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Transgenic mice harboring a full-length human mutant *DRPLA* gene exhibit age-dependent intergenerational and somatic instabilities of CAG repeats comparable with those in DRPLA patients

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Dentatorubral-pallidoluysian atrophy (DRPLA) is one among an increasing number of hereditary neurodegenerative diseases determined as being caused by unstable expansion of CAG repeats coding for polyglutamine stretches. To investigate the molecular mechanisms underlying CAG repeat instability, we established three transgenic lines each harboring a single copy of a full-length human mutant *DRPLA* gene carrying a CAG repeat expansion. These transgenic mice exhibited an age-dependent increase (+0.31 per year) in male transmission and an age-dependent contraction (–1.21 per year) in female transmission. Similar tendencies in intergenerational instabilities were also observed in human DRPLA parent–offspring pairs. The intergenerational instabilities of the CAG repeats may be interpreted as being derived from the instability occurring during continuous cell division of spermatogonia in the male, and that occurring during the period of meiotic arrest in the female. The transgenic mice also exhibited an age-dependent increase in the degree of somatic mosaicism which occurred in a cell lineage-dependent manner, with the size range of CAG repeats being smaller in the cerebellum than in other tissues including the cerebrum, consistent with observations in autopsied tissues of DRPLA patients. Thus, the transgenic mice described in this study exhibited age-dependent intergenerational as well as somatic instabilities of expanded CAG repeats comparable with those observed in human DRPLA patients, and are therefore expected to serve as good models for investigating the molecular mechanisms of instabilities of CAG repeats.

INTRODUCTION

Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disease caused by unstable expansion of CAG repeats in the *DRPLA* gene on chromosome 12p13.31 (1–3). Unstable expansion of CAG repeats has been

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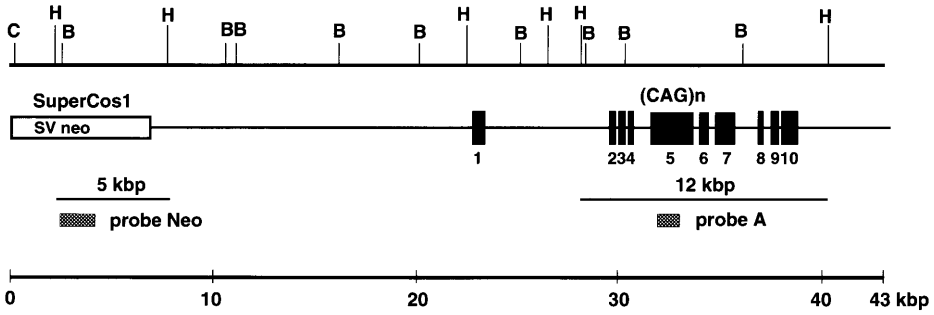

Figure 1. Schematic diagram illustrating the structure of the transgene (DRPLA18E), positions of probe A and Neo (stippled boxes) and *Hin*dIII restriction fragments (lines) used to identify the transgenic mice. DRPLA18E containing the entire mutant *DRPLA* gene was cloned by screening a cosmid genomic DNA library derived from a DRPLA patient. The exons of the *DRPLA* gene are depicted by solid boxes, and exon numbers are given below the boxes. Flanking regions and introns are depicted by lines. The restriction map for *Hin*dIII (H), *Bgl*II (B) and *Cla*I (C) is shown at the top of the figure. This construct includes regions flanking the *DRPLA* gene (17 kb upstream and 5 kb downstream) and the SuperCos1 cosmid vector containing the SV40 promoter and neomycin gene (open box).

identified as a mutation common to eight neurodegenerative diseases including spinal and bulbar muscular atrophy (SBMA), Huntington's disease (HD), spinocerebellar ataxia type 1 (SCA1), Machado–Joseph disease (MJD), spinocerebellar ataxia type 2 (SCA2), spinocerebellar ataxia type 6 (SCA6) and spinocerebellar ataxia type 7 (SCA7); many other neurodegenerative diseases are also speculated to be caused by similar mutations (4–12).

In these diseases, the CAG repeat instability is related intimately to various clinical and genetic features. First, inverse correlations exist between the size of expanded CAG repeats and the age at onset. Secondly, intergenerational increases in the sizes of expanded CAG repeats result in genetic anticipation and accelerated age at onset in successive generations. Thirdly, the broad spectra of clinical presentations are a function of the size of the expanded CAG repeats. The CAG repeat instability is observed not only in germline cells but also in somatic cells. The latter leads to somatic mosaicism resulting in considerable variations in the size and range of expanded CAG repeats among various tissues (13–17).

The *DRPLA* gene contains one of the most highly unstable CAG repeats identified, with intergenerational changes of $+5.81 \pm 0.92$ and $+1.25 \pm 1.55$ in paternal and maternal transmissions, respectively (18). As a result of the intergenerational instability of expanded CAG repeats, a mean acceleration of age at onset of 25.6 ± 2.4 and 14.0 ± 4.0 years per descendant has been observed in paternal and maternal transmissions, respectively (18). On the other hand, the involvement of somatic mosaicism in the pathogenesis of DRPLA remains to be determined, because no direct relationship between the degree of somatic mosaicism and the distribution of neuropathological changes in this disease has been established (15,16). Interestingly, it has been demonstrated that the expanded CAG repeats in the cerebellum exhibit the smallest degree of somatic mosaicism in HD, SCA1, MJD and DRPLA. Furthermore, we proposed that the degree of somatic mosaicism increases with age, on the basis of the analysis of somatic mosaicism in autopsied brains of DRPLA patients at various ages at death (16). These data raise the possibility that somatic mosaicism of expanded CAG repeats changes in a cell lineage-dependent and time-dependent manner.

Although the molecular mechanisms underlying CAG repeat instability are not well understood, it has been demonstrated by haplotype analysis that founder chromosomes are present in HD (19–21), MJD (22,23), DRPLA (24) and SCA7 chromosomes (25). The common haplotypes in HD, MJD and DRPLA are also shared with large CAG repeats within the normal range which represent intermediate alleles. Based on these observations, *cis*-elements in the genomic structure are presumed to be associated with CAG repeat instability. In addition, previous studies have shown that transgenic mice carrying cDNAs with expanded CAG repeats do not exhibit intergenerational instability (26–28). On the other hand, intergenerational instability has been observed in transgenic mice carrying a genomic fragment with expanded CAG repeats (29,30). These results also suggest that the genomic structure and the *cis*-elements in the genomic DNA are prerequisites for CAG repeat instability.

With this perspective, we determined that transgenic mice harboring an entire mutant gene including flanking regions derived from a mutant allele would be required to investigate the molecular mechanisms of CAG repeat instability. Generation of such mice has, however, not been successful due to difficulties in the cloning and introduction of the large genes for these diseases. On the other hand, the *DRPLA* gene spans only 20 kb (31), and the disease is among those such as SCA7 characterized by large intergenerational changes (18,32–34). Thus, we determined that the *DRPLA* gene was quite suitable for investigating the molecular mechanisms of CAG repeat instability, and generated transgenic mice harboring a single copy of a mutant *DRPLA* gene.

RESULTS

Intergenerational instability of CAG repeats in transgenic mice

We cloned 24 overlapping cosmid clones by screening a cosmid genomic DNA library constructed using the genomic DNA of a DRPLA patient. A cosmid clone (DRPLA18E) carrying a 36 kb genomic insert was selected for further analysis, because the clone contained the entire mutant *DRPLA* gene with 78 CAG repeats and the largest upstream region in the *DRPLA* gene (Fig. 1). Indeed, DRPLA18E included the genes for neuron-specific enolase 4.6 kb upstream of the *DRPLA* gene and for snRNA U7 1.3 kb downstream (35). This result confirmed that DRPLA18E contained the entire mutant *DRPLA* gene.

To generate transgenic mice harboring a single copy of a full-length human mutant *DRPLA* gene, we used embryonic stem (ES) cells as a route for transferring DRPLA18E. Five ES clones

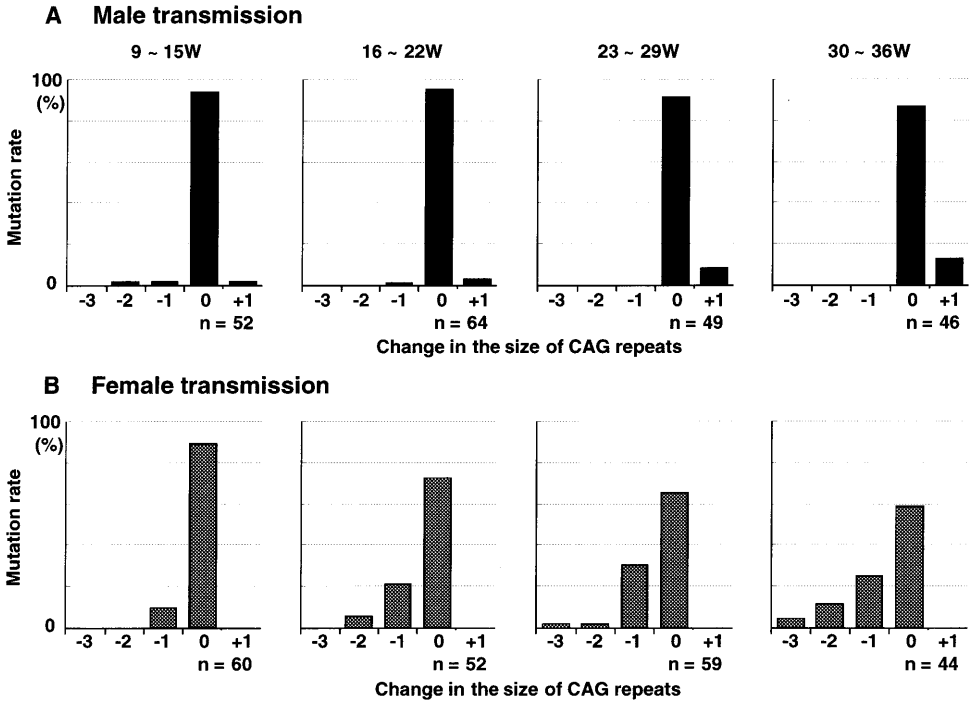

Figure 2. Mutation rates (%) per descendant in the Drm21 line. Parental ages at the time of birth of offspring are shown above, and numbers of transgenic offspring in each subgroup are shown below the histograms. (**A**) In male transmission, the expansion rate increased with age from 3.8 to 13%. (**B**) In female transmission, the contraction rate increased from 10 to 41%.

each carrying a single copy of the transgene were obtained from 24 independent G418-resistant clones. Further analysis with the probe Neo which was a 1321 bp *Ava*I and *Hin*dIII fragment of the SuperCos1 cosmid vector excluded the possibility of integration of a truncated insert including the neomycin gene or additional rearrangements (unpublished data). From these ES clones, we generated three transgenic lines, Drm12, Drm21 and Drm22, carrying 78, 76 and 77 CAG repeats, respectively. Although the expression of transgenes in the brain in each of the three lines was confirmed by RT–PCR analysis, these transgenic mice revealed no obvious phenotype for over a year (unpublished data).

Intergenerational changes in the sizes of expanded CAG repeats were analyzed mainly in the Drm21 line. The segregation ratios of transgenes were 51% in both male and female transmissions. In male transmission, expansion and contraction of CAG repeats were observed in 6.2 and 1.4% per descendant, respectively (n = 211). In female transmission, contraction was observed in 27% (n = 215), while expansion was not observed at all. The mean increases in the sizes of CAG repeats per generation were +0.043 ± 0.020 and –0.340 ± 0.043 in male and female transmissions, respectively. This parental bias in the intergenerational changes in the sizes of CAG repeats was highly significant ($P < 0.001$).

In order to investigate the effect of aging, the intergenerational changes were compared among four subgroups classified on the basis of parental ages at the birth of the offspring. Interestingly, the mutation rate per descendant increased with the parental age. In male transmission, the expansion rate increased with age from 3.8 to 13%. In contrast, in female transmission, the contraction rate increased from 10 to 41% (Fig. 2). Furthermore, these correlations between the intergenerational changes and parental ages were significant in both male ($rs = 0.16$, $P < 0.05$) and female ($rs = –0.29$, $P < 0.001$) transmissions (Fig. 3).

Intergenerational instability in DRPLA patients

To investigate if similar intergenerational instabilities underlie transmission of the mutant *DRPLA* gene in DRPLA patients, we analyzed 83 transmissions, including 56 paternal and 27 maternal transmissions, in 56 DRPLA pedigrees (Fig. 4). The intergenerational changes were +7.29 ± 0.69 (n = 56) and +1.37 ± 0.58 (n = 27) in paternal and maternal transmissions, respectively, and this difference between paternal and maternal transmissions was significant ($P < 0.001$). In paternal transmission, there was a significant correlation between the intergenerational changes and the paternal age ($r = 0.30$, $P < 0.05$). Furthermore, there was a significant increase in variance at paternal ages ≥35, compared with that at paternal ages < 35 ($P < 0.005$) and a similar increase in variance was observed from paternal ages 32–39. In maternal transmission, there was an increasing trend toward contraction of CAG repeats ($r = –0.19$), but this was not significant ($P = 0.338$). It should be noted, however, that contraction events were observed exclusively in maternal transmissions, suggesting that contraction events are phenomena highly specific to oogenesis.

Somatic mosaicism in transgenic mice

At the age of 64 weeks, genomic DNAs from various tissues were analyzed and the sizes and ranges of the CAG repeat expansions were determined in all three lines. Among the tissues, considerable variations in the size ranges of CAG repeats similarly were observed in both male and female mice of the Drm21 line (Fig.

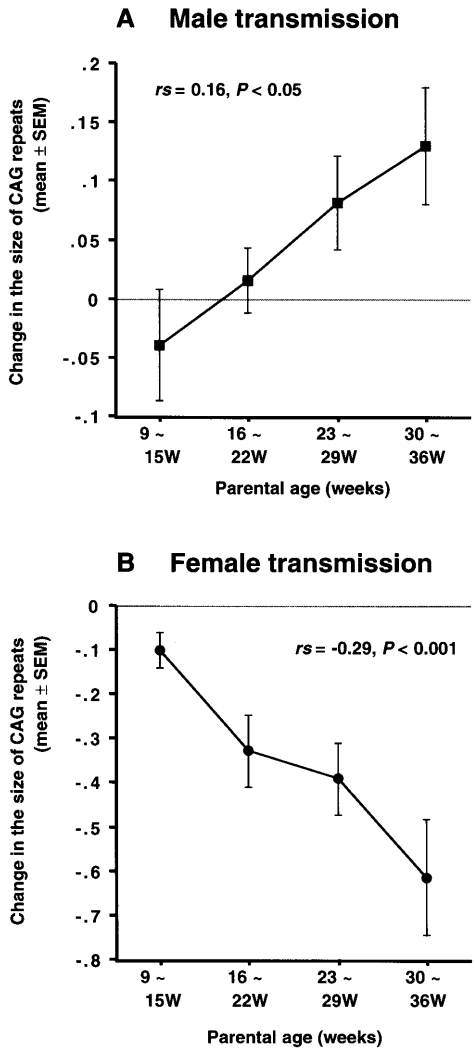

Figure 3. Relationships between the intergenerational changes in the sizes of CAG repeats and parental ages in the Drm21 line. Each value indicates the mean ± SEM for the subgroup corresponding to that in Figure 2. Significantly positive and inverse correlations were observed in male ($rs = 0.16$, $P < 0.05$) (**A**) and female ($rs = –0.29$, $P < 0.001$) (**B**) transmissions, respectively. The mean changes in the sizes of CAG repeats per year (regression coefficients) were +0.31 and –1.21 in male and female transmissions, respectively.

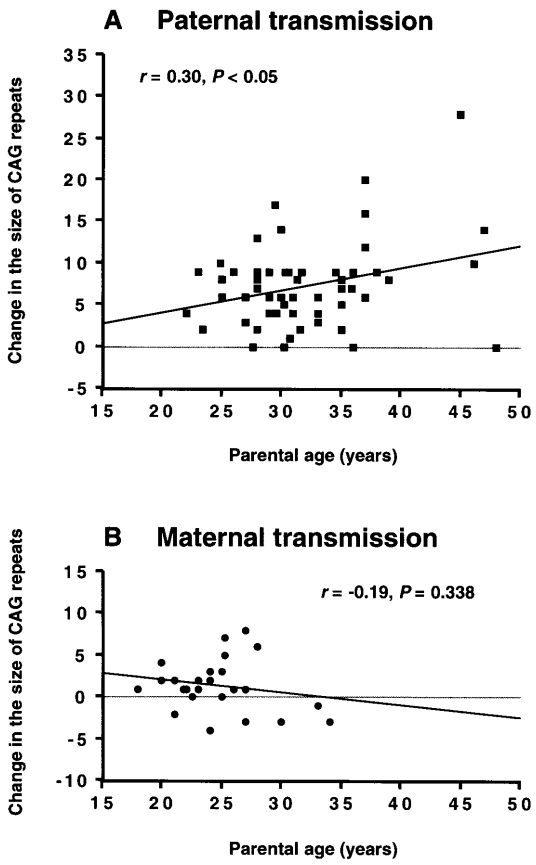

Figure 4. Relationships between the intergenerational changes in the sizes of expanded CAG repeats and parental ages in human DRPLA patients. There was a significant correlation between the intergenerational changes in the sizes of the expanded CAG repeats and the paternal age ($r = 0.30$, $P < 0.05$). The mean changes in the sizes of CAG repeats per year (regression coefficients) were +0.27 and –0.15 in paternal and maternal transmissions, respectively. Although there was a trend toward inverse correlation between the intergenerational changes in the sizes of expanded CAG repeats and the maternal age, it was not significant ($r = –0.19$, $P = 0.338$).

5A). In the other two lines, an identical pattern was observed reproducibly (unpublished data). Of particular note was the much smaller size range of CAG repeats in the cerebellum compared with the cerebrum and other somatic tissues. Similarly, a small size range of CAG repeats was observed in the heart. To evaluate the age-dependent changes in the sizes of CAG repeats, we analyzed the genomic DNAs from various tissues at the age of 3 weeks. The size ranges of CAG repeats were much smaller at 3 weeks than at 64 weeks, and no tissue-specific differences were observed in the size ranges at 3 weeks, suggesting that the degree of somatic mosaicism of the CAG repeats increases with age. To confirm this, the age-dependent increase in the somatic mosaicism of the CAG repeats was demonstrated in the tail DNAs obtained at 3 and 64 weeks from the same mice (Fig. 5B).

DISCUSSION

We established three transgenic lines each harboring a single copy of an entire human mutant *DRPLA* gene. These transgenic mice exhibited a strong parental bias with respect to the intergenerational changes in the sizes of CAG repeats. Furthermore, we observed somatic mosaicism which varied among tissues in an age-dependent manner, and the pattern was identical in the three lines. These data suggest that the instabilities of the CAG repeats are independent of the integration sites of the transgene.

The transgenic mice described in the present study exhibited CAG repeat instabilities comparable with those observed in DRPLA patients. First, these mice exhibited intergenerational instability of CAG repeats. Secondly, there was a significant difference in the intergenerational changes in the sizes of CAG repeats between male and female transmissions, with the male transmission being associated with a greater increase in the size of CAG repeats than female transmission. Thirdly, somatic mosaicism of CAG repeats was demonstrated. Particularly, the size range of CAG repeats in the cerebellum was much smaller than that in

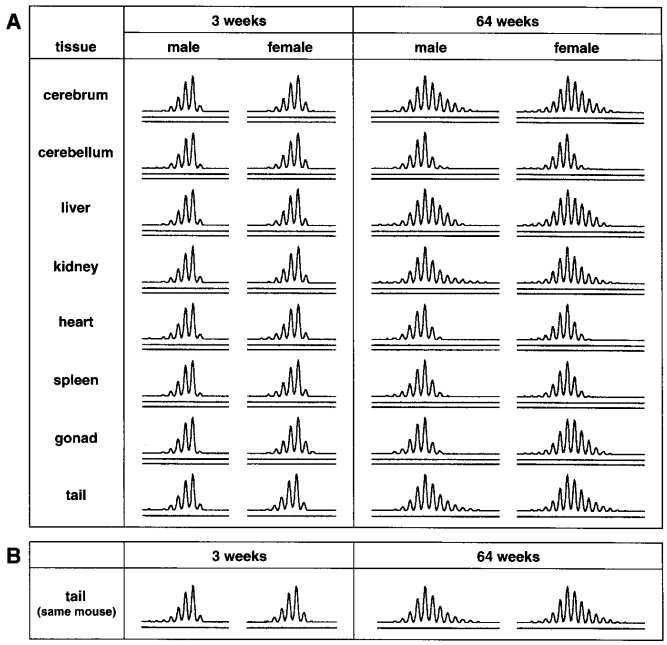

Figure 5. Somatic mosaicism in the Drm21 line. (**A**) Genescan traces of the CAG repeats from eight tissues of male and female mice at the ages of 3 and 64 weeks. At the age of 64 weeks, the size ranges of CAG repeats were much smaller in the cerebellum and heart compared with those in the cerebrum, liver, kidney, ovary and tail. In contrast, the size ranges of CAG repeats were much smaller at 3 weeks, with no tissue-specific differences in the size ranges. (**B**) The age-dependent changes in the sizes of CAG repeats were also observed in the tail DNAs obtained at 3 and 64 weeks from the same mice.

the cerebrum or other somatic tissues as determined in autopsied brains of DRPLA patients (16). Thus, the transgenic mice harboring a single copy of a human *DRPLA* gene used in this study more closely replicate the intergenerational as well as somatic instabilities of the expanded CAG repeats observed in human DRPLA patients than previously described transgenic mice. In the transgenic mice harboring a single copy of a full-length *SCA1* cDNA driven by a Purkinje cell-specific promoter (36), only contraction of the CAG repeats was observed in female transmission, while expansion of the CAG repeats and somatic mosaicism were not observed. Thus, our results emphasize the importance of the genomic structure for the expression of CAG repeat instabilities, especially the expansion of CAG repeats.

In the transgenic mice used in this study, there was a significant age-dependent increase in the intergenerational changes in the sizes of expanded CAG repeats similar to that described in one transgenic line for HD (29). To investigate whether the age-dependent increase in the intergenerational changes in the sizes of the CAG repeats observed in the transgenic mice is also observed in DRPLA patients, we analyzed 83 parent–offspring pairs of DRPLA patients (56 paternal and 27 maternal transmissions) and found a significant correlation between the intergenerational changes in the sizes of the expanded CAG repeats and the paternal age. Although an age-dependent increase in the degree of anticipation has been suspected previously based on clinical observations (37), this study is the first to demonstrate the age-dependent increase in the intergenerational changes in the sizes of the expanded CAG repeats in human CAG repeat diseases.

It is known that spermatogenesis continues throughout adult life; the spermatogenesis cycles are estimated to be 42 cycles per year in mice and 23 cycles per year in humans (38,39). As shown in Figure 3, the linear regression fits quite well with the age-dependent increase in the intergenerational instability of CAG repeats in transgenic mice. Furthermore, a similar age-dependent increase in the intergenerational instability of CAG repeats was confirmed in human paternal transmission. Based on the linear regression model and the continuous cell division required for spermatogenesis throughout adult life, the mean increase in the size of CAG repeats in mouse male transmission was calculated to be +0.31 per year and +0.0073 per spermatogenesis cycle. Interestingly, despite the fact that the actual sizes of the instabilities are very small in the transgenic mice, these values were comparable with those observed in DRPLA patients, which were calculated to be +0.27 and +0.012, respectively, on the basis of the aforementioned assumptions (Fig. 6). These results strongly suggest that the difference in the actual intergenerational changes between humans and mice is due to the different reproductive lifespans and that a common mechanism underlies the age-dependent increase in the sizes of CAG repeats in both humans and mice.

In contrast to spermatogenesis, considering that oogenesis in the mouse occurs only during fetal life and ceases at the diplotene stage of the first meiotic prophase by 5 days after birth (40), the aforementioned age-dependent contraction of CAG repeats is considered to occur after the cessation of meiotic DNA replication (Fig. 6). Similar observations have been made in mice transgenic for SCA1 and SBMA (30,36). These results strongly suggest that contraction of the CAG repeats occurs during the prolonged resting stage, and mechanisms such as repair of damaged DNA or selective degeneration of the primary oocyte with larger CAG repeats might be involved in the contraction process. Therefore, we would need to consider two processes to explain CAG repeat instability in female transmission: (i) instability in successive cell division from the zygote to primary oocyte and (ii) instability in the prolonged resting stage. Given the age-dependent contraction of the CAG repeats in female transmission, the latter process is considered to be involved in the contraction of the CAG repeats in transgenic mice. Although there is no direct evidence, one hypothesis is that successive cell division from the zygote to the primary oocyte may result in the expansion of CAG repeats. In human maternal transmission, an intergenerational change in size of $+1.37 \pm 0.58$ ($n = 27$) was observed, while in the female transmission of the transgenic mice, it was -0.36 ± 0.043 ($n = 215$). Since the number of cell divisions occurring between the zygote and primary oocyte stages is much larger in humans than in mice, this difference could account for the slightly increased intergenerational change of the CAG repeats observed in human maternal transmission. Interestingly, this trend toward age-dependent contraction was also suggested in the case of maternal transmission in DRPLA patients (Fig. 4), although it did not reach a statistically significant level. To explore this possibility further, a much greater number of cases resulting from maternal transmission will be required, but this is difficult in practice due to the low prevalence rate of DRPLA and the relatively narrow reproductive span in female DRPLA patients in contrast to the broad reproductive span in male DRPLA patients.

In conclusion, the present study clearly demonstrates that different mechanisms for CAG repeat instability are involved

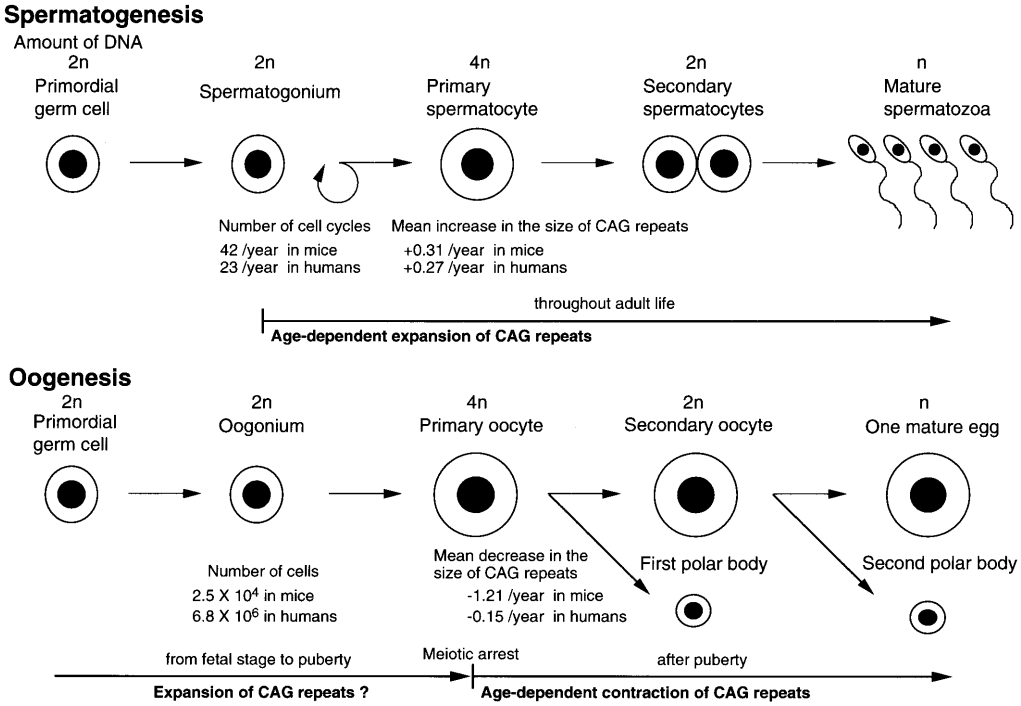

Figure 6. Gametogenesis and CAG repeat instability. The number of cell divisions required to produce a spermatozoon is much larger than that required to produce an egg, which could account for the difference in the intergenerational instability between male and female transmissions. During spermatogenesis, spermatogonia, as stem cells, undergo continuous cell division throughout adult life at 42 cycles per year in mice and 23 cycles per year in humans. Considering the number of cell divisions per year, the mean changes in the sizes of CAG repeats per year were calculated to be +0.31 (+0.0073 per spermatogenesis cycle) and +0.27 (+0.012 per spermatogenesis cycle) in Drm21 mice and DRPLA patients, respectively. In the case of oogenesis, primordial germ cells continuously divide to produce oogonia during fetal life and give rise to the maximum populations of 2.5×10^4 ($\approx 2^{15}$) and 6.8×10^6 ($\approx 2^{23}$) in mice and humans, respectively. Up to puberty, all female germ cells (primary oocytes) containing a tetraploid amount of DNA (4n) arrested at the diplotene stage of the first meiotic prophase are in the prolonged resting stage. Thus the aforementioned observations suggest that age-dependent contraction of CAG repeats occurs after the cessation of meiotic DNA replication, especially during the prolonged resting stage. In addition, expansion of CAG repeats may occur during the process of cell division taking place between the zygote and primary oocyte stages.

during spermatogenesis and oogenesis, and also that common mechanisms are involved in humans and mice.

This study also demonstrated somatic instabilities of CAG repeats, comparable with those observed in DRPLA patients. First, the size range of the CAG repeats was smaller in the cerebellum than in the cerebrum and various somatic tissues. This observation has been well documented in HD, SCA1, MJD and DRPLA (13–17). Since the cerebellum contains a dense population of granule cells which are neurons, it is assumed that neurons exhibit the lowest instability because they do not undergo cell division and that cell division is required for the development of somatic instabilities of CAG repeats (16). Similar phenomena were observed in the granular layers of the cerebellar cortex and hippocampal formation in autopsied DRPLA brains (17). Interestingly, heart muscle also exhibited a small size range of CAG repeats in the transgenic mice. The postmitotic nature of heart muscle cells could account for this phenomenon. To confirm that neurons show the lowest variability in CAG repeat size, direct analysis of individual neurons would be required. Another interesting finding of the present study is the age-dependent increase in the degree of somatic mosaicism. As shown in Figure 5, the size ranges of CAG repeats were much larger at 64 weeks compared with those at 3 weeks. In our previous study on somatic mosaicism in DRPLA patients, we found that the size range of CAG repeats exhibited a strong correlation with the age at death. Based on these findings, we proposed the hypothesis that the degree of somatic mosaicism increases with the patients' age (16). The results of our study strongly support this hypothesis.

It has been clearly demonstrated that transgenic mice harboring a single copy of an entire human mutant *DRPLA* gene carrying an expanded CAG repeat show age-dependent intergenerational as well as somatic instabilities of CAG repeats. Since instabilities of expanded CAG repeats in transgenic mice are comparable with those observed in human DRPLA patients, such transgenic mice are expected to be good models for exploring the molecular mechanisms that underlie instabilities of CAG repeats.

MATERIALS AND METHODS

Cloning of the mutant *DRPLA* gene

A high molecular weight genomic DNA fragment extracted from a lymphoblastoid cell line of a DRPLA patient with 79 CAG repeats was partially digested with *Sau*3AI and size-fractionated by pulse-field agarose gel electrophoresis. The genomic DNA fragments ranging in size from 30 to 42 kb were purified from the gel by electroelution, dephosphorylated by alkaline phosphatase and ligated into the *Bam*HI-cleaved SuperCos1 cosmid vector (Stratagene). The ligation products were *in vitro* packaged using Gigapack III gold packaging extract (Stratagene) and propagated through *Escherichia coli* XL1-Blue MR. Cosmid clones carrying the mutant *DRPLA* gene were isolated by colony hybridization

using a fragment of the *DRPLA* cDNA as the probe (probe A in Fig. 1), which was the 648 bp *Acc*I–*Eco*RI fragment of human *DRPLA* cDNA (41).

Generation of transgenic mice

A cosmid clone (DRPLA18E) containing the entire mutant *DRPLA* gene with 78 CAG repeats was linearized by *Cla*I digestion. Ten micrograms of the linearized cosmid DNA was transferred into 1×10^7 ES cells (CCE) by electroporation as described previously (42). The copy numbers of transgenes were determined by Southern blot analysis of *Hin*dIII-digested ES cell DNAs using probe A, followed by densitometric quantitation of the bands derived from the transgenes compared with that of the endogenous mouse *DRPLA* gene on the autoradiograms using a Fuji Bioimaging Analyzer BAS2000. ES cells containing a single copy of the transgene were selected and injected into C57BL/6J blastocysts as described previously (42). Transgenic mice were generated from chimeric founders through germlines. Each of the hemizygous transgenic offspring, 12 male and 12 female mice, were mated with C57BL/6J mice and the intergenerational changes in the sizes of expanded CAG repeats were analyzed in both male and female transmissions. Somatic mosaicism was analyzed using genomic DNAs extracted from various tissues at the ages of 3 and 64 weeks.

Analysis of the CAG repeat expansion

Mouse genomic DNAs were extracted from various tissues using the QIAamp Tissue Kit (Qiagen). The CAG repeat size was determined by PCR using previously described methods with minor modifications (2,16). Briefly, PCR was performed in a total volume of 30 µl containing 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 2.0 M *N,N,N*-trimethylglycine, 250 µM dNTP and 10 pmol of each primer with one primer labeled with FAM. The PCR products were electrophoresed through denaturing 6% polyacrylamide gels. The GS500 TAMRA-labeled size standard (Perkin Elmer) and PCR products obtained by amplification of a mouse genomic DNA carrying 76 CAG repeats with the primer labeled with TET were also run simultaneously in the same lanes. The CAG repeat size was determined using the ABI 377 DNA sequencer and Genescan version 2.1 software.

Analysis of intergenerational changes in DRPLA parent–offspring pairs

We analyzed 56 paternal and 27 maternal transmissions in 36 and 20 DRPLA pedigrees, respectively. The diagnosis of DRPLA was made on the basis of molecular testing. Informed consent was obtained from all of the subjects. The sizes of expanded CAG repeats were determined as described previously (2,32).

Statistical analysis

Mann–Whitney *U* test and Spearman rank correlation analysis were used to determine the significances of differences between male and female transmissions and the correlations between the intergenerational changes and parental ages, respectively. For the analyses of correlation and variance in human subjects, Pearson's correlation analysis and *F* test were used, respectively. These statistical analyses were performed using the SPSS version 6.01 for Windows. A *P*-value <0.05 was considered statistically significant.

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REFERENCES

1. Naito, H. and Oyanagi, S. (1982) Familial myoclonus epilepsy and choreoathetosis: hereditary dentatorubral-pallidoluysian atrophy. *Neurology*, **32**, 798–807.
2. Koide, R., Ikeuchi, T., Onodera, O., Tanaka, H., Igarashi, S., Endo, K., Takahashi, H., Kondo, R., Ishikawa, A., Hayashi, T., Saito, M., Tomoda, A., Miike, T., Naito, H., Ikuta, F. and Tsuji, S. (1994) Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). *Nature Genet.*, **6**, 9–13.
3. Nagafuchi, S., Yanagisawa, H., Sato, K., Shirayama, T., Ohsaki, E., Bundo, M., Takeda, T., Tadokoro, K., Kondo, I., Murayama, N., Tanaka, Y., Kikushima, H., Umino, K., Kurosawa, H., Furukawa, T., Nihei, K., Inoue, T., Sano, A., Komure, O., Takahashi, M., Yoshizawa, T., Kanazawa, I. and Yamada, M. (1994) Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG trinucleotide on chromosome 12p. *Nature Genet.*, **6**, 14–18.
4. La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature*, **352**, 77–79.
5. Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, **72**, 971–983.
6. Orr, H.T., Chung, M.-y., Banfi, S., Kwiatkowski, T.J. Jr., Servadio, A., Beaudet, A.L., McCall, A.E., Duvick, L.A., Ranum, L.P.W. and Zoghbi, H.Y. (1993) Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nature Genet.*, **4**, 221–226.
7. Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S. and Kakizuka, A. (1994) CAG expansions in a novel gene for Machado–Joseph disease at chromosome 14q32.1. *Nature Genet.*, **8**, 221–228.
8. Pulst, S.-M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X.-N., Lopes-Cendes, I., Pearlman, S., Starkman, S., Orozco-Diaz, G., Lunkes, A., DeJong, P., Rouleau, G.A., Auburger, G., Korenberg, J.R., Figueroa, C. and Sahba, S. (1996) Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. *Nature Genet.*, **14**, 269–276.
9. Sanpei, K., Takano, H., Igarashi, S., Sato, T., Oyake, M., Sasaki, H., Wakisaka, A., Tashiro, K., Ishida, Y., Ikeuchi, T., Koide, R., Saito, M., Sato, A., Tanaka, T., Hanyu, S., Takiyama, Y., Nishizawa, M., Shimizu, N., Nomura, Y., Segawa, M., Iwabuchi, K., Eguchi, I., Tanaka, H., Takahashi, H. and Tsuji, S. (1996) Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, DIRECT. *Nature Genet.*, **14**, 277–284.
10. Imbert, G., Saudou, F., Yvert, G., Devys, D., Trottier, Y., Garnier, J.-M., Weber, C., Mandel, J.-L., Cancel, G., Abbas, N., Dürr, A., Didierjean, O., Stevanin, G., Agid, Y. and Brice, A. (1996) Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. *Nature Genet.*, **14**, 285–291.

11. Zhuchenko, O., Bailey, J., Bonnen, P., Ashizawa, T., Stockton, D.W., Amos, C., Dobyns, W.B., Subramony, S.H., Zoghbi, H.Y. and Lee, C.C. (1997) Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the α_{1A}-voltage-dependent calcium channel. *Nature Genet.*, **15**, 62–69.
12. David, G., Abbas, N., Stevanin, G., Dürr, A., Yvert, G., Cancel, G., Weber, C., Imbert, G., Saudou, F., Antoniou, E., Drabkin, H., Gemmill, R., Giunti, P., Benomar, A., Wood, N., Ruberg, M., Agid, Y., Mandel, J.-L. and Brice, A. (1997) Cloning of the *SCA7* gene reveals a highly unstable CAG repeat expansion. *Nature Genet.*, **17**, 65–70.
13. Telenius, H., Kremer, B., Goldberg, Y.P., Theilmann, J., Andrew, S.E., Zeisler, J., Adam, S., Greenberg, C., Ives, E.J., Clarke, L.A. and Hayden, M.R. (1994) Somatic and gonadal mosaicism of the Huntington disease gene CAG repeat in brain and sperm. *Nature Genet.*, **6**, 409–414.
14. Chong, S.S., McCall, A.E., Cota, J., Subramony, S.H., Orr, H.T., Hughes, M.R. and Zoghbi, H.Y. (1995) Gametic and somatic tissue-specific heterogeneity of the expanded *SCA1* CAG repeat in spinocerebellar ataxia type 1. *Nature Genet.*, **10**, 344–350.
15. Ueno, S., Kondoh, K., Kotani, Y., Komure, O., Kuno, S., Kawai, J., Hazama, F. and Sano, A. (1995) Somatic mosaicism of CAG repeat in dentatorubral-pallidoluysian atrophy (DRPLA). *Hum. Mol. Genet.*, **4**, 663–666.
16. Takano, H., Onodera, O., Takahashi, H., Igarashi, S., Yamada, M., Oyake, M., Ikeuchi, T., Koide, R., Tanaka, H., Iwabuchi, K. and Tsuji, S. (1996) Somatic mosaicism of expanded CAG repeats in brains of patients with dentatorubral-pallidoluysian atrophy: cellular population-dependent dynamics of mitotic instability. *Am. J. Hum. Genet.*, **58**, 1212–1222.
17. Hashida, H., Goto, J., Kurisaki, H., Mizusawa, H. and Kanazawa, I. (1997) Brain regional differences in the expansion of a CAG repeat in the spinocerebellar ataxias: dentatorubral-pallidoluysian atrophy, Machado–Joseph disease, and spinocerebellar ataxia type 1. *Ann. Neurol.*, **41**, 505–511.
18. Ikeuchi, T., Onodera, O., Oyake, M., Koide, R., Tanaka, H. and Tsuji, S. (1995) Dentatorubral-pallidoluysian atrophy (DRPLA): close correlation of CAG repeat expansions with the wide spectrum of clinical presentations and prominent anticipation. *Semin. Cell Biol.*, **6**, 37–44.
19. Squitieri, F., Andrew, S.E., Goldberg, Y.P., Kremer, B., Spence, N., Zeisler, J., Nichol, K., Theilmann, J., Greenberg, J., Goto, J., Kanazawa, I., Vesa, J., Peltonen, L., Almqvist, E., Anvret, M., Telenius, H., Lin, B., Napolitano, G., Morgan, K. and Hayden, M.R. (1994) DNA haplotype analysis of Huntington disease reveals clues to the origins and mechanisms of CAG expansion and reasons for geographic variations of prevalence. *Hum. Mol. Genet.*, **3**, 2103–2114.
20. Rubinsztein, D.C., Leggo, J., Goodburn, S., Barton, D.E. and Ferguson-Smith, M.A. (1995) Haplotype analysis of the Δ2642 and $(CAG)_n$ polymorphisms in the Huntington's disease (HD) gene provides an explanation for an apparent 'founder' HD haplotype. *Hum. Mol. Genet.*, **4**, 203–206.
21. Almqvist, E., Spence, N., Nichol, K., Andrew, S.E., Vesa, J., Peltonen, L., Anvret, M., Goto, J., Kanazawa, I., Goldberg, Y.P. and Hayden, M.R. (1995) Ancestral differences in the distribution of the Δ2642 glutamic acid polymorphism is associated with varying CAG repeat lengths on normal chromosomes: insights into the genetic evolution of Huntington disease. *Hum. Mol. Genet.*, **4**, 207–214.
22. Takiyama, Y., Igarashi, S., Rogaeva, E.A., Endo, K., Rogaev, E.I., Tanaka, H., Sherrington, R., Sanpei, K., Liang, Y., Saito, M., Tsuda, T., Takano, H., Ikeda, M., Lin, C., Chi, H., Kennedy, J.L., Lang, A.E., Wherrett, J.R., Segawa, M., Nomura, Y., Yuasa, T., Weissenbach, J., Yoshida, M., Nishizawa, M., Kidd, K.K., Tsuji, S. and St George-Hyslop, P.H. (1995) Evidence for inter-generational instability in the CAG repeat in the *MJD1* gene and for conserved haplotypes at flanking markers amongst Japanese and Caucasian subjects with Machado–Joseph disease. *Hum. Mol. Genet.*, **4**, 1137–1146.
23. Stevanin, G., Cancel, G., Didierjean, O., Dürr, A., Abbas, N., Cassa, E., Feingold, J., Agid, Y. and Brice, A. (1995) Linkage disequilibrium at the Machado–Joseph disease/spinal cerebellar ataxia 3 locus: evidence for a common founder effect in French and Portuguese–Brazilian families as well as a second ancestral Portuguese–Azorean mutation. *Am. J. Hum. Genet.*, **57**, 1247–1250.
24. Yanagisawa, H., Fujii, K., Nagafuchi, S., Nakahori, Y., Nakagome, Y., Akane, A., Nakamura, M., Sano, A., Komure, O., Kondo, I., Jin, D.K., Sørensen, S.A., Potter, N.T., Young, S.R., Nakamura, K., Nukina, N., Nagao, Y., Tadokoro, K., Okuyama, T., Miyashita, T., Inoue, T., Kanazawa, I. and Yamada, M. (1996) A unique origin and multistep process for the generation of expanded DRPLA triplet repeats. *Hum. Mol. Genet.*, **5**, 373–379.
25. Johansson, J., Forsgren, L., Sandgren, O., Brice, A., Holmgren, G. and Holmberg, M. (1998) Expanded CAG repeats in Swedish spinocerebellar ataxia type 7 (SCA7) patients: effect of CAG repeat length on the clinical manifestation. *Hum. Mol. Genet.*, **7**, 171–176.
26. Bingham, P.M., Scott, M.O., Wang, S., McPhaul, M.J., Wilson, E.M., Garbern, J.Y., Merry, D.E. and Fischbeck, K.H. (1995) Stability of an expanded trinucleotide repeat in the androgen receptor gene in transgenic mice. *Nature Genet.*, **9**, 191–196.
27. Goldberg, Y.P., Kalchman, M.A., Metzler, M., Nasir, J., Zeisler, J., Graham, R., Koide, H.B., O'Kusky, J., Sharp, A.H., Ross, C.A., Jirik, F. and Hayden, M.R. (1996) Absence of disease phenotype and intergenerational stability of the CAG repeat in transgenic mice expressing the human Huntington disease transcript. *Hum. Mol. Genet.*, **5**, 177–185.
28. Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S. and Kakizuka, A. (1996) Expanded polyglutamine in the Machado–Joseph disease protein induces cell death *in vitro* and *in vivo*. *Nature Genet.*, **13**, 196–202.
29. Mangiarini, L., Sathasivam, K., Mahal, A., Mott, R., Seller, M. and Bates, G.P. (1997) Instability of highly expanded CAG repeats in mice transgenic for the Huntington's disease mutation. *Nature Genet.*, **15**, 197–200.
30. La Spada, A.R., Peterson, K.R., Meadows, S.A., McClain, M.E., Jeng, G., Chmelar, R.S., Haugen, H.A., Chen, K., Singer, M.J., Moore, D., Trask, B.J., Fischbeck, K.H., Clegg, C.H. and McKnight, G.S. (1998) Androgen receptor YAC transgenic mice carrying CAG 45 alleles show trinucleotide repeat instability. *Hum. Mol. Genet.*, **7**, 959–967.
31. Nagafuchi, S., Yanagisawa, H., Ohsaki, E., Shirayama, T., Tadokoro, K., Inoue, T. and Yamada, M. (1994) Structure and expression of the gene responsible for the triplet repeat disorder, dentatorubral and pallidoluysian atrophy (DRPLA). *Nature Genet.*, **8**, 177–182.
32. Komure, O., Sano, A., Nishino, N., Yamauchi, N., Ueno, S., Kondoh, K., Sano, N., Takahashi, M., Murayama, N., Kondo, I., Nagafuchi, S., Yamada, M. and Kanazawa, I. (1995) DNA analysis in hereditary dentatorubral-pallidoluysian atrophy: correlation between CAG repeat length and phenotypic variation and the molecular basis of anticipation. *Neurology*, **45**, 143–149.
33. David, G., Dürr, A., Stevanin, G., Cancel, G., Abbas, N., Benomar, A., Belal, S., Lebre, A.-S., Abada-Bendib, M., Grid, D., Holmberg, M., Yahyaoui, M., Hentati, F., Chkili, T., Agid, Y. and Brice, A. (1998) Molecular and clinical correlations in autosomal dominant cerebellar ataxia with progressive macular dystrophy (SCA7). *Hum. Mol. Genet.*, **7**, 165–170.
34. Gouw, L.G., Castañeda, M.A., McKenna, C.K., Digre, K.B., Pulst, S.-M., Perlman, S., Lee, M.S., Gomez, C., Fischbeck, K., Gagnon, D., Storey, E., Bird, T., Jeri, F.R. and Ptácek, L.J. (1998) Analysis of the dynamic mutation in the SCA7 gene shows marked parental effects on CAG repeat transmission. *Hum. Mol. Genet.*, **7**, 525–532.
35. Ishida, Y., Sato, T., Takano, H. and Tsuji, S. (1996) Identification of neuron-specific enolase (NSE) gene near the dentatorubral-pallidoluysian atrophy (DRPLA) gene and its implication in neuronal degeneration. *Am. J. Hum. Genet.*, **59** (suppl.), A265.
36. Kaytor, M.D., Burright, E.N., Duvick, L.A., Zoghbi, H.Y. and Orr, H.T. (1997) Increased trinucleotide repeat instability with advanced maternal age. *Hum. Mol. Genet.*, **6**, 2135–2139.
37. Farrer, L.A., Cupples, L.A., Kiely, D.K., Conneally, P.M. and Myers, R.H. (1992) Inverse relationship between age at onset of Huntington disease and paternal age suggests involvement of genetic imprinting. *Am. J. Hum. Genet.*, **50**, 528–535.
38. Oakberg, E.F. (1956) A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.*, **99**, 391–413.
39. Strachan, T. and Read, A.P. (1996) *Human Molecular Genetics*. BIOS Scientific Publishers, Oxford.
40. Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
41. Onodera, O., Oyake, M., Takano, H., Ikeuchi, T., Igarashi, S. and Tsuji, S. (1995) Molecular cloning of a full-length cDNA for dentatorubral-pallidoluysian atrophy and regional expressions of the expanded alleles in the CNS. *Am. J. Hum. Genet.*, **57**, 1050–1060.
42. Gondo, Y., Nakamura, K., Nakao, K., Sasaoka, T., Ito, K., Kimura, M. and Katsuki, M. (1994) Gene replacement of the p53 gene with the *lacZ* gene in mouse embryonic stem cells and mice by using two steps of homologous recombination. *Biochem. Biophys. Res. Commun.*, **202**, 830–837.