Characterization of a new transgenic mouse model of the Spinocerebellar Ataxia type 2 disease

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ABSTRACT

The objective of this work was the generation of an animal model of the SCA2 disease for future studies on the benefits of therapeutic molecules and the underlying neuropathological mechanisms in this human disorder. The transgenic fragment was microinjected into pronuclei of B6D2F1 X OF1 mouse hybrid strain. For Northern blots, RNAs were hybridized with a human cDNA fragment from the SCA2 gene and a mouse b-actin cDNA fragment. Monoclonal antibodies directed at the N-terminal of the ataxin 2 protein with 22Q were used for Western blot analysis. A rotating rod apparatus was utilized to measure motor coordination of mice. Immunohistochemical detection of Purkinje neurons was performed with anti-calbindin 28K as the primary antibody. An ubiquitous expression of the SCA2 transgene with 75 CAG repeats regulated by the SCA2 self promoter was obtained after the generation of our transgenic mice. The analysis of transgenic mice revealed significant differences of motor coordination compared with the wild type littermates. A specific degeneration of Purkinje neurons and transgene over-expression in the brain, liver and skeletal muscle, rather than in lungs and kidneys was also observed, resembling the expression pattern of the ataxin 2 in humans.

Key words: SCA2 disease, transgenic mice, ubiquitous expression

Introduction

CAG repeat expansion in different genes encoding unrelated proteins with polyglutamine expanded domains causes several late-onset progressive neurodegenerative disorders including Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), dentatorubral and pallidoluysian atrophy (DRPLA), and spinocerebellar ataxias (SCA's) [1]. SCA2 disease is a member of the increasing number of spinocerebellar ataxias characterized by the wide range of expression of the SCA2 gene, whereas the primary specific degeneration target are the cerebellum Purkinje neurons [2-4]. The promoter region of the SCA2 gene was identified as a part of a CpG island using promoter prediction algorithms and luciferase expression experiments [5].

Ataxin 2, the protein product of the SCA2 gene, is composed of 1312 amino acids residues with an estimated molecular weight of 140 kD. Wild type ataxin 2 contains 22 glutamine repeats at the amino-terminus as the most common form of the protein. Expansions of 32 or more glutamines without CAA repeat interruptions cause the SCA2 phenotype in patients [6]. Northern blot analysis revealed that the human SCA2 gene is expressed in the brain, heart, placenta, liver, pancreas and skeletal muscle [2-4], suggesting its ubiquitous expression with no preference for affected regions [4]. The immunohistochemical analysis of the brain tissue demonstrated that human ataxin 2 had a cytoplasmic location in normal individuals, and that the SCA2 gene is expressed in Purkinje cells and some specific groups of brainstem and cortical neurons [7]. Except for the polyglutamine expansions, ataxin 2 has no similarity with any other polyglutamine protein [2-4]. The study of the normal and diseased human brains can provide important insights into the pathogenesis of polyglutamine disorders, but such observations are frequently limited to the terminal stages of the disease process [8]. The generation of mouse models of neurodegenerative disorders can help circumvent this problem, but many of them are based on the expression of truncated constructs to produce neurodegeneration [9-11]. In addition, most polyglutamine disease models use heterologous promoters to direct the expression of the transgene to the primary target of these diseases [8,10-12]. Here, we provide a transgenic mouse model of a polyglutamine disease based on the expression of the full-length SCA2 cDNA with a CAG repeat expansion under the regulation of the self human SCA2 promoter. Given the ubiquitous expression of the human SCA2 transgene together with the specific impairment of Purkinje cell functions, this is the first report of transgenic mice that share features similar to those of SCA2 patients.

Material and methods

Isolation and cloning of the human full-length cDNA with 75 CAG repeat expansion

Using RT-PCR we amplified a full-length cDNA from a Cuban SCA2 patient with 75 CAG repeats. Total blood sample was homogenized in Tri-Reagent (Sigma) and total RNA was isolated as described in the manufacturer's protocol. The first strand cDNA was synthesized from 5 mg of total RNA using the Promega RT-PCR system. PCR was performed using the following primers. Forward primer: 5´-GCC CTC CGA TGC GCT CAG CG-3´, and reverse primer: 5´-AGC AGT AAT AGC AGC AAG AAT C-3´, which

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TGC GCT CAG CG-3´, and reverse primer: 5´-AGC AGT AAT AGC AGC AAG AAT C-3´, which span the human full-length cDNA generating an amplicon of 4.477 Kb. The amplicon was subcloned using Sac I and Nhe I restriction enzymes in the previous clone 7-pGL3 (derivate of the pGL3 basic vector, Promega), that contains the human SCA2 promoter [5]. The resulting construct used for transgenesis (plasmid 775) was confirmed by sequence analysis (data not shown).

Generation of transgenic mice

To linearize the transgenic construct, plasmid 775 was digested with Cla I and a 5.28 Kb fragment (figure 1) was isolated in an agarose gel. The purified SCA2 transgenic fragment was microinjected into pronuclei of a B6D2F1 X OF1 mouse hybrid strain. To determine the transgenic phenotype, one centimeter pieces of the tail were cut from the mice. DNA was isolated using proteinase K (0.5 mg/ml) and saline precipitation to eliminate most proteins. Finally, we precipitated the DNA with isopropanol. After isolation, 500 ng of the tail DNAs were resuspended in sterile water, and analysed by PCR using human specific primers [3] that flank the human CAG repeat region: DAN1 (5´-CGT GCG AGC CGG TGT ATG GG-3´) and DAN2 (5´-GGC GAC GCT AGA AGG CCG CT-3´). PCR protocol was as follows: 4 min at 94 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 62 ºC and 1 min at 72 ºC. PCR products generated a 327 bp fragment containing 75 CAG repeats from SCA2 cDNA. We identified 2 mutant founder mice F050 and F066. Homozygous mice were also obtained from the founder mouse F066. For Southern blots, equal amounts of genomic DNAs from control and transgenic mice were digested with appropriate restriction enzymes and probed with a human specific SCA2 cDNA fragment labeled with ^{32}P. Probes included the 3´ end of the human exon 1 (immediately after the CAG repeat) and human exons 2, 3, 4 and 5.

RNA isolation and Northern blots

Five month old mouse tissues were homogenized in Tri-Reagent (Sigma) and total RNA were isolated as described in the manufacturer's protocol. Five micrograms of RNA were run on 1.5% agarose gels and transferred overnight to nylon membranes (Hybond N+, Amersham). RNAs were hybridized overnight at 65 ºC simultaneously with a human cDNA fragment from the SCA2 gene (the same probe used for Southern blots) and a cDNA fragment from the mouse β-actin gene. The expected size for the human SCA2 transgene was 3.9 Kb and for the mouse β-actin gene 1.8 Kb.

Figure 1. Configuration of the microinjection fragment used for transgenesis. The thin line depicts the 5´ upstream regulatory region of the human SCA2 gene. The white box indicates the human full-length SCA2 cDNA with 75 CAG repeats. The black box indicates the poly(A) addition site from SV40. Primers for PCR DAN 1 and DAN 2 are indicated. Recognition sites for the restriction enzymes Kpn I, Sac I, Eco RI and Nhe I are also indicated as K, S, E, and N respectively.

Western blot

Triple detergent buffer (1% Triton X-100, 0.5% SDS, 0.5% deoxycholate, 100 µM Tris HCl PH=8, 150 µM NaCl) and the Sigma mammalian protease inhibitors cocktail were added to the frozen five month old mouse cerebella, mixed with polytrone for 1 min and kept on ice. Protein extracts were centrifuged at 10 500 rpm in an Eppendorf microfuge at 4 ºC for 30 min. After centrifugation samples were stored at -70 ºC. For Western blot analysis 100 µg of total proteins were pre-incubated in sample buffer for 15 min at 95 ºC. Samples were run in 10% SDS-PAGE gels. Proteins were detected with a monoclonal antibody against the amino-terminal region of the human ataxin 2 protein with 22Q.

Clasping test

Six week old mice were held by the tail for at least one minute. Clasping posture, defined as one or two hindleg folding close to the body was recorded.

Footprinting analysis

After painting the one-year-old animals´hind feet with nontoxic black paint, mice were allowed to walk through a dark 30 cm-long, 9 cm-wide, and 6 cm-high tunnel. Footprint patterns were scored for 2 parameters. Step length: Calculated by measuring the distance of the walk through the tunnel divided by the number of steps. Linear movement: Calculated by drawing a line perpendicular to the direction of the walk, starting at the first right footprint. The angle between this perpendicular line and each subsequent right footprint was determined, and differences in angle were calculated between each consecutive step pair. The absolute value of all angle differences were added and divided by the number of steps scored. A large linear movement measurement would be indicative of non-linear movement through the tunnel.

Rotarod performance

Motor coordination was tested using a rotating rod apparatus (Ugo Basile, Varese, Italy). Mice were placed on an accelerating rod with a rotating speed from 4-40 rpm for a maximum 10 min. The time spent on the rod without falling was recorded. The test was performed for 5 consecutive days with 4 individual trials per day. Resting time between trials was 30 min.

Immunohistochemistry

The one-year-old mouse cerebella were fixed in 10% formalin and then embedded in paraffin. Sections of 5 µm width were mounted on chromalum coated slides and exposed to 56 ºC for 20 minutes. Specimens were dewaxed, rehydrated, rinsed and washed in PBS (pH 7.4) for 30 minutes. Once endogenous peroxidase was quenched, specimens were washed and treated with Dako target retrieval solution equilibrated at 99 ºC. Tissue samples were then incubated for 30 minutes with anti-calbindin-28K (1:200, Chemicon International) in Dako background reducing solution. The immunohistochemical reactions were carried out using the labeled streptavidin-biotin/HRP conjugate

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method (LSAB, HRP kit, Dako, Carpinteria, USA) according to the manufacturer's instructions. The peroxidase reaction was developed with the 3-3'-diamino-benzidine-tetra-hydrochloride (DAB) and counterstaining was done with hematoxylin.

Statistical analysis

Quantitative data was expressed as the mean ± standard deviation. T-test and ANOVA with Tukey-Kramer post-test were used to calculate the statistical significance of differences between groups respectively. P values of less than 0.05 were considered statistically significant.

Results

Intergenerational stability of CAG repeat in transgenic mice

PCR products were obtained from 10 F1 descendents of the founder F050 and 14 F1 descendents of the founder F066. PCR products were also obtained from 32 F2 descendents of the founder F066. In all animal examined, the primary PCR product showed no change in repeat size as compared with the microinjected transgene. Representative results are shown in figure 2. Southern blot analyses comparing signal intensities demonstrated that all SCA2 transgenic lines had integrated one or two copies of the SCA2 cDNA (figure 3). The similar migration of detected fragments in Southern blots also indicated the conservation of the CAG repeat size through subsequent generations.

Transgene expression

Northern blot analyses revealed transcripts of 3.9 kb corresponding to the human SCA2-$(CAG)_{75}$ transgene from the cerebellum (figure 4A), brain, liver, skeletal muscle, lung and kidney of the transgenic mice (figure 4B). Western blot analysis of protein extracts demonstrated the transgene expression in the cerebellum at the protein level (figure 4C). A 140 kD band corresponding to mouse ataxin 2 protein was detected in both wild type and transgenic animals whereas a 190 kD band corresponding to human ataxin 2-75 Q was found only on the latter. Bands of higher and lower molecular weight were detected in the blots suggesting aggregation and degradation of the 190 kD transgenic protein, respectively.

Functional testing of transgenic mice

The ataxic neurological phenotype as demonstrated using clasping, footprinting and rotarod tests provides evidence for disrupted Purkinje cell function.

Clasping was observed in founders (F0), F1 and homozygous F2 at 24, 12 and 6 weeks of age respectively. Representative results are shown in figure 5.

At 1 year of age, the footprint patterns of the founders differed dramatically in the two measured parameters from the patterns generated by 1-year-old non-transgenic littermates. Instead of walking along a straight line with a smooth alternating gait as did the wild type animals, 1-year-old founders weaved from side to side while moving through the tunnel, using shorter steps (figure 6).

Rotarod analysis of the founder animals F050 and F066 at the earliest age examined (20 weeks) revealed significant differences of motor performance compared with the wild type littermates (p<0.001, ANOVA with Tukey-Kramer post-test) (figure 7A). These differences were maintained until the last age tested (one year, data not shown).

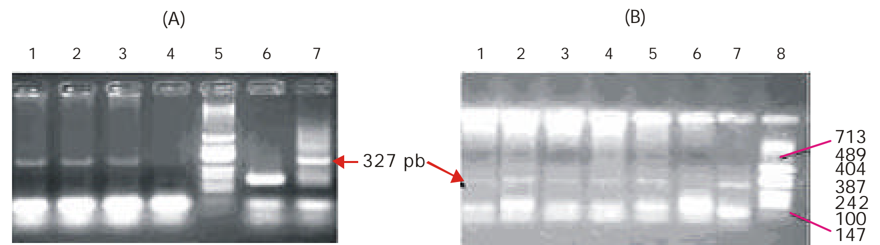

Figure 2. PCR products from control and SCA2 transgenic mouse genomic DNA. Arrows indicate the expected size of the expansions. (A) Lane 1-3. F1 generations from founders F050 and FO66. Lane 4. Control non-transgenic mouse. Lane 5. Molecular Weight Standard. Lane 6. PCR product containing 22 CAG repeats. Lane 7. PCR product containing 75 CAG repeats; (B) Lane 1-6: F2 animals from founder F066. Lane 7. Control non-transgenic mouse. Lane 8. Cuban SCA2 patient carrying a 41 CAG expansion and a normal allele of 22 CAG units. Lane 9. PCR product containing 75 CAG repeats. Lane 10. Molecular Weight Standard.

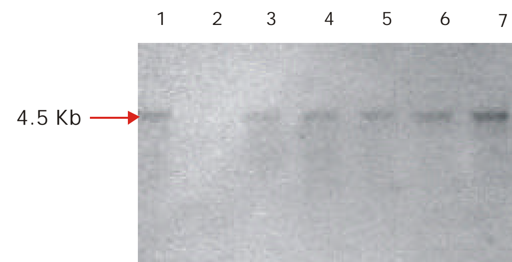

Figure 3. Southern blots of genomic DNA from founders F050 and F066, and a number of F1 and F2 progeny. Sizes of the expected digestion fragments are indicated with arrows. (A) DNA was digested with the combination Sac I-EcoR I. Lane 1. F050. Lane 2. F066. Lane 3. Non-transgenic control mouse. Lane 4. Sac I-EcoR I fragment of the plasmid 775 (0.1 ng); (B) DNA was digested with Kpn I. Lane 1. KpnI fragment of the plasmid 775 (0.01 ng). Lane 2. Non-transgenic control mouse. Lane 3. F1/10. Lane 4. F1/14. Lane 5. F2/5. Lane 6. F2/6. Lane 7. F2/13.

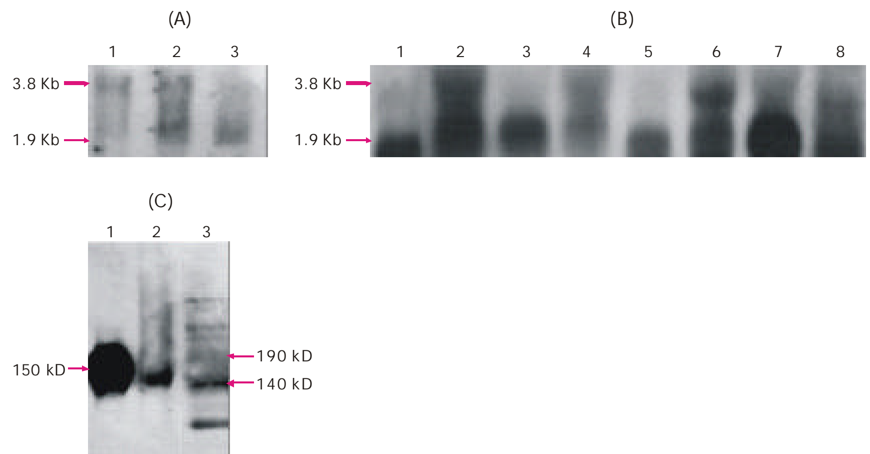

Figure 4. Northern (A and B) and Western blot analysis (C) of transgenic mice tissues. (A) Cerebellum. 1-homozygous, 2- heterozygous, 3- wild type; (B) 2,4,6,7,8- Brain, liver, skeletal muscle, lung and kidney from heterozygous mice. 1,3,5- Brain, liver and skeletal muscle from wild type mice. The expected size of the human SCA2 transgene (3.9 Kb) and the mouse â-Actin gene (1.8Kb) are indicated; (C) 1-Monoclonal antibody of 150 KD size used as Molecular Weight Standard, 2- Cerebellum from a wild type mouse, 3- Cerebellum from transgenic a mouse. The expected size of the human ataxin 2 (190 KD) and the mouse ataxin 2 (140 KD) are indicated.

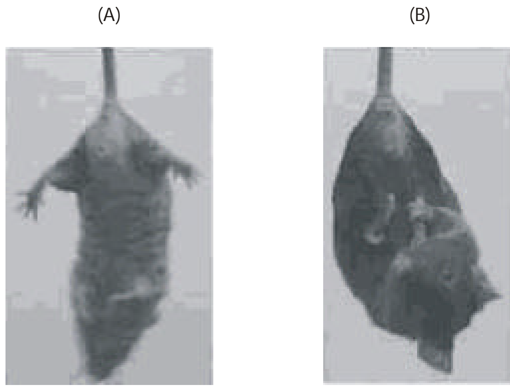

Figure 5. Clasping test. (A) Non-transgenic mouse; (B) Homozygous transgenic mouse showing the feet-clasping posture when suspended by the tail.

Rotarod testing of F066 transgenic progeny revealed impaired motor performance at 12 weeks as compared to controls ($p < 0.001$, ANOVA with Tukey-Kramer post-test). However, when testing animals at 6 weeks only homozygous mice ($p < 0.001$, ANOVA with Tukey-Kramer post-test) showed motor coordination impairment, suggesting that expression levels of expanded ataxin 2 influences the onset time of ataxic symptoms. We also noted a greater dispersion of ataxic symptom onset time in heterozygous compared to homozygous mice (data not shown).

Purkinje cell degeneration in founder transgenic mice

Most Purkinje cells from both founder mice examined had lost calbindin-28K expression as compared to the age-matched control mice. Furthermore, Purkinje cells of both founders appeared devoid of the dendritic arbour while experiencing shrinkage of their cell bodies (figure 8).

Discussion

We generated transgenic mouse lines expressing the full-length human SCA2 gene with 75 reiterations of CAG under the control of the human SCA2 promoter.

SCA2 transgene products were detected in the brain, liver and skeletal muscle, and to a lesser extent in the lung and kidney. Although the ubiquitous expression of the transgene was shown at RNA level using Northern blot analysis, we observed specific degeneration of Purkinje neurons from the cerebellar cortex using immunohistochemistry techniques. This expression pattern is similar to that observed in humans [2-4], but different to that described in mice [13].

A previous study has reported the use of the murine PcP2 (L7) promoter to direct the expression of the human SCA2 gene with an expanded allele of 58 CAG repeats [8]. They described the insertion of 2 copies of the transgene as in to the present study. However, they found that the outcome of the ataxic phenotype, as recorded using the rotarod test, was at 26 and 16 weeks for the heterozygous and homozygous transgenic mice, respectively. Using the same motor coordination test, our transgenic mice developed the neurological phenotype in 12 weeks for heterozygotes, whereas the homozygous mice did it in 6 weeks. These results suggest that the promoter elements of the human SCA2 gene used in the present work are very effi-

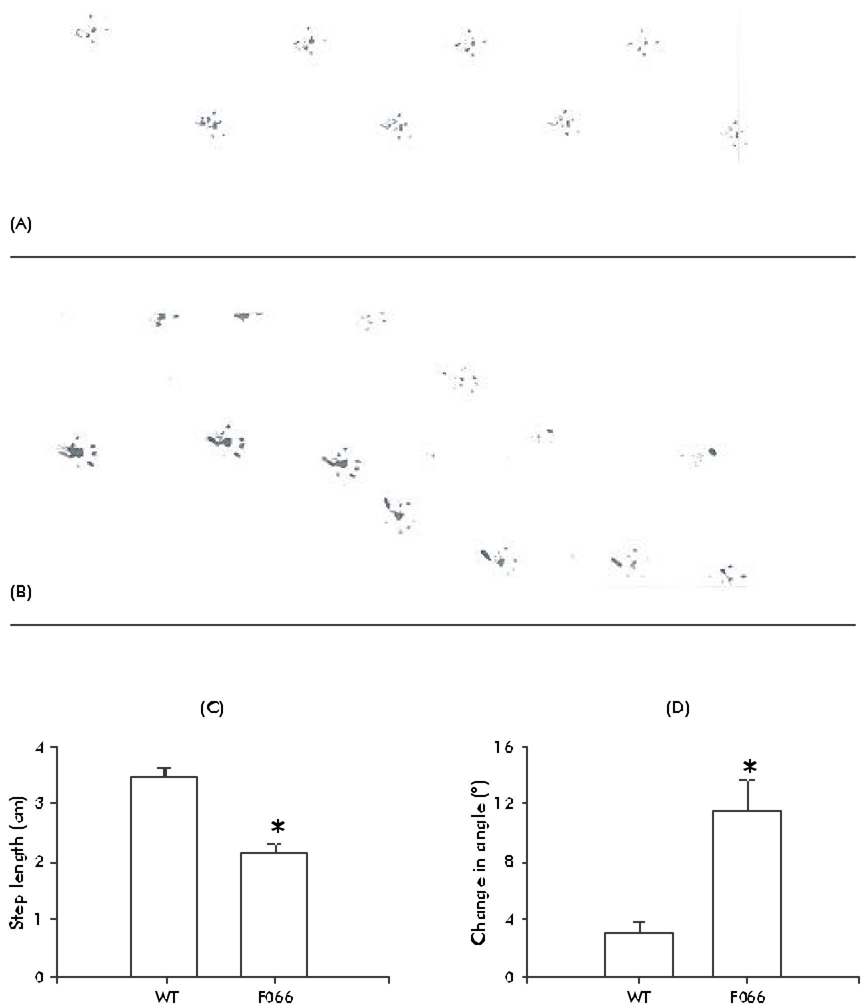

Figure 6. Footprint pattern analysis. (A) Wild type; (B) Founder F066. Footprint patterns of wild type and founder F066 were quantitatively assessed for step length (C) and linearity of the movements (D). Statistical significance ($p < 0.001$, t-test) is indicated with asterisks.

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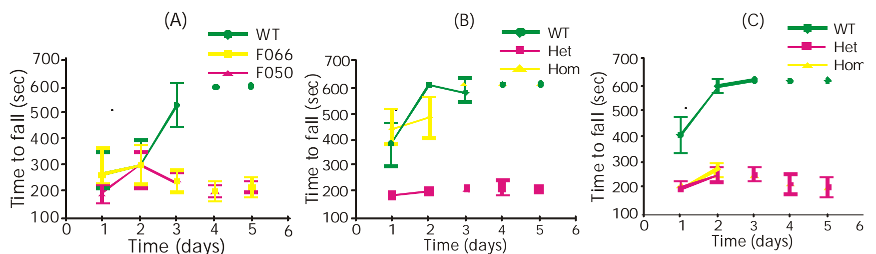

Figure 7. Average performance on a rotarod apparatus. (A) Twenty-week-old wild type and founder animals. F066 and F050 animals failed to improve their performance. (B) Six-week-old wild type and F066 transgenic progeny. Homozygotes showed impaired performance improvement while heterozygotes improved day by day in a manner similar to the wild type animals. (C) Twelve-week-old wild type and F066 transgenic progeny. Both, homozygotes and heterozygotes failed to improve performance as compared to age-matched wild type animals.

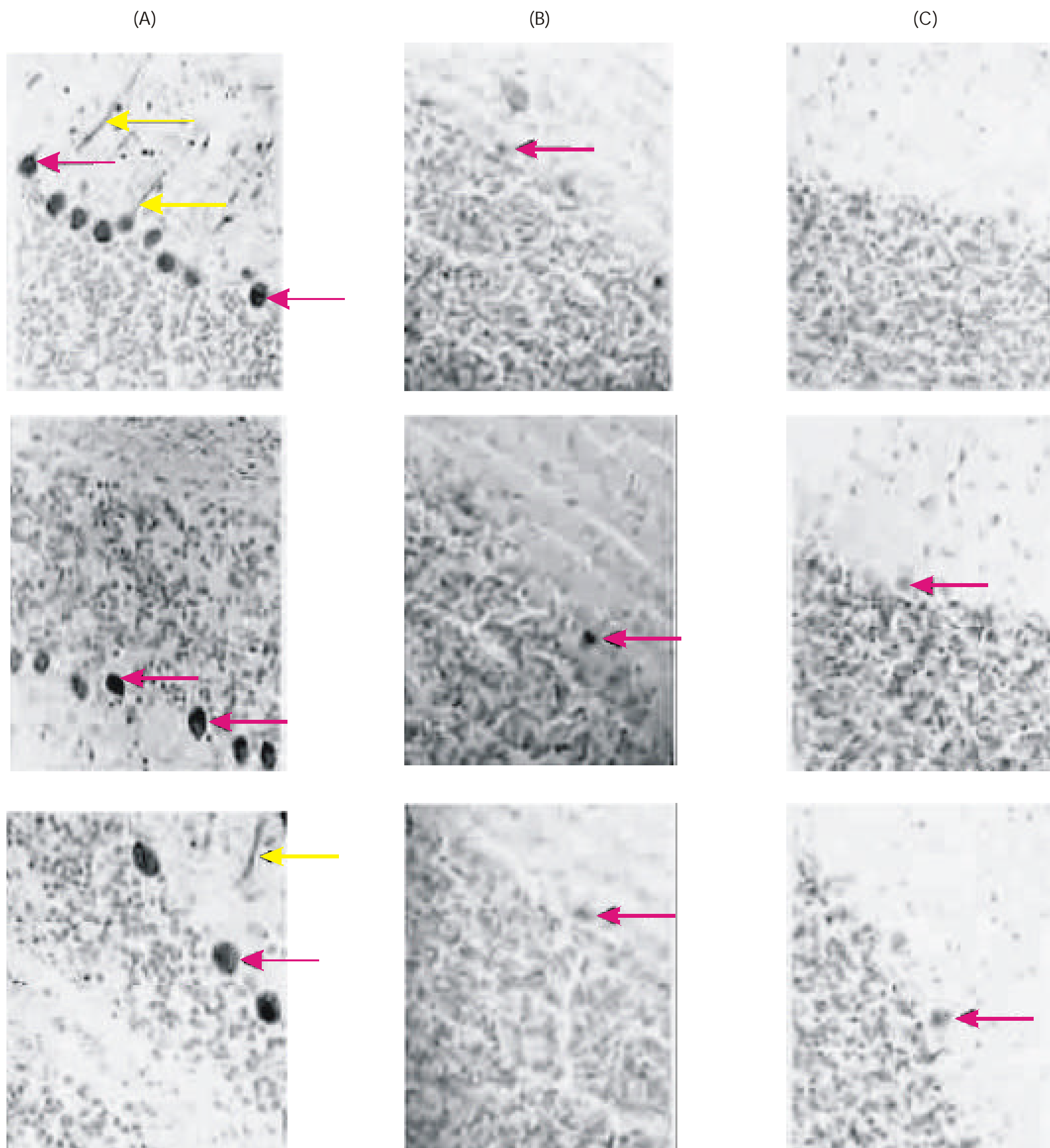

Figure 8. Calbindin-28K labeling of cerebella from wild type (column A), founder F050 (column B) and F066 (column C). Purkinje cell bodies (indicated by bold arrows) of both founders appeared devoid of the dendritic arbour and presents shrinkage of their cell bodies as compared to the control mice. Purkinje cell dendrytes are indicated by thin arrows in non transgenic mice.

cient to direct the expression of the transgene in our mouse model.

Several transgenic mice lines that express human genes with CAG repeat expansions under the control of their own promoters have been generated in the last decade [9,14-16]. Although in most of these mice models a neurological phenotype was observed the lack of the specific degeneration of the disease target neurons has also been reported. For example, the expression of the exon 1 of the HD gene [9], does not lead to specific striatal neurodegeneration as occurs in HD patients [14]. Actually, it is well understood that polyglutamine expansions provokes a dominant gain of function leading to a neurological phenotype, but the rest of the protein modulates the neurotoxicity as well [17-20]. Accordingly, in our SCA2 mouse model we considered not only the CAG repeat, but also the rest of the human SCA2 gene coding region.

On the other hand, the expression of a large genomic DNA fragment containing full-length human genes in mice have led to instabilities of CAG repeat sequences over generations. For instance, mice expressing DRPLA and SCA7 genes with introns and exons have produced intergenerational CAG repeat instabilities [15, 16]. However, the same paper [16] described that during the expression of SCA7 cDNA fragments, the CAG repeat expansion is intergenerationally stable. In our study SCA2 transgenic mice stability of the CAG repeat was also observed until the F2 generation. Thus our results support the idea that genomic instability could be minimized when utilizing constructions lacking non-coding regions.

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In conclusion, we have generated a unique SCA2 transgenic animal model that combines the ubiquitous expression of the full-length SCA2 cDNA-$(CAG)_{75}$ with a distribution pattern resembling that observed in humans, the specific degeneration of Purkinje neurons that lead to the early onset of ataxic phenotype, and generational stability of the expanded CAG repeats. Accordingly, this model is suitable for the evaluation of different therapeutic candidates to avoid or delay the onset of this incurable disease in humans.

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