

Appendix A from Matsuura et al., ‘Interruptions in the Expanded ATTCT Repeat of Spinocerebellar Ataxia Type 10: Repeat Purity as a Disease Modifier?’

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Methods

The study was approved by the Baylor College of Medicine institutional review board, and the appropriate informed consent was obtained from human subjects.

SCA10 Analysis

DNA was extracted from whole blood by conventional methods. The ATTCT region was PCR amplified using the primers attct-L (5'-AGAAAACAGATGGCAGAAATGA-3') and attct-R⁶ (5'-GCCTGGGCAACATAGAGAGA-3'), as described previously. DNA samples with a single normal allele by PCR underwent Southern blot analysis to assess large expansions, by use of 10 μ g of *Eco*RI-digested genomic DNA and the 800-bp SCA10 probe (Matsuura et al. 2000).

Long-Range PCR of the 280-Repeat Allele

We amplified the ATTCT repeat region from genomic DNA, by using the primers L1 (5'-ACAAAGGATCAGAATCCCTGGAAAAGTTAAATATATATGGG-3') and R1 (5'-AGCATATAATTGAGTCTTGCCTTTCAAATCCAGT-3'). Reactions were 50- μ l volumes, 1 \times reaction buffer, 200- μ M dNTPs, and 5 units KOD XL DNA polymerase (Novagen). The PCR conditions consisted of an initial denaturing at 94°C for 2 min, 30 PCR cycles (94°C for 15 s, 67°C for 5 s, and 70°C for 4 min), and an additional extension at 74°C for 10 min. We could successfully amplify the expanded allele (3.4 kb) equally with the normal allele (2.0 kb), corresponding to the Southern blot data. We then sequenced the expanded allele, using an Applied Biosystems (ABI) prism 377 automated sequencer with fluorescent dideoxynucleotides, after separation on a 1.0% agarose gel.

ATTCT Repeat-Primed PCR (RP)

The reaction was performed with 1 μ M of the ³²P-end-labeled forward primer P1 (5'-GAAGACAAATAGAAAACAGATGGCACA-3') corresponding to a unique sequence of the ATTCT repeat, 0.1 μ M of the reverse primer P2 (TACGCATCCCAGTTTGAGACGG(AATAG)₈-3') consisting of a repeat sequence with a hanging tail at its 5' end, and 1 μ M of the hanging primer P3 (TACGCATCCCAGTTTGAGACG) modified from that described elsewhere (Matsuura and Ashizawa 2002). The RP products were electrophoresed on 6% denaturing polyacrylamide gels for >4 h for the investigation of patterns within the repeat tracts and were visualized by autoradiography.

Cloning of RP Products

We gel-extracted RP products of SCA10 patients, with lengths corresponding to 80–100 repeats, after electrophoresis on 3% agarose gels. Our experience suggested that repeat lengths above ~100 cannot be stably maintained in *E. coli*; thus, this is the upper limit of fragment lengths that can be isolated. If the RP products from an individual showed the pattern indicative of interruptions, we gel-purified the upper bands above the first interruption, to allow determination of the sequence involved in the interruption. RP products were cloned into pSMART LC vector (Lucigen). Colonies were size selected by preliminary PCR with the use of P1 and P3 and were subsequently sequenced. Because of the inherent instability of the ATTCT repeat when propagated in *E.*

coli, the plasmids were grown at 30°C and with minimal agitation, which we found successfully reduced the deletion frequency of the repeat in clones. At least two independent clones from each PCR product were bidirectionally sequenced using an ABI prism 377 automated DNA sequencer with the two sequencing primers of the pSMART LC vector, following miniprep purification of plasmid DNA. If sequence data derived from clones was not consistent (presumably because of polymerase errors), then additional independent clones were sequenced.

Cloning of Normal Alleles

We amplified the ATTCT region from normal individuals or unassigned ataxia patients, whose number of repeats was already determined. We selected 38 individuals (23 Caucasian, 9 Mexican, and 6 Japanese) who had >17 repeats in either allele. PCR was performed in the reaction of 20- μ l HotStarTaq Master Mix (Qiagen), with use of 0.2- μ M primers L2 (5'-AATTGCCCAAAGATTAAAC-3') and R1 (5'-TTCAGAGACTGCAGTAAAGACAGG-3'). PCR conditions were 95°C for 15 min, 30 PCR cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min), and an additional extension at 72°C for 10 min. Amplified products were cloned into pCRII-TOPO vector (Invitrogen). After PCR size selection using primers attct-L and attct-R⁶, colonies with different sizes were sequenced respectively. At least two independent clones of the same size for each PCR product were sequenced for the verification of sequence accuracy. We added the previously obtained data of 40 normal alleles (20 homozygous individuals) (Matsuura et al. 2000) and analyzed all the data together.