ISOLATION OF A (ATTTT)N POSITIVE LOCUS BY CLONING, PCR CYCLE SEQUENCING AND NONRADIOACTIVE DIRECT BLOTTING ELECTROPHORESIS

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Introduction:

Hypervariable human DNA markers have a profound impact on medicine. Most DNA typing systems use tandem repetitive minisatellites or VNTR loci, which can show extreme levels of allelic variability in repeat copy number and therefore DNA fragment length. To create a single locus probe, we were working with the minisatellite family (ATTTT)n. This is the only repetitive sequence family with a pentanucleotide sequence unit, described in the human genome (Vogel et al. 1990). We want to demonstrate the isolation of a (ATTTT)n positive locus by cloning, PCR cycle sequencing and nonradioactive Direct Blotting Electrophoresis. The isolated locus is hypervariable and can be used for forensic investigations.

Material and methods:

Oligonucleotide synthesis: Synthetic oligonucleotides were synthesized according to the phosphoramidite method, using a Gene Assembler (Pharmacia). The oligonucleotides were deprotected and desalted by NAP 10 columns (Pharmacia).
Isolation and digestion of DNA: Human genomic DNA was isolated according to Miller et al. (1988). The DNA was digested with ALU I (Boehringer).
Electrophoresis: Electrophoresis was performed in 0,9% agarose gels (20x20 cm), 30h, 40 V.
Hybridization: 3'OH digoxigenated oligonucleotides (ATTTT)5 and Oligo 2 were used as hybridization probes (5xSSPE, 5xDenhardts solution, 0,1% SDS, 10 g/ml denatured E. coli DNA).
Detection: Nonradioactive immunological detection was performed according to the Lumigen manual (Boehringer).
Cloning: Alu I digested 3-4 kb DNA fragments were isolated from Agarosegel (Quiaex / Quiagen) and cloned in Sma I digested pGEM-3Zf(+) vector (Promega). Recombinant clones were randomly selected and screened with (ATTTT)5. DNA from (ATTTT)5 positive clones were isolated with QIA prep-spin Plasmid Kit (Quiagen).
Sequence analysis: PCR cycle sequencing was performed with the Dig Taq DNA Sequencing Kit (Boehringer). To separate the sequence reaction we used the direct blotter from MWG.

Results and Discussion:

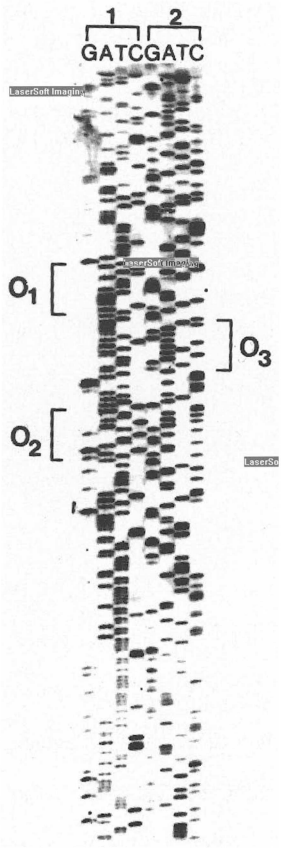
Synthetic oligonucleotides, which contain very much A and T such as TT(ATTT)5 and (ATTTT)5 were synthesized. Hybridization of (ATTTT)5 on DNA, digested by Alu I, results in a multilocus fingerprint pattern. This oligonucleotide generates individual specific DNA banding pattern in humans.

Developing a single locus probe, individual (ATTTT)5 bearing DNA loci from the 3 to 4 kb region of such an ALU I digested human DNA fingerprint were isolated. These 3 or 4 kb DNA fragments were cloned in vector pGEM-3Zf(+), which was digested with Sma I. Six (ATTTT)5 positive clones were randomly selected and showed a polymorphic pattern with ALU I digested DNA of unrelated persons.

To identify the sequence of the loci nonradioactive sequencing and directblotting electrophoresis were performed. PCR cycle sequencing was used to produce DNA single strands.A digoxigenated M13 primer was used and given to the DNA of the recombinant vectors. This circular vector was subjected to a normal PCR with therefore dd nucleotides in 4 caps respectively. After 25 PCR cycles the sequencing reaction and DNA single strands were produced at the same time.
PCR cycle sequencing presents many advantages to normal PCR:
- direct sequencing of template DNA is possible without additional effort to make single stranded DNA.
- 25 PCR cycles result in a huge quantity of single stranded DNA, this takes effect on the signal strength of the banding pattern in nonradioactive detection. So it is possible to read 500 bp in most cases.
- cycle sequencing is a very good method to sequence small quantities of ds DNA. Considerably less DNA template is required than in normal didesoxy sequencing.

Direct blotting electrophoresis was used to seperate the sequence reaction. This method requires a special sequencing device (Pohl et al. 1992): During the electrophoresis a DNA binding membrane passes by under the edge of the gel plates. So the DNA fragments leave the gel and automatically bind on the nylon membrane. This method doesn't need the efforts in blotting, is very quick and minimizes the loss of DNA in transfer, because the DNA is carried over directly from the gel to the membrane. With PCR cycle sequencing , direct blotting electrophoresis and nonradioactive detection of the sequence patterns, the results can be obtained within one day.

All of our six recombinant, (ATTTT)5 positive clones were partially sequenced (FIG.: 1) and we found that they contain the same DNA fragment. No repetitive sequence is seen in this part of the DNA fragment, however in this sequence A and T nucleotides appear very often (76,4%). Perhaps we will be able to identify repetitive minisatellites in further sequencing. Comparison of Genebank and EMBL sequences showed that we dealt with unpublished sequences. To verify if the sequence is the flanking region of a new detected minisatellite an oligonucleotide, homologous to parts of the sequenced DNA was sythesized (5'CATTACATGCTTAGCATGA3'). These oligonucleotide (Oligo 2) was reprime labeled with Digoxigenin and then hybridized to Alu I digested DNA. The DNA fragments, isolated and cloned in vector pGEM-3Zf(+) were detected. Every person shows individual specific banding pattern (Fig.: 2). So we discovered a hypervariable DNA locus and found out that the oligonucleotide can be used as a single locus probe for forensic investigations. Now we are sequencing the whole cloned DNA fragment. Future investigations will show the allelic frequency of this new detected minisatellite in different population samples.



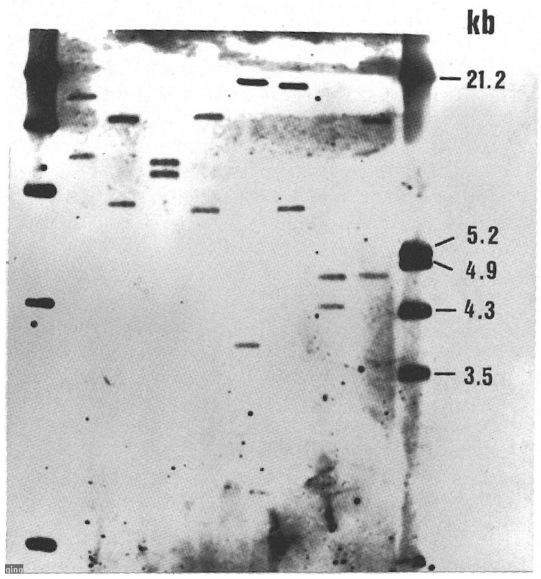


Fig.2: DNA profile of Alu I digested DNA, revealed by Oligo 2 DIG.

Fig.1: Nonradioactive sequence ladder of a recombinant (ATTTT)5 positive clone. (1) sequencing with M13 DIG forward primer, (2) sequencing with M13 DIG reverse primer

Literature:

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