INVESTIGATIONS TO IMPROVE ALLELE DEFINITION IN THE "COLLAGEN 2A1" SYSTEM (AMP-FLP)

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INTRODUCTION

Continuous distributions of fragments are still characteristic of the highly polymorphic single locus VNTR systems (Puers et al. 1991). To solve these problems different bin approaches and match criteria have been elaborated (Baird et al. 1986, Brenner and Morris 1990, Gill et al. 1990). Another inherent problem lies in the use of λ-DNA as marker DNA whose sequences differ considerably from those to be measured. The electrophoretic mobility of DNA is also influenced by sequence and composition. Therefore, λ-DNA is basically not suitable for estimating size of (human) fragments. One concept for a more exact estimation of allele size is to compare the unknown human fragment with a human DNA standard composed of as many alleles from the system under investigation as possible (Puers et al. 1991).
The construction of such a standard is also possible with AMP-FLPs (Amplified Fragment Length Polymorphism) where PCR can be used to construct an allelic ladder.

One such system is "COL 2A1" (12 q 14.3) which is located in the 3'-flanking region of the Collagen gene and was initially sequenced by Stoker et al. (1985). Two types of primers have been applied to amplification (Wu et al. 1990, Priestley et al. 1990), therefore fragments recovered by Priestley et al. are 72 bp longer than those of Wu et al.. Priestley et al. described 5 alleles, in the range between 650 and 890 bp while Wu et al. described 4 alleles in the range 600 - 700 bp. The repeat sequence can be 31 or 34 bp long (Stoker et al. 1985) and is inherited in a mendelian fashion. This situation can therefore lead to fragments which are different in composition but identical in their electrophoretic mobility.
In spite of these considerations the aim of this investigation was to construct a human DNA standard to achieve better comparability.

MATERIALS AND METHODS

Blood samples were extracted as previously described (Brinkmann et al. 1991). The amplification was carried out in a Thermocycler (Biometra) with primers (20mers) described by Wu et al. (1990) under the following conditions: Denaturing: 94°C, 1 min. Annealing: 60°C, 1 min. Extension: 72°C, 1.5 min. Cycles: 25
Each amplification sample contained 100 ng human genomic DNA, 200 μM dNTP, 1x Taq buffer (Promega), 2 U Taq polymerase (Promega) and primers (0.5 μM). The total assay volume was 50 μl and this was covered by 30 μl mineral oil.

The first electrophoretical separation of the amplified fragments (Fig. 1) was carried out in PA-gels (PA = polyacrylamide) (6% T, 3% C; 400 μm; 10 cm; horizontal) with piperazine diacrylamide as cross-linker. The leading buffer was 140 mM borate solution and the tracking buffer was 35 mM sulfate solution.
The final separations (Figs. 2 - 5) were performed in PA-gels (5% T, 2,5% C; 400 μm; 20 cm; horizontal) with piperazine diacrylamide as cross-linker, leading buffer 280 mM borate solution and tracking buffer 80 mM formate solution (Budowle, pers. com. 1991). All buffers were adjusted with TRIS to pH 9.0. To eliminate any artefacts due to amplification efficiency, equimolar mixtures of human DNA amplifications were prepared.

RESULTS AND DISCUSSION

The first standard (Fig. 1) consisted of 6 alleles taken from amplified DNA from 6 individuals using 6 % PAG for separation. However, using this gel, the separation was insufficient. The band no. 4 (lane S) was a combination of corresponding alleles from individuals I-IV but 2 individual samples (III, IV) exhibit 2 alleles in this region (Fig. 1). It was therefore necessary to improve the electrophoretic conditions for separation. A modification of the running conditions led to the production of a second standard ladder (Figs. 2; see materials and methods).

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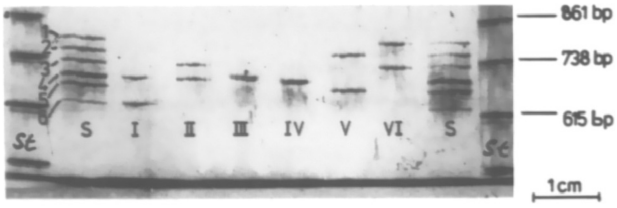

Fig. 1: First standard for the AMP-FLP system "COL 2A1" (**S**) and PCR amplified human DNA samples (**I - IV**) from which it was constructed. Separation of DNA fragments was performed in a 6 % PA-Gel using 140 mM borate solution as leading buffer and 35 mM sulfate solution as tracking buffer. TRIS was used in both buffers to adjust the pH to 9.0. The gel was silver stained (Budowle et al. 1991). The original distance between the smallest and largest standard fragment (lane M) was 1.9 cm. **St** = 123 bp ladder (Gibco)

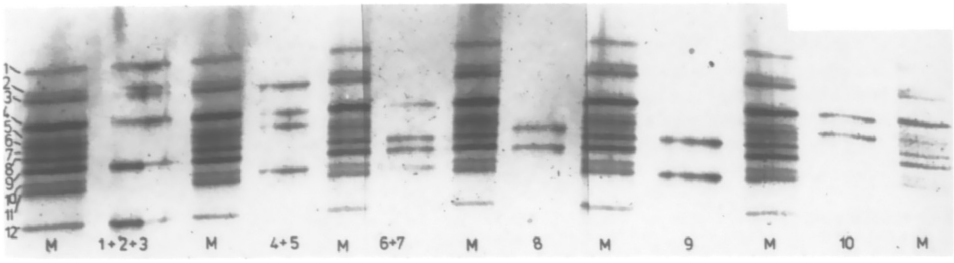

Fig. 2: Magnified section of the PA gel showing the second improved standard for the AMP-FLP system "COL 2A1" (**M**) and the PCR amplified human DNA samples (**1-10**) from which it was constructed. Separation of DNA fragments was performed in a 5 % PA gel using 280 mM borate solution as leading buffer and 80 mM formate solution as tracking buffer adjusted with TRIS to pH 9.0 (Budowle, pers. com. 1991). The gel was silver stained (Budowle et al. 1991). **1+2+3**: Mixture of the "COL 2A1" DNA fragments of the individuals 1,2 and 3; **4+5**: Mixture of the "COL 2A1" DNA fragments of the individuals 4 and 5; **6+7**: Mixture of the "COL 2A1" DNA fragments of the individuals 6 and 7; **8,9,10**: Separate "COL 2A1" DNA fragments of the individuals 8,9 and 10. The original distance between the smallest and largest standard fragment (lane M) was 1.9 cm

One criterion for the quality of separation is the distance between the smallest and largest standard fragment. This measurement of 1 cm (Fig. 1) could be improved to 1,9 cm using the final method of separation (Fig. 2; see materials and methods).
The second standard finally contained 13 definable alleles (Fig. 3) but sometimes only 12 could be differentiated (Fig. 2).
Figure 3 impressively demonstrates the problems with this type of electrophoresis.
It can be seen that the separate DNA fragments in lane A and B cannot be clearly assigned to the corresponding bands of the allelic ladder, although they are components of this ladder.
PA gels cannot yet be produced with a unified quality so that the results are not reproducible.
It is necessary to obtain an even higher quality of PAG electrophoresis and resolution of fragments in this system from which a larger number of alleles can be assumed.
The smallest distance between the fragment lengths which can be distinguished by the BioImage Video measuring system (Millipore) is only 3 bp but this is difficult to resolve.
One possible improvement would be to label the "COL 2A1" fragments radioactively and to separate them on a sequencing gel (40 cm PAG, denaturing conditions). Sequencing of the individual alleles to verify the gene model would also be desirable.
The "COL 2A1" system seems to be much more complex than was originally assumed in previous publications (Stoker et al. 1985; Priestley et al. 1990; Wu et al. 1990).
Nevertheless this system can be a useful tool for identification and paternity analysis using a binning approach.

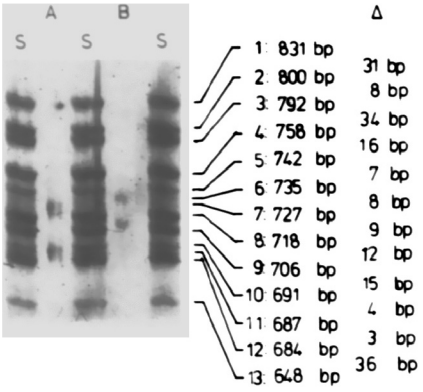

Fig. 3: Magnification of the second improved human DNA standard (**S**) separated in a second gel using the same method as presented in Figs. 2 - 4. Here it is possible to differentiate 13 alleles instead of 12 in the standard. The DNA fragment lengths were estimated by comparison to the 123 bp ladder using the BioImage system. In lanes **A** and **B** amplified "COL 2A1" fragments from the separate individuals from which the standard is constructed are only recognizable with difficulty. The fragments in lane **B** demonstrate the problems with the gels presently in use. The correct assignment of unknown alleles in such gels is impossible, although they give a high resolution for the human standard

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