosaicism has not been shown directly in females but similarities to the majority of male transmissions suggest that maternal instability is likely to occur during oogenesis.

To explore HD instability in an experimental animal, we have inserted a single CAG tract into the mouse germline to optimize detection of subtle repeat fluctuations in offspring. Mice harboring a single random integration of an *HD* exon 1 segment from a typical HD patient with 48 CAGs displayed CAG alterations in only ~2% of transmissions, indicating dramatic differences in CAG repeat instability between mouse and man. As an optimal test, we inserted CAG tracts of different lengths (18–109 units) into the appropriate genomic location, in exon 1 of the murine *HD* gene homolog, *Hdh* (39,40), creating a related set of genetically precise HD mouse models (41). These mice revealed CAG length-dependent intergenerational instability that reproduces many of the features of human transmissions but at longer repeat lengths.

## RESULTS

### *HD* exon 1 transgenic and *Hdh* CAG knock-in mice

HD CAG repeat instability typically constitutes small fluctuations in the modestly sized repeat tracts (40–50 units) that cause adult onset HD, although occasionally larger jumps occur giving rise to extreme repeats (~55–130 units) that trigger juvenile disease. To study this subtle pattern of repeat behavior we have generated lines of mice with single *HD* CAG tracts that can be followed accurately in progeny. Initially, we used embryonic stem (ES) cell technology to create lines of transgenic mice in which an *HD* exon 1 fragment with either 18 or 48 CAGs, typical of normal and HD chromosomes, respectively, is inserted at a random location in the mouse genome (Materials and Methods). Two of the lines from this experiment, $HD^{Tex1CAG18}$ and $HD^{Tex1CAG48}$, were shown by Southern blot analyses to possess a single copy of the *HD* exon 1 fragment with 18 and 48 CAGs, respectively (data not shown). The transgene was not designed as an expression cassette and immunoblot studies confirmed that neither line produces stable *HD* exon 1-encoded polypeptides, although RT–PCR analysis revealed very low levels of RNA product in most tissues of $HD^{Tex1CAG48}$ mice (data not shown).

Subsequently, to provide an accurate genetic model of the *HD* defect, we used homologous recombination in ES cells to create *Hdh* knock-in lines with distinct CAG tracts appropriately inserted in exon 1 of the murine *HD* homolog (41) on mouse chromosome 5 (39,40). Four knock-in lines from these experiments, $Hdh^{Q20}$, $Hdh^{Q50}$, $Hdh^{Q92}$ and $Hdh^{Q111}$, are precise genetic models of HD that accurately express mutant huntingtin (41; data not shown) and differ only by the length of the CAG repeat tract (18, 48, 90 and 109 CAGs, respectively).

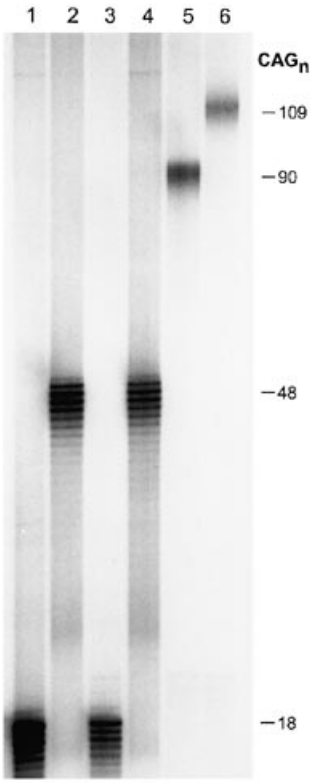


**Figure 1.** PCR amplification of the *HD* CAG repeat from *HD* exon 1 transgenic and *Hdh* CAG knock-in mice. DNA from tail clips obtained from typical $HD^{Tex1CAG18}$ (lane 1), $HD^{Tex1CAG48}$ (lane 2), $Hdh^{Q20}$ (lane 3), $Hdh^{Q50}$ (lane 4), $Hdh^{Q92}$ (lane 5) and $Hdh^{Q111}$ (lane 6) heterozygotes were used for PCR amplification of the *HD* CAG repeat (48). No PCR product is amplified from the endogenous mouse *Hdh* allele because its DNA sequence is not conserved in this region (39,40). The location of size standards of 18, 48, 90 and 109 CAGs, is indicated.

CAG repeat length can be monitored in each of the six *HD* transgenic and knock-in mouse lines by standard PCR amplification assays, developed for genotyping in man, that depend on oligonucleotide primer sets specific for the human exon 1 sequences flanking the repeat (3,42,43). As shown in Figure 1, the mobility of the PCR product obtained with tail clip DNA decreases with increasing CAG number, from 18 to 109 units, comprising in each case a single nested set of bands that allows assignment of repeat size relative to cloned, sequenced arrays. Because the adjacent CCG-rich segment is mildly polymorphic in man (44), we also assessed its size in members of each of the transgenic and knock-in mouse lines and found it to be unchanged at seven repeat units (data not shown).

### Inheritance of normal and adult onset *HD* CAG repeat lengths

We have monitored intergenerational CAG repeat instability in these *HD* transgenic and *Hdh* knock-in lines by directly

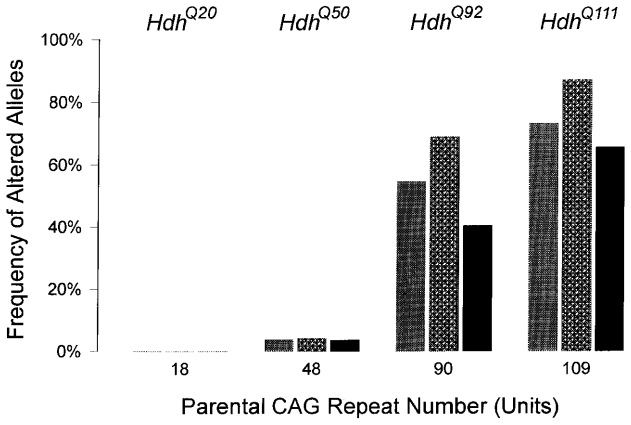


**Figure 2.** Frequency of altered *HD* CAG repeat alleles transmitted by *Hdh* knock-in parents to progeny. For each *Hdh* knock-in line, $Hdh^{Q20}$ (18), $Hdh^{Q50}$ (48), $Hdh^{Q92}$ (90) and $Hdh^{Q111}$ (109), the frequency of altered *HD* CAG alleles occurring during transmission from either mothers or fathers (gray bar), as well as maternally (hatched bar) and paternally (black bar) altered, 18, 48, 90 and 109 repeats is indicated. The proportion of altered alleles was: $Hdh^{Q20}$, 0/46 (0/26 female and 0/20 male); $Hdh^{Q50}$, 6/155 (2/47 female and 4/108 male); $Hdh^{Q92}$, 46/84 (29/42 female and 17/42 male); $Hdh^{Q111}$, 69/95 (27/31 female and 42/64 male).

comparing the length of the repeat tract in each mother or father with that observed in their progeny. These assessments were performed over a period of time on matings involving a few heterozygous males and females (three to four) of each line. Parental mice were of mixed 129SvEv/CD1 genetic background and were typically bred to CD1 partners, to examine transmissions from the same, rather than different, parents.

We initially studied the inheritance of 18 and 48 unit CAG repeat tracts from $HD^{Tex1CAG18}$ and $HD^{Tex1CAG48}$ transgenic mice and examined repeat size in a total of 110 and 102 transgene-positive offspring, respectively. No changes in the 18 CAG repeat were observed, whereas the 48 unit array was altered in two progeny. The differential stability of the normal and expanded *HD* CAG tracts is therefore reproduced in the mouse. However, the low frequency of instability of the 48 CAG array (2% of meioses) is in stark contrast to its high mutation rate (>80% of meioses) in man. This might indicate that the pathways leading to instability in man do not operate similarly in the mouse or that instability is suppressed by the site of transgene integration or some other peculiarity of the $HD^{Tex1CAG48}$ line. Alternatively, 48 CAGs may not be a good substrate for instability in the context of only a short, non-expressed 5′ segment of the *HD* gene.

To explore these scenarios, we assessed the inheritance of 18 and 48 repeat units located appropriately in exon 1 of the endogenous mouse locus (Fig. 2). In transmissions from $Hdh^{Q20}$ and $Hdh^{Q50}$ knock-in mice we observed no alterations in the small number of meioses involving the 18 CAG repeat that we monitored (0/59 events), whereas the 48 repeat tract changed in ~4% of meioses (6/155 events). These results are similar to the mutation frequencies observed in the transgenic mice, suggesting that the distinct behavior of 48 CAGs in mouse and man is not due to inherent differences in chromosomal context or transcriptional activity of the repeat.

## CAG length-dependent increase in mutation frequency

The consistent mild instability of 48 but not 18 CAGs in both the transgenic and knock-in models demonstrates a repeat length-dependent mechanism. The response of this mechanism to even longer CAG tracts was tested in transmissions from $Hdh^{Q92}$ and $Hdh^{Q111}$ knock-in mice. These displayed a striking, graded increase in instability (Fig. 2) to a level comparable with that exhibited by the majority of HD chromosomes in man. The 90 unit tract was altered about half the time (46/84 meioses) and 109 CAGs changed in 73% of offspring (69/95 meioses). In a proportion of the mice with altered 48, 90 and 109 CAG repeats, we also assessed the length of the adjacent CCG repeat stretch and did not observe changes to the seven CCGs present in each of the *Hdh* knock-in mice (data not shown). These findings clearly demonstrate that CAG repeat length is a primary determinant of instability in the mouse and that this instability occurs by a process that has no discernible impact on the downstream CCG-rich sequences.

## Sex of parent effect on frequency of instability

Examination of the data to determine whether the sex of the transmitting parent influences the mutation rate revealed a tendency for changes to occur more frequently when the repeat tract is inherited from females (Fig. 2). This effect is not apparent at 48 repeats, with about equal proportions of male and female transmissions resulting in changes (4/108 and 2/47 events, respectively), but is evident for both the 90 and 109 repeat arrays. For the $Hdh^{Q92}$ line, the mutation frequency in male transmissions was 41% (17/42 events) versus 69% (29/42 events) in female transmissions. For the longer array, alterations were observed in 66% (42/64 events) and 87% (27/31 events) of the progeny of heterozygous $Hdh^{Q111}$ males and females, respectively. These data demonstrate that the longer repeat tracts change in size more often when transmitted through the female rather than the male germline ($\chi^2 = 7.45$, 1 df, $P = 0.006$).

## Lack of parental age effect on frequency of instability

We also examined the data to determine whether the age of the dams and sires at the time of conception influenced the mutation frequency and found no clear evidence of an effect in either sex at the ages tested. Females were 2–17 months and bore two to three litters, while the males were 1–11 months of age and fathered two to five litters each. For example, the 90 CAG tract was altered about two thirds of the time when transmitted by an $Hdh^{Q92}$ female at ~6 (4/6 meioses), 7 (2/3 meioses) and 8 (5/6 meioses) months of age. An $Hdh^{Q92}$ male transmitted altered repeats about one third of the time when he was 3 (2/7 meioses) and 6 (2/6 meioses) months old. The majority of 109 repeat tracts transmitted by five $Hdh^{Q111}$ females were changed, whether pups were conceived when the mothers were 2 (8/10), 5 (3/3) or 16 (5/5) months of age. This array was altered ~60% of the time during transmissions from an $Hdh^{Q111}$ male at ~1 month (3/5 meioses) and from the same male at 4 months of age (4/6 meioses). Thus, in these experiments CAG repeat instability in the *Hdh* CAG knock-in mice is not dramatically influenced by the age of the parent. However, we have yet to test a large number of transmissions from either very young or elderly mice of each line.

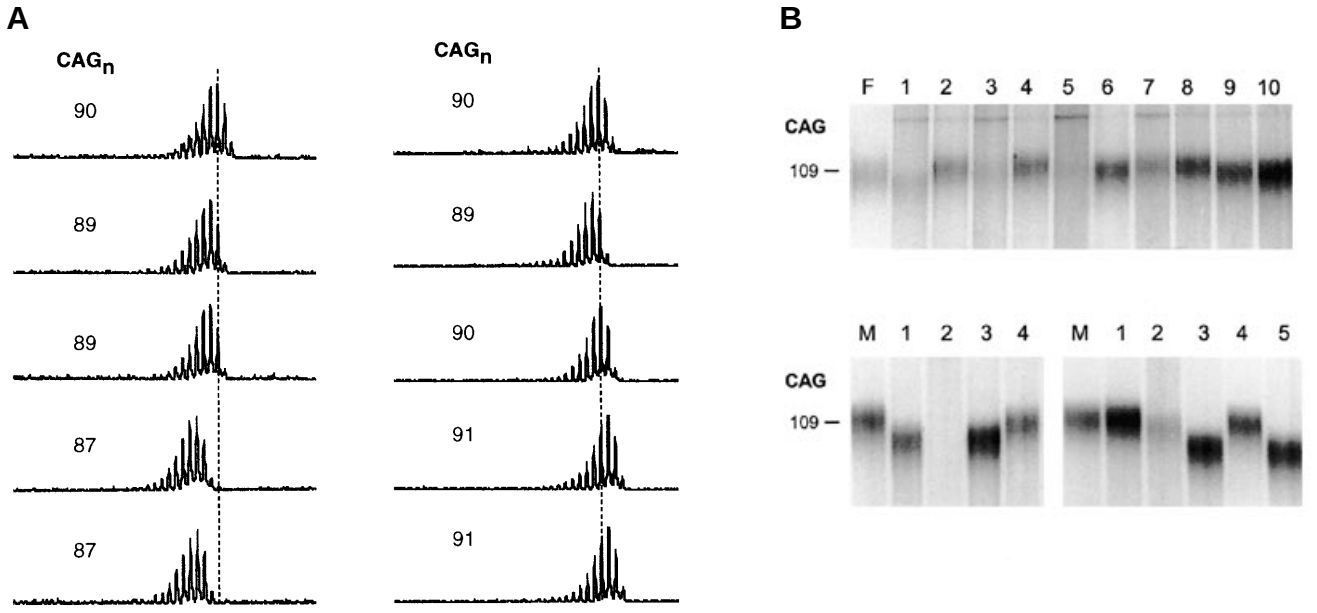


**Figure 3.** Relationship of *HD* CAG repeat size in $Hdh^{Q92}$ and $Hdh^{Q111}$ parents and corresponding progeny. (**A**) Examples of ABI 377 automated genotyping results obtained for PCR analysis of tail clip DNAs from an $Hdh^{Q92}$ mother (left) and father (right) and four of their progeny. The dashed vertical line in each trace indicates the position of the 90 CAG repeat size standard. In each sibship, the CAG allele size (units) in the parent (top trace) and each offspring (traces below) is indicated. (**B**) Examples of PCR products amplified and displayed using the radioactive format to ascertain transmission of the longer 109 CAG repeat tract from an $Hdh^{Q111}$ father (F) (top) and two mothers (M) (bottom) to offspring (numbered sequentially to the right of the parent). The position of the 109 CAG repeat size standard is indicated. The father possesses 108 CAGs, rather than 109, and transmitted alleles of 107 (progeny 1), 110 (2), 108 (3), 110 (4), 109 (6), 109 (7), 109 (8), 108 (9) and 108 (10) repeat units. No result was obtained on this gel for progeny 5. The first mother, with 109 CAGs, transmitted alleles of 104 (progeny 1), 105 (3) and 107 (4) repeats, with no PCR product amplified in the DNA of progeny 2. The second mother, also with 109 CAGs, transmitted alleles of 109 (progeny 1), 106 (2), 102 (3), 108 (4) and 101 (5) repeat units.

### Magnitude of repeat expansions and contractions

In examining the inheritance of the 48, 90 and 109 CAG arrays of the *Hdh* knock-in mice we identified a total of 121 altered arrays, 63 deriving from paternal transmissions and 58 produced in maternal transmissions. Typical examples of parents and their offspring are shown in Figure 3. The majority of cases involved subtle changes of only one or a few CAGs. In no case did we observe a large expansion akin to the dramatic jumps in size that are inherited from fathers in HD. Indeed, the largest expansion exhibited an increase of only three CAGs. Most contractions were of similar small magnitude, with the largest constituting a change of eight repeat units.

### Sex of parent effect on direction of change

While the magnitude of the size change was typically small, there was a strong bias toward either expansion or contraction determined by the sex of the transmitting parent (Fig. 4). Only six mutant alleles were produced in matings with $Hdh^{Q50}$ knock-in mice, four from paternal and two from maternal transmissions. The former were evenly split between increases and decreases of one CAG unit whereas the latter two maternal changes were both decreases of one and two CAGs. For the 90 CAG tract transmitted by $Hdh^{Q92}$ knock-in mice, the majority of paternally altered repeat tracts (11/17 events) were expansions of a single CAG, while the remaining third (6/17) involved the loss of one unit. In contrast, all but two of the arrays that changed in maternal transmissions were contractions of one to six repeat units (27/29), although increases of one and three CAGs were also identified. Our $Hdh^{Q111}$ breeding has involved predominantly inheritance of the 109 repeat tract from males. The vast majority of the paternally altered repeats (34/42 events) were increases of one or two units, with the remainder (8/42) being decreases of one or two CAGs. In the smaller number of female transmissions, almost all of the altered 109 arrays entailed the loss of one to eight repeats (24/27 events), while the three exceptions had increased by either one or two units. Consistent with the lack of an age effect on frequency of instability, there was no obvious age of parent effect in either sex on the magnitude or the direction of the change for any of the repeat sizes (data not shown).

### CAG repeat variability in somatic tissues of *Hdh* CAG knock-in mice

In HD, somatic CAG repeat variation is obvious only in the post-mortem brain and peripheral tissues of some juvenile onset cases with extreme CAG expansions (32,37). We observed no mosaicism in genotyping tail clip DNAs from any of the *Hdh* CAG knock-in mice. Similarly, when we examined the size of the repeat tract in DNAs extracted from the brains and peripheral organs of each line we found little or no somatic variability in the 48 CAG tract at 15 months of age (Fig. 5). However, male and female $Hdh^{Q92}$ and $Hdh^{Q111}$ mice at ~9 and 5 months of age, respectively, show mosaicism that is detectable in spleen and cortex but which is striking in liver and striatum. As shown in the examples in Figure 5, the broad PCR signal in the latter tissues suggests that the 90 and 109 repeat tracts both display a bias

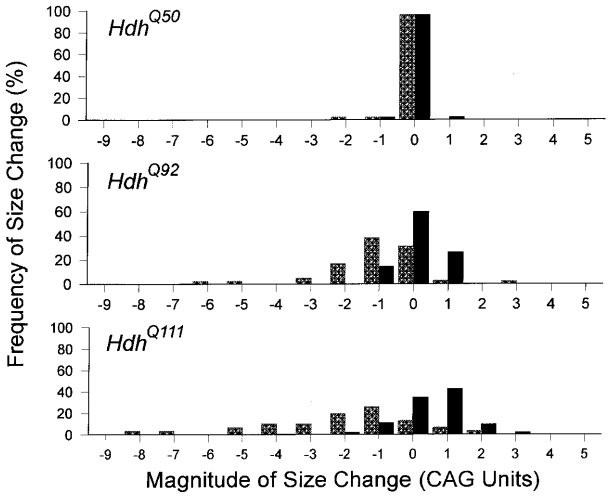


**Figure 4.** Size distribution of altered *HD* CAG repeats. The frequencies of maternally (hatched bar) and paternally (black bar) inherited size changes, varying in magnitude from a loss of eight units to a gain of three repeats, associated with the transmission of 48, 90 and 109 repeat units by $Hdh^{Q50}$, $Hdh^{Q92}$ and $Hdh^{Q111}$ knock-in mice, respectively, are shown. The number of maternally and paternally altered alleles was: $Hdh^{Q50}$, 4 and 2; $Hdh^{Q92}$, 17 and 29; $Hdh^{Q111}$, 27 and 42, respectively.

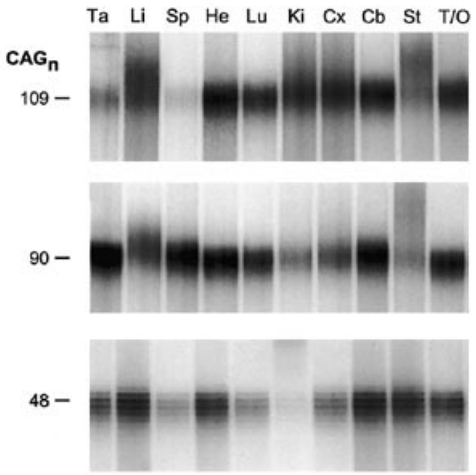


**Figure 5.** Comparison of *HD* CAG repeat length in somatic tissues of older *Hdh* knock-in mice. The *HD* CAG repeat was amplified by PCR from DNA samples extracted from tissues dissected from a single $Hdh^{Q111}$ male at 5 months of age (top), a 9-month-old $Hdh^{Q92}$ male (middle) and a $Hdh^{Q50}$ female mouse at 15 months of age (bottom). Ta, tail; Li, liver; Sp, spleen; He, heart; Lu, lung; Ki, kidney; Cx, cerebral cortex; Cb, cerebellum; St, striatum; T/O, testis or ovary. As PCR analyses were carried out using bulk tissue DNA, the modest changes in repeat size of the germ cells in the testis and ovary DNA samples are not apparent. In each case the position of the 48, 90 and 109 unit CAG size-standard is given. The band above the 48 unit PCR product in the kidney DNA appears inconsistently only in this sample and is due to contamination.

toward expansion, particularly apparent in the striatal DNA, which tends to be a smear of larger allele sizes. Remarkably, this dramatic pattern of somatic instability was not observed in female $Hdh^{Q92}$ and $Hdh^{Q111}$ mice at 5 weeks of age (data not shown). Thus, repeat instability occurs in select somatic tissues and is dependent on the length of the CAG tract and the age, but not the sex, of the mouse. Notably, we did not observe repeat tracts that were severely reduced in size in any of the tissues surveyed, including the cerebellum which, in some juvenile onset HD cases, exhibits a peculiar single allele dramatically smaller than that found in other tissues (32).

## DISCUSSION

We have demonstrated that two distinct HD mouse models which differ in the chromosomal location and expression of a typical adult onset HD CAG tract (48 units) both display similar low levels of CAG repeat instability, ~2–4% of meioses. Thus, in these mice, the precise genomic context of the array, including accurate transcription/translation, is not primary to the underlying mutational process(es). Indeed, by comparing the behavior of repeat tracts in a series of *Hdh* knock-in mice which differ only in the size of the array (18–109 units), we have established that the length of the CAG repeat itself is the major determinant of intergenerational instability. In addition, in agreement with previous studies (45–49), our data reveal that high mutation frequencies (>70% of meioses) are achieved only by large arrays, akin to those at the extreme high end of the range causing juvenile onset HD. Thus, the *HD* repeat, appropriately inserted into the mouse *HD* homolog, reveals length-dependent instability similar to that in man but operating at longer CAG repeat numbers.

Notably, adult and juvenile length HD CAG repeats in our knock-in mice yield levels of instability similar to those observed in a variety of transgenic mouse lines (45–49). CAG repeats of 45 and 54 units, in genomic DNA segments carrying the entire androgen receptor (*AR*) (45) and DM protein kinase (*DMPK*) (46) genes, respectively, are altered in ~7–10% of transmissions. In contrast, longer arrays of ~120–150 repeats in *HD* exon 1 (47) and *DMPK* 3′-UTR (48) transgenic mice change in ~60–70% of meioses. In addition, extremely high, age-dependent, mutation rates were observed upon female, but not male, transmission of an 82 CAG repeat tract in a spinocerebellar ataxia 1 (*SCA1*) cDNA transgenic line (49). These results, therefore, are consistent with the view that CAG repeat number, rather than *cis* or *trans* sequence elements (45–48) or transcriptional state (47), is the predominant determinant of instability. Moreover, as suggested previously (46), these data also support the notion that the underlying process(es) acts at a greater repeat length in mouse than in man.

The uniformity of the CAG repeat in the somatic tissues of young adult mice strongly argues that intergenerational instability occurs in gametogenesis. This notion is further supported by parental sex effects that are evident in a higher mutation frequency in females (by ~20%) and a strong bias toward male expansions and female contractions. Moreover, these data indicate that the fundamental mechanisms operating to produce gametic mosaicism in males and females may be distinct.

In contrast to reports of repeat instability in HD (47) and SCA 1 (49) transgenic mice with long CAG tracts, we did not observe a parental age effect in either male or female transmissions, suggesting a different behavior of the repeat arrays in *Hdh* knock-in mice. However, this feature merits further study as our parental males and females were well into adulthood, averaging 5–6 months of age, when the majority of their pups were conceived. The striking age-related increase in contractions of a long CAG repeat in SCA 1 transgenic mice is already evident in younger mothers (49). A similar female-specific pathway might be found to contribute to the higher mutation frequency and bias

toward contractions that we observed in female transmissions in *Hdh* knock-in mice when additional younger females are tested.

The distinctive age-dependent pattern of somatic mosaicism in the liver and striatum of $Hdh^{Q92}$ and $Hdh^{Q111}$ knock-in mice is also noteworthy. It is reminiscent of instability reported in the transgenic *HD* 140–160 CAG exon 1 mouse model, distinguished by expression of a truncated N-terminal huntingtin fragment which rapidly causes neurologic disease and death (47,50). In these mice, striking mosaicism in the liver and striatum, not seen in young mice, is severe in older mice, indicating that age is a factor (47). However, our *Hdh* knock-in mice do not rapidly develop disease or display evidence of abnormal cell proliferation, such as reactive gliosis (unpublished data), suggesting that the age-dependent instability is due to repeated rounds of DNA repair in post-mitotic cells, rather than proliferative alterations in the cell population. In any case, it is evident that the underlying mechanism(s) is critically dependent on CAG repeat length, as we did not readily detect mosaicism in *Hdh* knock-in mice with the shorter repeat tract. Thus, long CAG tracts may form structures that are targets for processes, active in liver and striatal cells, that produce DNA damage, leading to repeated rounds of DNA repair that, over time, yield a smear of CAG repeat sizes in these tissues.

Overall our results indicate both similarities and differences in the behavior of *HD* CAG repeat arrays in *Hdh* knock-in mice and HD patients. In both species, gametogenesis is the primary target of the underlying CAG length-dependent processes and distinct mechanisms are likely to produce instability in males and females. In addition, altered arrays transmitted from mothers and fathers are typically subtle variants of the parental repeat tract, with size increases tending to come from males. However, the two species exhibit different precise sensitivity to the length of the CAG tract. Extreme repeat numbers are required to achieve high levels of gametic instability in the mouse, where the impact of each additional repeat appears to be mild. This contrasts with the situation in man, where there is a steep increase in intergenerational instability going from CAG repeats in the normal range (~6–34 units) to those in the HD disease range (~44–48 units). Moreover, the large jumps in repeat size that characterize paternal transmission in HD do not appear to occur in the mouse. By the same token, female *Hdh* knock-in mice display higher levels of instability than males, although there is no readily apparent sex of parent effect on the frequency of instability in HD.

The direct comparison of *Hdh* knock-in mice and HD patients is complicated by numerous inherent distinctions, including both genetic and environmental factors, and some qualitative and quantitative differences are therefore to be expected. Even in man, some of these confounding variables, such as the multiplicity of distinct disease chromosomes and diversity of races and nationalities, limit the interpretation of many HD family studies. Our results suggest that the mechanism that generates extreme repeats during transmission from fathers, which explains some of the perplexing genetic features of HD, is apparently not shared by the knock-in mouse. However, the preponderance of small fluctuations in both species suggests the possibility of a common mechanism for the bulk of CAG repeat alterations. Indeed, relatively minor species-specific differences in a process common to both man and mouse could account for the apparent quantitative and qualitative differences in the patterns of repeat instability. Consequently, the *Hdh* CAG knock-in mouse, with its marked CAG length-sensitive intergenerational instability, provides a potential system in which to explore the fundamental processes underlying the most frequent alterations affecting HD disease alleles in man.

## MATERIALS AND METHODS

### Generation of *HD* exon 1 transgenic and *Hdh* knock-in mice

Mice transgenic for *HD* exon 1 possessing 18 and 48 CAG repeat units were created using *HD* exon 1 CAG18 and *HD* exon 1 CAG48, two clone constructs generated by inserting a 4.1 kb *Eco*RI restriction fragment of the *HD* gene (accession no. L34020) from a normal individual and a typical adult onset HD case, respectively, into pGEM7(KJ1)-Sal upstream of a pGKneo selection cassette. The number of CAG repeats in these clones was determined by DNA sequence analysis (51). In each case, the genomic fragment comprises *HD* exon 1, 3.6 kb of DNA sequence 5′ of the ATG initiation codon and 269 bp of downstream intron 1 sequence. These constructs were linearized at a unique *Sal*I restriction site and, in separate experiments, introduced into the R1 line of ES cells by electroporation (52). Selection of ES cell colonies in G418, extraction of DNA and Southern blot analyses were as previously described (41,53). DNA isolated from G418-resistant ES cell clones was assessed by Southern blot analysis to identify transformants with a single randomly integrated copy of either pGEM7(KJ1)Sal *HD* exon 1 CAG18 or pGEM7(KJ1)Sal *HD* exon 1 CAG48. The integrity of the *HD* exon 1 fragment was confirmed by restriction with *Eco*RI, while enzymes that do not cut within the constructs, *Xho*I, *Hin*dIII and *Eco*RV, permitted assessment of transgene copy number when blots were hybridized with a *neo*-specific hybridization probe (41) and a single copy probe, 191F1B10A, located at the extreme 5′-end of the *HD* gene fragment (accession no. L34020). Founder chimeric males were generated in aggregation experiments (53,54) and, in each case, two were bred with CD1 females to establish the $HD^{Tex1CAG18}$ and $HD^{Tex1CAG48}$ lines, maintained on a 129SvEv/CD1 mixed genetic background used in this study. Transgenic mice were subsequently identified by PCR analysis of the *HD* CAG repeat using standard protocols (3,42,43).

Generation of $Hdh^{Q20}$, $Hdh^{Q50}$, $Hdh^{Q92}$ and $Hdh^{Q111}$ knock-in mice with 18, 48, 90 and 109 consecutive CAG repeats, inserted in the appropriate position of the murine *HD* gene, by targeted replacement of *Hdh* exon 1 with a chimeric *Hdh*:*HD* exon 1, was accomplished using the ES cell-homologous recombination strategy described previously for $Hdh^{Q50}$ (41). The $Hdh^{Q20}$ and $Hdh^{Q50}$ lines were established from two germline transmitting founders while the $Hdh^{Q92}$ and $Hdh^{Q111}$ lines, in each case, were generated from a single germline transmitting male aggregation chimera. The presence of the appropriate targeted allele in each knock-in mouse was determined by specific Southern blot assay of tail clip DNA as described (41). For all the *Hdh* knock-in lines, the male founders were bred to CD1 females and the lines were maintained on a mixed 129SvEv/CD1 genetic background. Male and female parental mice were second or third generation from the germline transmitting founder. In each case, the length and uninterrupted nature of the CAG repeat was confirmed by DNA sequencing of PCR products amplified from tail clip DNAs. The polyglutamine stretch in the mutant huntingtin product, denoted in the name of the knock-in allele, is two residues longer than the number of CAG units in the repeat due to invariant, penultimate exon 1 CAA, CAG codons.

### RNA and protein analyses

Expression of the transgene in the $HD^{Tex1CAG18}$ and $HD^{Tex1CAG48}$ lines was assessed by RT–PCR of template RNA (DNase I-treated) extracted from brain, liver, kidney, spleen, heart and lung. First strand cDNA, synthesized using random hexamer oligonucleotide primers, was amplified by PCR using *HD* exon 1 primers (3,42,43) according to the manufacturer's protocol (RT–PCR kit; Stratagene). Immunoblot analysis of protein extracts from brain, liver, kidney, spleen, heart and lung of the *HD* exon 1 transgenic and *Hdh* knock-in mice were as described, using anti-huntingtin antisera that detect the N-terminus of huntingtin (HP12 and 1F8) (41).

### DNA extraction and PCR amplification of *HD* CAG repeats

Genomic DNA was isolated from tail biopsies taken at birth or at weaning and from tissues dissected from adult mice as described previously (41). *HD* CAG repeat length, for assessment of intergenerational and somatic instability, was determined using ~40 ng of genomic DNA in one of two specific PCR assays (3,42,43). The shorter repeats (18 and 48 units) were amplified with oligonucleotide primers CAG1 and CAG2, using incorporation of [$\alpha$-$^{32}$P]dGTP and displayed in a denaturing polyacrylamide gel as described (43). For the longer repeats (90 and 109 units) greater sensitivity was obtained by amplification with primers CAG1 and CAG3 (42) using previously reported conditions (47). For the 90 CAG stretch, primer CAG1 was fluorescently labeled with 6-FAM (Perkin Elmer) and products were displayed on an ABI 377 automated DNA sequencer (Applied Biosystems Division, Perkin Elmer) (48). For the 109 repeat segment, PCR products were labeled by incorporation of [$\alpha$-$^{32}$P]dGTP and displayed in a radioactive format (42).

### Determination of *HD* CAG repeat length

PCR products were resolved in denaturing 6% polyacrylamide gels and their sizes were determined relative to the migration of PCR products generated from parental DNAs and a set of DNA standards of known CAG repeat length, previously determined by DNA sequencing. For the 18, 48 and 109 repeat lengths, assessed in the two radioactive formats, a nested set of amplified products was obtained and the most intense PCR product, as determined by eye, was taken to represent the size of the repeat. For analyses of the $Hdh^{Q92}$ 90 repeat unit in the automated ABI377 format, Genescan and Genotyper software packages were used to assign repeat size and Genescan 500-TAMRA (Perkin Elmer) acted as an internal size standard in each lane. Because the size changes are modest, each DNA sample was assessed using five PCR reactions that typically produced the same allele size, determined as the highest peak of the Genescan trace. Maximum peaks differed by multiples of one CAG unit. Notably, for the $Hdh^{Q111}$ mice, the 109 CAG repeat was found to be highly unstable and some of the parents possessed alleles of 107 and 111 CAGs.

### Comparison with parental CAG repeat size

For all assay formats the *HD* CAG allele sizes of parents and offspring were compared directly by displaying PCR products amplified in the same experiments in adjacent lanes of the same gel. The size of the allele in offspring was determined relative to that of the parent using the highest peak in the Genescan traces or the most intense band(s) in the smear of radioactively labeled products and, in the latter, calling the alleles conservatively, i.e. differing the least from the parental alleles.

### Statistical analyses

Statistical analyses were performed using the Sigma Stat analysis package (Jandel Scientific), assessing differences in frequency of allele alterations by the $\chi^2$ test and associations between frequency or magnitude of instability and sex or age of parent by ANOVA.

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