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Large CAG/CTG repeat templates produced by PCR, usefulness for the DIRECT method of cloning genes with CAG/CTG repeat expansions

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ABSTRACT

We report here a simple method for generating large CAG/CTG repeat sequences. We have applied this method to clone the genomic sequence containing the CAG/CTG repeat and its upstream intronic sequence present in spinocerebellar ataxia type 3 or Machado–Joseph disease (SCA3/MJD) by a modified DIRECT method. With these modifications we have considerably simplified the generation of the repeat probe used to screen for anomalous bands. This method will facilitate the molecular approach to other genetic disorders where expansions of repeat sequences could be involved.

CAG/CTG repeat expansions are dynamic mutations (1) present in at least eight genes responsible for neuropathological diseases (2–12). The disease alleles are present when a threshold of typically 37–40 repeats is exceeded with alleles ranging between 35 and 130 repeats, except for the small expansions present in spinocerebellar ataxia type 6 (SCA6) (11). Several strategies have been used to clone these genes but a recently developed DIRECT method has been presented as a powerful technique (10,13). We present here a simple PCR based method to generate large CAG/CTG repeat sequences. We have simplified the generation of the relatively large CAG probes required in the DIRECT method and applied it to clone the spinocerebellar ataxia type 3 or Machado–Joseph disease (SCA3/MJD) repeat expansion. The technique could be applicable to other neurological diseases, where genomic alterations due to repeat sequence expansions could be involved.

Large CAG/CTG products were generated by PCR when CAG and CTG 8mer primers crossannealed at low annealing temperature. Under these conditions a smear of repeat concatamers is produced. We used a size range of 150–250 bp PCR products to generate large probes (between 50 and 80 repeats) suitable for detecting repeat expansions at highly stringent hybridisation conditions. The PCR reaction was performed in a final volume of 50 µl containing 10% DMSO, 200 µM of each dNTP, 50 pmol of CAG and CTG 8mer primers, 0.5 U *Taq* DNA polymerase in 1× PCR buffer (Boehringer Mannheim). The reaction consisted of 35 cycles of PCR with 30 s at 94°C, 30 s at 40°C and 60 s at 74°C. The PCR products were loaded in 1% low melting agarose and a slice corresponding to ~150–250 bp of repeat concatemers was directly used to produce the probe by the Megaprime DNA labelling system (Amersham). The probe was labelled to a specific radioactivity of $5–10 \times 10^8$ c.p.m./µg. The labelled probe was purified on a Sephadex G-50 (Pharmacia) column, denatured and added to the hybridisation solution described below.

High molecular weight genomic DNA (3 µg) from SCA3/MJD affected subjects and their healthy relatives (Fig. 1) was digested with 30 U of relatively frequent cutting blunt-end restriction enzymes (*Bst*UI, *Eco*RV, *Hin*cII, *Nla*IV, *Pvu*II, *Ssp*I; New England Biolabs), electrophoresed through a 1% agarose gel and transfered to nylon membranes (Hybond N+, Amersham). The filters were hybridised in a solution containing 5× Denhardt's reagent, 50 µg/ml denatured sonicated salmon sperm DNA, 0.2 M phosphate buffer pH 6.8, 0.1% pyrophosphate, 4× SSC, 0.5% SDS, 0.002% heparin and 10% dextran sulphate (14). The hybridisation was performed for 16 h at 65°C. After the hybridisation the membranes were washed at 65°C to a stringency of 0.01× SSC and 0.2% SDS for 2 h. The filters were autoradiographed for 2–5 days with Curix RP2 film (Agfa) at –80°C using an intensifying screen.

After digestion with *Nla*IV a 1.2 kb band was clearly visible in samples from SCA3/MJD individuals and absent in samples from unaffected individuals (Fig. 1). Then 30 µg of high molecular weight genomic DNA from an affected subject was digested with 100 U of *Nla*IV and subjected to preparative gel electrophoresis. A 0.3 cm wide agarose slice, corresponding to a size ~1.2 kb was cut, and the genomic DNA was purified using a GeneClean II Kit (BIO 101, Inc.) and cloned into λZAPII vector (Stratagene) using phosphorylated *Eco*RI linkers (New England Biolabs). The cloned DNA was packaged *in vitro* (Stratagene) and a total of 10^5 plaques were screened with an 8mer CAG primer labelled with T4 polynucleotide kinase (Amersham). Nine positive clones were detected, which were sequenced and the sequences were compared in public databases using blastN and blastX. Three clones corresponded to a portion of the SCA3/MJD gene that contains the CAG/CTG repeat sequence. The new identified sequence contains part of the intron upstream of the CAG/CTG repeat (Fig. 1). The new sequence was lodged in the EMBL with accession number AJ000501. The other six clones corresponded to AJ002191, previously reported as 14 kDa subunit protein, and AJ002192, two clones each; and AJ002193 and AJ002194, detected once each.

We have presented here a modified DIRECT method that simplifies the generation of large CAG probes. The previously described method make use of the biotin/streptavidin system to

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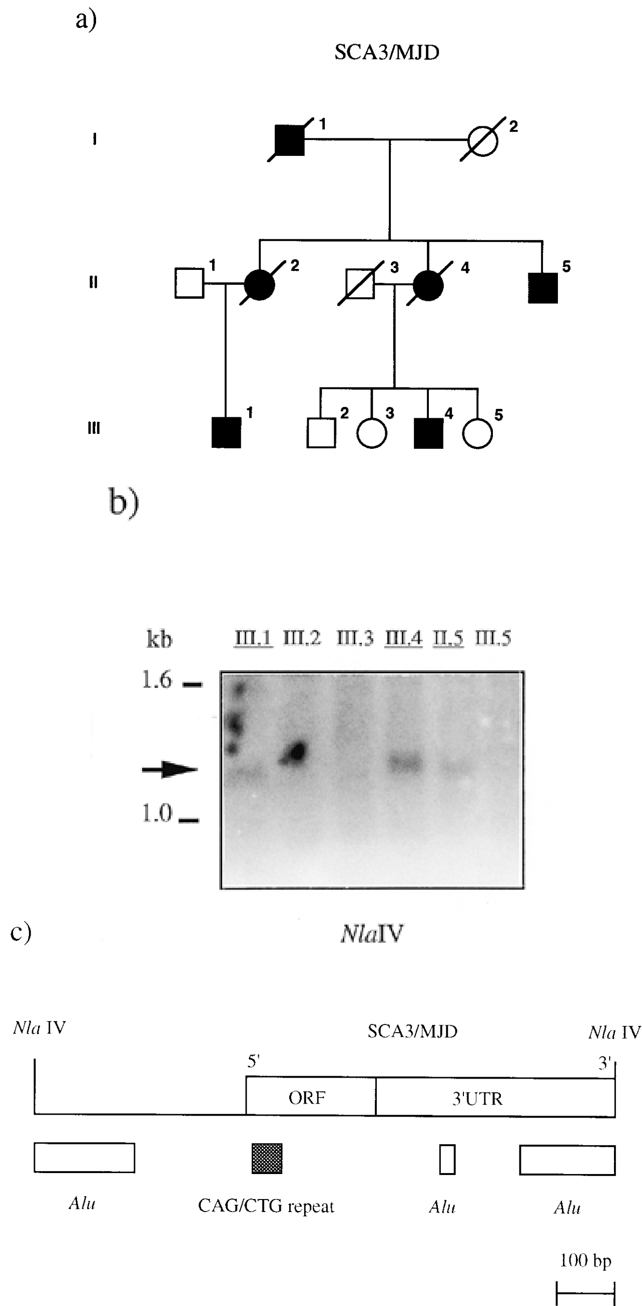

Figure 1. DIRECT analysis of SCA3/MJD. (**a**) SCA3/MJD pedigree: the filled symbols indicate affected individuals and the unfilled symbols indicate non-affected subjects. The SCA3/MJD repeat expansion present in the affected subjects II.5, III.1 and III.4 contain 70, 69 and 72 repeats, respectively. (**b**) Autoradiogram results when a filter containing 3 µg genomic DNA from members of the SCA3/MJD family were digested with *Nla*IV and hybridised with a large CAG probe produced as described in the text. A 1.2 kb band can be seen in the affected subjects only. (**c**) Schematic representation of the cloned region of the SCA3/MJD gene.

purify CAG probes produced by PCR on a known subcloned CAG/CTG repeat expansion of 55 units. We have considerably simplified this first step of the DIRECT method and successfully applied it to clone the SCA3/MJD repeat expansion. Also we have used different frequent cutting enzymes which facilitates the screening for anomalous bands as they produce relatively short fragments. By its simplicity, the DIRECT method represents a great advantage with respect to other cloning strategies. It represents an important option to consider in cloning efforts, especially for neurological diseases where different unstable sequences have been found to be involved, such as CGG in Fragile X syndrome (15–17), GAA in Friedrerich's ataxia (18), and progressive myoclonus epilepsy type 1 (19–21).

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