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PCR-amplification of D2S44 alleles

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Summary. A strategy for PCR-amplification and sequencing of the flanking regions in the polymorphism D2S44 (YNH24) has been developed based on the investigations of Edwards et al. (1991). The flanking regions of the YNH24 probe were successfully amplified and two distinct PCR products with fragment sizes of 180 and 250 bp obtained. After asymmetric PCR and didesoxy-sequencing 60 bp could be determined for every PCR fragment. D2S44-specific primers were constructed which were located at the transition between the flanking and repeat regions. Amplification conditions were optimized using the YNH24 probe, different nuclease S1 concentrations and incubation times. Optimized conditions were applied to the amplification assay of human D2S44 alleles which had been investigated by RFLP analysis.

Key words: D2S44 polymorphism – Single locus polymorphism – Sequencing of flanking regions – Amplification – D2S44 alleles

Zusammenfassung. Basierend auf den Ergebnissen von Edwards et al. (1991) wurde eine Strategie zur PCR-Amplifikation und Sequenzierung flankierender Regionen im Polymorphismus D2S44 (YNH24) entwickelt. Die flankierenden Regionen der YNH24-Sonde wurden amplifiziert und zwei distinkte PCR-Produkte mit Fragmentgrößen von 180 und 250 bp erhalten. Nach asymmetrischer PCR und Didesoxy-Sequenzierung konnten für jedes PCR-Fragment 60 bp bestimmt werden. D2S44 spezifische Primer wurden konstruiert, die sich am Übergang von der flankierenden zur Repeat-Region befinden. Die Amplifikationsbedingungen wurden unter Verwendung der YNH24-Sonde, unterschiedlicher Nuklease S1-Konzentrationen und Inkubationszeiten optimiert. Die optimierten Bedingungen wurden für die Amplifikation humaner D2S44 Allele eingesetzt, die mittels der RFLP Analyse untersucht worden waren.

Schlüsselwörter: D2S44 Polymorphismus – Single Locus Polymorphismus – Sequenzierung flankierender Regionen – Amplifikation – D2S44 Allele

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Introduction

VNTR (variable number of tandem repeat) polymorphisms represent efficient tools for human identification. The locus D2S44 (YNH24) (Nakamura et al. 1987; Puers et al. 1991) which is investigated by RFLP analysis has fragment sizes from 1 to 3 kb (HaeIII) and 2 to 5 kb (HinfI) (Promega Gene Print 1990) with repeat sizes of 31 bp (Nakamura et al. 1987).

Contrary to PCR-VNTRs (AmpFLPs and STRs) with fragment sizes ranging from 130 to 1000 bp (Brinkmann

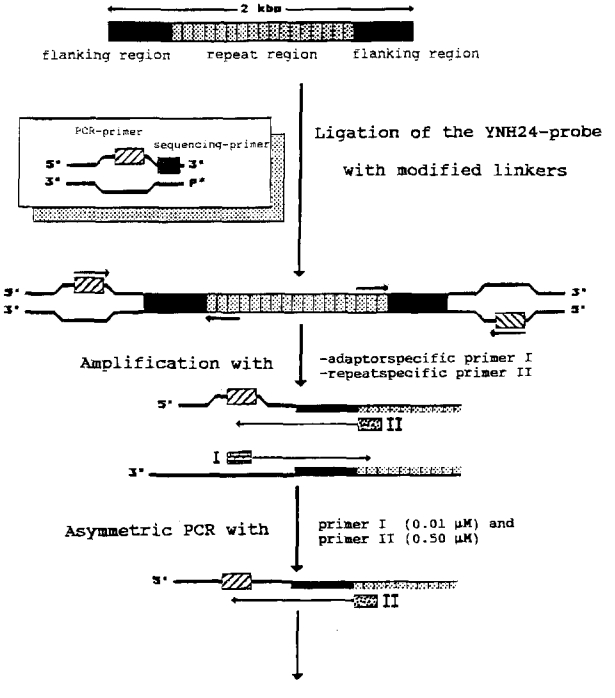

Fig. 1. Strategy for the determination of flanking sequences in the polymorphism D2S44 (according to Edwards et al. 1991); P* = the strand which contains the PCR and sequencing primer side is dephosphorylated at the 5′ end, whereas the complementary strand is 5′-phosphorylated to allow an unidirectional ligation of the YNH24 probe with the adaptors. For exact description of the experimental conditions see Materials and methods

et al. 1992; Edwards et al. 1992; Kimpton et al. 1992; Wiegand et al. 1993; Boerwinkle et al. 1989; Rand et al. 1992; Skowasch et al. 1992) D2S44 has not yet been examined by PCR-amplification. Due to the advantage of PCR-based DNA typing by applying human cocktails composed of different alleles, the aim of this study was to determine the terminal sequences of the YNH24 probe according to the methods described by Edwards et al. (1991; Fig. 1), the construction of D2S44 specific primers and the optimization of amplification conditions for human D2S44 alleles.

Materials and methods

The HaeIII-digested YNH24 probe (Promega Corporation, USA; 2 kbp) was used to determine the sequence of the flanking regions.

Blunt end ligation

Blunt end ligation was carried out using 140 ng (0.11 pmol, 2 kbp) YNH24 probe and 1 µg (27 pmol, 52´mer) adaptor in a 10 µl reaction assay consisting of 1 µl 10× ligase buffer (Boehringer, Mannheim, FRG) and 1 µl T4 DNA ligase (Boehringer, Mannheim, FRG) at 14°C overnight.

Amplification of the flanking regions

PCR was carried out with one adaptor- and 2 repeat-specific primers.

Primer sequences: Ampl (adaptor specific): 5´-CGA ATC GTA ACC GTT CGT ACG AGA ATC GCT-3´; Repri 1 (repeat-specific, identical to the 5´–3´-repeat sequence): 5´-CAG GAG CAG TGG GAA GTA CAG TGG GGT TGT-3´; Repri 2 (repeat-specific, complementary to the 5´–3´-repeat sequence): 5´-ACA ACC CCA CTG TAC TTC CCA CTG CTC CTG-3´.

PCR protocol: 0.005 pg (3×10^{-9} pmol) YNH24 probe, 0.015 µM each primer, 0.2 mM each dNTP, 10 µl 10× PCR buffer (Promega Corporation, USA) and 2.5 U Taq DNA polymerase (Promega Corporation, USA) diluted with double distilled sterile water to a final volume of 100 µl.

Amplification conditions: 1 min – 94°C; 1 min – 58°C; 1 min – 72°C; 30 cycles; final extension phase at 72°C for 7 min (Biometra Triothermoblock, Germany).

Gel electrophoresis: PCR products were separated in a preparative 1.8% agarose gel for 2–3 h at 100 V. Visualization of the DNA bands was performed under UV light after ethidium bromide staining. DNA fragments were cut out of the gel. DNA was isolated from the gel by electroelution, desalted and concentrated twice with centricon 100 filtration (1000 g, 20 min; Amicon Beverly, USA).

Asymmetric PCR (Gyllensten and Erlich 1987)

Asymmetric PCR was carried out as described above with 5, 10 and 20 µl centricon eluate, 0.05 µM repeat- and 0.01 µM adaptor-specific primers. Single stranded DNA was separated on a 12% polyacrylamide gel and visualized by silver staining (Budowle et al. 1991).

Sequencing reactions (Sanger 1977)

Sequencing reactions were carried out with 100 ng (1.5 pmol) single stranded DNA, 10 pmol adaptor-specific sequencing primer and the T7-Deaza-Sequencing-Kit (Fa. Pharmacia, Freiburg, FRG) according to the standard protocol.

Seqpri 1: 5´-TAC GAG AAT CGC TGT CTC TGC AGT-3´.

Amplification of human DNA with D2S44-specific primers

DNA was extracted from blood according to the method of Brinkmann et al. (1991) and quantified using the slot blot technique and the human specific probe D17Z1 (Gibco BRL) (Waye et al. 1989).

Primer sequences: P1 (22´mer): 5´-CAG TGA GGG AGG CTG AGT TCA A-3´; P2 (29´mer): 5´-TGC TTG CTA TAG CAA ATA GAA TTT ACT GC-3´.

PCR protocol: 2 ng template DNA, 0.05 µM each primer, 10 µl 10× PCR buffer (Promega Corporation, USA), 0.2 mM each dNTP and 2.5 U Taq DNA polymerase (Promega Corporation, USA) diluted with double distilled sterile water to a final volume of 100 µl. All PCR-amplifications were performed with negative (without DNA) and positive (cell line K562, YNH24 probe) control samples.

Amplification conditions: 1 min – 94°C, 1 min – 52°C, 4 min – 72°C; 30 cycles with a final extension phase at 72°C for 7 min (Biometra Triothermoblock).

Nuclease S1 treatment

50 µl of the PCR assay were mixed with 10 µl 10× nuclease S1 buffer (Maniatis et al. 1989) and 1 U/µl nuclease S1 (Boehringer Mannheim, FRG), diluted with double distilled sterile water to a final volume of 100 µl and incubated at 37°C for 60 min. After electrophoresis (1% agarose gel, 1× Tris/Borate/EDTA buffer, 35 V, overnight) DNA was transferred to nylon membranes (Hybond-N, Amersham, FRG) by Southern blotting (20× SSC, Southern 1975). Hybridization was carried out according to the NICE standard protocol (ICI) using the GenePrint Light probe YNH24 (D2S44) (Promega Corporation, USA). Fragment sizes were determined automatically using the Bioimage (Millipore).

Results

Sequencing and determination of the terminal sequences

Amplification of the flanking regions was optimized by testing serial dilutions of primer concentrations, and the best result was obtained with 0.015 µM of each primer (Fig. 2). Determination of the sizes by agarose gel electrophoresis revealed PCR fragments 180 and 250 bp long.

The flanking regions were successfully sequenced and the passage from the repeat to the flanking region could be demonstrated. A total of 60 bp could be determined for each PCR fragment. D2S44 specific primers were directly located at the transition between the repeat and flanking region (Fig. 3).

Experimental studies with the YNH24 probe

PCR-amplification was optimized using 0,005 pg (3×10^{-9} pmol) YNH24 probe, different primer concentrations (0.05–0.25 µM) and various annealing temperatures. The best result was obtained with 0.05 µM each primer and 52°C

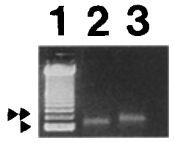

Fig. 2. Amplification of the flanking regions in the polymorphism D2S44. Amplification was carried out with 0.005 pg YNH24 probe and 0.001 µM each primer according to the described standard protocol. PCR fragments separated on a 1.8% agarose gel and visualized using ethidium bromide staining. *(1)* 123 bp ladder (▶ 123 bp, ▶▶ 246 bp); *(2)* 180 bp PCR product of the 5′-flanking region; *(3)* 250 bp PCR product of the 3′-flanking region

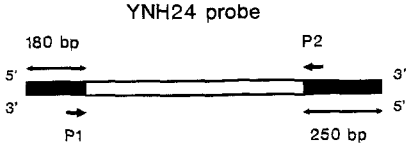

Fig. 3. YNH24 probe with the position of the D2S44 specific primers (P1 and P2) and the fragment sizes of the flanking regions (▬ flanking region; ═ repeat region (1430 bp))

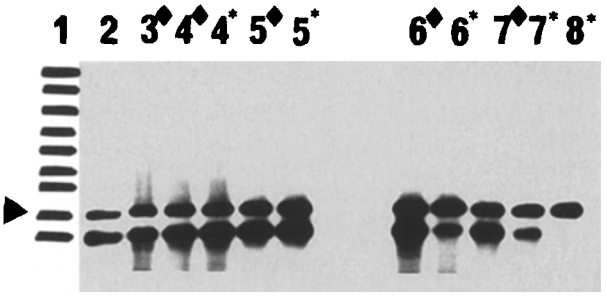

Fig. 4. Amplification pattern of the YNH24 probe using different nuclease S1 concentrations and incubation times. 0.005 pg YNH24 probe was amplified and the PCR products separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with the GenePrint Light probe YNH24. *(1)* NICE ladder (Gibco BRL); *(2)* positive control (YNH24 probe without nuclease S1 treatment); *(3)* YNH24 probe with 1 U nuclease S1; *(4)* YNH24 probe with 2 U nuclease S1; *(5)* YNH24 probe with 5 U nuclease S1; *(6)* YNH24 probe with 20 U nuclease S1; *(7)* YNH24 probe with 50 U nuclease S1; *(8)* YNH24 probe with 100 U nuclease S1; (◆) 30 min incubation time; (*) 60 min incubation time; (▶) 1431 bp fragment

annealing temperature. Amplification of the YNH24 probe resulted in the expected 1400 bp PCR fragment and an additional smaller DNA band (Fig. 4, lane 2). Optimization of the nuclease S1 treatment was performed with half of the PCR assay, different nuclease S1 concentrations and different incubation times (Fig. 4) Increased enzyme concentration and incubation time was paralleled by a weakening of the additional band which completely disappeared at an incubation time of 60 min and 1 U/µl nuclease S1 (Fig. 4, lane 8*).

Amplification and nuclease S1 treatment of human D2S44 alleles

The same experimental conditions as previously described were used for amplification and nuclease S1 treatment of human D2S44 alleles except that the amount of template DNA was 2 ng (1×10^{-9} pmol) (Fig. 5).

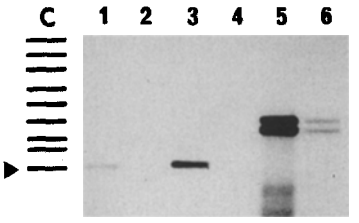

Fig. 5. Locus D2S44: Amplification pattern of a heterozygous individual with and without nuclease S1 (1 U/µl, 60 min) treatment. 2 ng of template DNA was amplified according to the described standard protocol and the alleles were visualized with the GenePrint Light probe YNH24 after electrophoresis and hybridization. Positive and negative samples were analyzed in separate lanes. *(C)* NICE ladder (Gibco BRL); *(1)* positive sample: 1400 bp PCR fragment of the YNH24 probe after nuclease S1 treatment; *(2)* negative sample (without DNA); *(3)* positive sample: YNH24 probe (2.5 ng); *(4)* negative sample: salmon DNA; *(5)* D2S44 alleles without nuclease S1 treatment; *(6)* D2S44 alleles with nuclease S1 treatment; (▶) 1431 bp fragment

Different heterozygous individuals which had been investigated by RFLP analysis were examined and the fragment lengths determined by comparison with known lambda DNA fragments. Sizes determined so far ranged from 1200 bp up to 3500 bp and were in general 1000 bp shorter than Hinf I-digested D2S44 alleles. Longer alleles (>4000 bp) could not been demonstrated.

Discussion

The strategy proposed by Edwards et al. (1991) also proved to be applicable to the D2S44 flanking regions. The adaptor-specific primer was 9 bp shorter. The second primers Repri1 and Repri 2 were selected from the repeat region of the locus D2S44.

Due to the high G/C content (55%) within the repeat region the electrophoresis temperature during sequencing was increased from 55° C to 70° C to prevent formation of secondary structures.

Although a variety of PCR parameters were applied, D2S44 alleles could not be directly visualized in agarose gels using ethidium bromide staining. This phenomenon was also described by Jeffreys et al. (1988) and Deka et al. (1992) especially for the amplification of longer alleles. Demonstration of alleles, however, was successfully carried out by non-radioactive hybridization with the alkaline phosphatase labelled YNH24 probe.

Shorter PCR fragments, in addition to the D2S44 alleles, could be eliminated by applying nuclease S1 treatment after PCR amplification. Jeffreys et al. (1988) observed the same phenomenon and attributed it to the existence of single stranded and incompletely extended alleles. Compared with the conditions described by Jeffreys et al. (1988) the nuclease S1 concentration was increased tenfold to 1 U/µl and the incubation time extended from 30 min to 60 min. Applying these modifications, isolated alleles of the polymorphism D2S44 could be demonstrated.

Thus, it is possible to analyze certain VNTR polymorphism, such as D2S44, not only by RFLP analysis, but also by initial amplification by PCR.

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