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Structural variation in the alleles of a short tandem repeat system at the human alpha fibrinogen locus

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Abstract This paper reports the sequences of 22 alleles identified at the HumFGA (human alpha fibrinogen) short tandem repeat locus in the British Caucasian and Afro-Caribbean populations. Alleles at the lower end of the observed size range were found to increase in size by 4-bp increments with the repeat unit following the pattern $TC(TCTT)_n$. However, 5 alleles were identified that differed in size by 2 bp from the 4 bp increment as a result of the deletion of a TC dinucleotide, or the addition of a TT dinucleotide, immediately prior to the 1st repeat unit. Alleles at the upper end of the observed size range were found to have a more complex repeat unit structure and also exhibited duplication of both 5′ and 3′ flanking sequences. A nomenclature for the designation of HumFGA alleles is proposed on the basis of this sequence data.

Key words Polymerase chain reaction · Short tandem repeat · HumFGA · Allelic designation · Sequence variation

Introduction

Short tandem repeat (STR) loci are widely distributed throughout the human genome, occurring with a frequency of 1 locus every 6–10 kb (Beckmann and Weber 1992). STRs are composed of tandemly repeated sequences of 2–5 bp in length, and many have been shown to be polymorphic, with alleles differing in the number of repeat units (Craig et al. 1988) and, in some cases, their base sequence (Adams et al. 1993; Urquhart et al. 1993; Möller and Brinkmann 1994; Möller et al. 1994). The polymorphic nature and accessibility of STR alleles to amplification using the polymerase chain reaction (PCR) has led to their recent introduction into forensic identity testing (Frégeau and Fourney 1993; Kimpton et al. 1993, 1994; Lygo et al. 1994).

This laboratory recently introduced a multiplex PCR-based DNA profiling system (Kimpton et al. 1994) into the analysis of casework samples. The system is based on the co-amplification of 4 tetranucleotide repeat loci, which are sized using an ABI 373A DNA sequencer, GENESCAN 672 software and a commercially available size standard. Development work is underway on a second multiplex system in order to increase the evidential value of PCR evidence currently presented in court (Urquhart et al. 1995; Oldroyd et al. 1995). This second-generation multiplex will be composed of the 6 STR loci HumFGA (Mills et al. 1992), D18S51 (Straub et al. 1993), D21S11 (Sharma and Litt 1992), D6S502 (selected from the Co-operative Human Linkage Centre Database, Oldroyd et al. 1995), HumvWA (Kimpton et al. 1992) and HumTHO1 (Edwards et al. 1991). A sex test, which makes use of a 6-bp deletion of the amelogenin gene on the X chromosome (Sullivan et al. 1993), is also included.

As part of the validation of the second-generation multiplex we have sequenced alleles identified at the HumFGA locus (GenBank acccession number M64982). The HumFGA locus is located in the third intron of the human alpha fibrinogen gene at chromosomal position 4q28 and has been shown to be co-dominantly inherited (Mills et al. 1992). In a limited population survey Mills and colleagues identified 9 alleles, ranging in size from 256 to 284 bp. The polymorphic repeat unit was reported to consist of the tetranucleotide sequence TCTT and alleles differed in size by 4 bp increments in most cases. However, an allele of 266 bp pairs was observed which, it was postulated, may have arisen from either the deletion of a dinucleotide from the 268 bp allele, or the insertion of a dinucleotide within the 264 bp allele. As a result of population databasing studies within this laboratory, and within those of the Forensic Science Service (Birmingham, UK), 22 HumFGA alleles have been identified. This paper reports the sequences of these alleles and data from the repeat unit, flanking regions and primer binding sites are presented. Allelic designations are proposed on the basis of this sequence data.

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Materials and methods

Sample preparation

Bloodstains were prepared on pieces of clean cotton cloth from donated blood samples and then allowed to dry at room temperature. The stains were stored at –70° C until ready for use. Genomic DNA was extracted from a bloodstain using the chelex procedure as described previously (Walsh et al. 1991). Recovered DNA was quantified by dot blot hybridisation to a higher primate specific probe (Walsh et al. 1992).

First round PCR amplification

A first round PCR amplification was performed with 10 ng of genomic DNA in a final concentration of 1 × GeneAmp PCR buffer (Perkin-Elmer), 200 µM dNTPs (Sigma), 0.25 µM each primer (Fig. 1 a) and 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer) in a total reaction volume of 50 µl. The reactions were performed in 0.5 ml GeneAmp thin-walled reaction tubes (Perkin-Elmer) and were overlaid with 50 µl of mineral oil (Sigma). Amplification was carried out in a Perkin-Elmer Cetus 9600 thermal cycling block for 32 cycles of 95° C for 60 s, 60° C for 60 s, 72° C for 60 s and a final extension at 72° C for 10 min.

Second round PCR amplification

Gel purification of individual STR alleles was carried out by horizontal gel electrophoresis in 4.5% Metaphor agarose (Flowgen) containing 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0, 0.5 µg/ml ethidium bromide). Electrophoresis was carried out at 10 V/cm for 5 h at 15° C in recirculated 0.5 × TAE running buffer. DNA bands, visualised under ultraviolet irradiation, were excised and then purified using a Mermaid gel purification kit (BIO 101) according to the manufacturers' instructions. An aliquot of the recovered PCR product containing approximately 1 ng of DNA was re-amplified over 12 cycles using the same conditions described above with the exception that primer concentration was reduced to 0.1 µM. The sequence data obtained in this study was that of the sense (TCTT) strand, therefore the PCR primers used in the second round amplification carried a 5′ biotin label on the antisense (AGAA) strand.

Solid phase sequencing reactions

An aliquot of the second round amplification containing 0.75–1.00 µg of DNA was added to 300 µg of Dynabeads M-280 Streptavidin (Dynal) in the presence of a final concentration of 2 M lithium chloride and incubated at 48° C for 15 min. All the following wash steps were carried out in a total volume of 500 µl with the aid of an MPC-E magnetic block (Dynal). The immobilised DNA was washed in TT buffer (250 mM Tris-HCl pH 8.0, 0.1% Tween 20) and then in double distilled water prior to being denatured in 1.5 M NaOH for 4 min at room temperature. The eluted non-biotinylated strand was removed and the DNA/bead complex washed sequentially in TT buffer and TET buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% Tween 20) before being resuspended in an appropriate volume of water.

Solid phase sequencing was carried out using a Prism Sequenase dye terminator single-stranded sequencing kit (Applied Biosystems) in a total reaction volume of 20 µl according to the manufacturers' instructions. The sequencing primer was the non-biotinylated primer used in the second round PCR amplification reaction. Unincorporated labelled terminators were removed by washing the DNA/bead complex once in TT buffer, twice in 80% ethanol containing 1.35 M ammonium acetate and once in TET buffer. The samples were then resuspended in 4 µl of loading buffer (deionised formamide containing 5 mM EDTA pH 8.0), denatured at 37° C for 4 min and placed on ice. The samples were loaded onto a 6% polyacrylamide gel containing 8.3 M urea and 1 × TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 0.125 mM EDTA, pH 8.3) and analysed on a model 373A automated DNA sequencer (Applied Biosystems). Electrophoresis was carried out at 2500 V, 40 mA, 30 W, 40° C for 12 h. Sequence data were analysed using 373A version 1.2.1 analysis software (Applied Biosystems). Consensus sequences were generated for each allele in this study from two separate experiments using SeqEd version 1.0.3 software (Applied Biosystems).

a	HumFGA 1	$^{5'}$ACTGGCATTCATGGAAGGCTGC$_{3'}$
	HumFGA 2	$^{5'}$TCGGTTGTAGGTATTATCACGGTCTG$_{3'}$
b	HumFGA 3	$^{5'}$Hex-GCCCCATAGGTTTTGAACTCA$_{3'}$
	HumFGA 4	$^{5'}$TGATTTGTCTGTAATTGCCAGC$_{3'}$

Fig. 1 **a** HumFGA primer sequences for the first and second round amplifications. **b** HumFGA primer sequences from the second generation multiplex system (Urquhart et al. 1995; Oldroyd et al. 1995). Hum FGA3 is labelled with hexachloro-6-carboxyfluorescein *(Hex)*

Results

Allelic size range

The 22 Hum FGA alleles, ranging in size from 168 to 294 bp, identified and sequenced in the present study are shown in Fig. 2. Of these alleles, 14 (184–220 bp) were identified in population databasing studies carried out in this laboratory using DNA from 216 unrelated Caucasian individuals (M. Greenhalgh, personal communication). A further 8 HumFGA alleles (168, 176, 180, 182, 224, 230, 246 and 294 bp) have been identified in individuals of Afro-Caribbean appearance and have not, as yet, been seen in individuals of Caucasian appearance. HumFGA alleles identified in this study differed in size (78 base pairs smaller) from those determined by Mills et al. (1992) as a result of using different PCR primer sequences.

Over the size range 168–224 bp, the majority of HumFGA alleles increase in size by regular 4 base pair increments. The gap between the 2 smallest alleles (168 and 176 bp) is, however, 8 bp and the existence of an allele of size 172 bp could be inferred, but has, as yet, not been observed. Within this range 5 alleles (182, 190, 198, 202 and 206 bp) differ in size by 2 bp from the regular 4 bp increment. In addition, 3 larger alleles were identified, of 230, 246 and 294 bp, which differed markedly from the regular 4 bp incremental pattern observed in the smaller alleles.

Repeat region

Sequence data from the repeat regions of these alleles, presented in Fig. 2, show that HumFGA alleles at the lower end of the observed size range (168–220 bp) exhibit a simple repeat unit structure based on the tetranucleotide

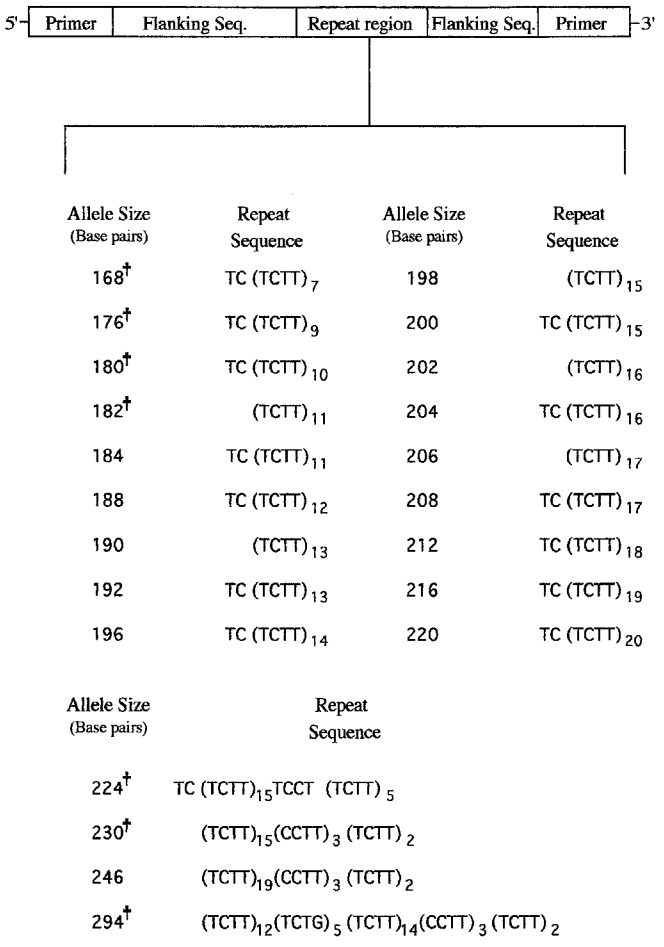

Fig. 2 Schematic representation of the 22 HumFGA alleles (TCTT strand) identified in this study, showing the repeat region sequence. †Alleles identified in databasing studies by the Forensic Science Service (A. Urquhart, personal communication)

TCTT. Most of these alleles increased in size by regular 4 bp increments corresponding to the addition of 1 extra repeat unit. In each of the 5 alleles which differed from the regular 4 bp increment, a TC dinucleotide was not observed prior to the 1st TCTT tetranucleotide repeat unit. This could have resulted from mutations such as the deletion of the TC dinucleotide or the addition of a TT dinucleotide to produce a complete TCTT repeat unit, although the mutational mechanism is unknown. Alleles at the upper end of the observed size range (224–292 bp) exhibit a more complex repeat pattern. The 224 bp allele possesses a non-consensus TCCT tetranucleotide at its 16th repeat unit. The 230 bp allele contains 17 consensus TCTT repeat units interrupted between the 15th and 16th repeats by 3 non-consensus CCTT tetranucleotides. The 246 bp allele contains 21 consecutive TCTT consensus repeat units which are interrupted between the 19th and 20th repeats by 3 non-consensus CCTT tetranucleotides. The repeat region of the 294 bp allele is even more complex. This allele has 28 consensus TCTT repeat units which are interrupted between the 12th and 13th repeats by a tract of 5 TCTG tetranucleotides and between the 26th and 27th repeats by a tract of 3 CCTT repeats. In addition, the 230, 246 and 294 bp alleles do not possess a TC dinucleotide prior to the 1st TCTT tetranucleotide repeat unit.

Alleles 168-224 bp 5'(Primer) CAGATTAAAC TGTAACCAAA ATAAAATTAG GCATATTTAC AAGCTAGTT **(TCTT)$_3$** TTT (Repeat region)

Alleles 230, 246 and 294 bp 5'(Primer) CAGATTAAAC TGTAACCAAA ATAAAATTAG GCATATTTAC AAGCTAGTT **(TCTT)$_4$** TTT (Repeat region)

Fig. 3 Sequence of the 5′ flanking sequence from the 5′ primer site (SGM) to the beginning of the repeat region, comparing alleles of 168–224 bp with the more complex alleles of 230, 246 and 294 bp

Alleles 168-224 bp (Repeat region) TCTC **(CTTC)$_2$** CTTT CTTC CTTT CTTTTTT (Primer)3'

Alleles 230, 246 and 294 bp (Repeat region) TCTC **(CTTC)$_4$** CTTT CTTC CTTT CTTTTTT (Primer)3'

Fig. 4 Sequence of the 3′ flanking sequence from the repeat region to the beginning of the 3′ primer site (SGM), comparing alleles of 168–224 bp with the more complex alleles of 230, 246 and 294 bp

Flanking regions

Alleles within the size range 168–224 bp exhibit a 5′ and 3′ flanking sequence identical to that deposited in the GenBank database. The 230, 246 and 294 bp alleles, however, all possess an additional consensus TCTT repeat unit inserted within the 5′ flanking sequence. The exact position of this insertion is not known as it occurs within, or adjacent to, a tract of 3 TCTT repeat unit motifs which are present in the 5′ flanking sequence of all HumFGA alleles as shown in Fig. 3. These 3 alleles also appear to have a duplication of 8 bp within the 3′ flanking sequence, as shown in Fig. 4.

Primer sites

The PCR primers used in the first and second round amplifications are shown in Fig. 1 a. They were designed to be external to the primers used to amplify HumFGA alleles in the second-generation multiplex system, which are shown in Fig. 1 b. This allowed for the possibility of sequence variation within the internal primer binding sites to be examined, which, it has been suggested may result in the non-amplification of alleles (Koorey et al. 1993). In all alleles examined no sequence variation was noted in either the 5′ or 3′ internal primer binding sites.

Fig. 5 The revised DNA sequence of the repeat region of HumFGA alleles and their corresponding allelic designations

Allele Size (Base pairs)	Repeat Region	Allelic Designation
168	$(TTTC)_3$ TTTT TTCT $(CTTT)_7$ CTCC $(TTCC)_2$	15
176	$(TTTC)_3$ TTTT TTCT $(CTTT)_9$ CTCC $(TTCC)_2$	17
180	$(TTTC)_3$ TTTT TTCT $(CTTT)_{10}$CTCC $(TTCC)_2$	18
182	$(TTTC)_3$ TTTT TT $(CTTT)_{11}$CTCC $(TTCC)_2$	18.2
184	$(TTTC)_3$ TTTT TTCT $(CTTT)_{11}$CTCC $(TTCC)_2$	19
188	$(TTTC)_3$ TTTT TTCT $(CTTT)_{12}$CTCC $(TTCC)_2$	20
190	$(TTTC)_3$ TTTT TT $(CTTT)_{13}$CTCC $(TTCC)_2$	20.2
192	$(TTTC)_3$ TTTT TTCT $(CTTT)_{13}$CTCC $(TTCC)_2$	21
196	$(TTTC)_3$ TTTT TTCT $(CTTT)_{14}$CTCC $(TTCC)_2$	22
198	$(TTTC)_3$ TTTT TT $(CTTT)_{15}$CTCC $(TTCC)_2$	22.2
200	$(TTTC)_3$ TTTT TTCT $(CTTT)_{15}$CTCC $(TTCC)_2$	23
202	$(TTTC)_3$ TTTT TT $(CTTT)_{16}$CTCC $(TTCC)_2$	23.2
204	$(TTTC)_3$ TTTT TTCT $(CTTT)_{16}$CTCC $(TTCC)_2$	24
206	$(TTTC)_3$ TTTT TT $(CTTT)_{17}$CTCC $(TTCC)_2$	24.2
208	$(TTTC)_3$ TTTT TTCT $(CTTT)_{17}$CTCC $(TTCC)_2$	25
212	$(TTTC)_3$ TTTT TTCT $(CTTT)_{18}$CTCC $(TTCC)_2$	26
216	$(TTTC)_3$ TTTT TTCT $(CTTT)_{19}$CTCC $(TTCC)_2$	27
220	$(TTTC)_3$ TTTT TTCT $(CTTT)_{20}$CTCC $(TTCC)_2$	28
224	$(TTTC)_3$ TTTT TTCT $(CTTT)_{15}$CCTT $(CTTT)_5$ CTCC $(TTCC)_2$	29
230	$(TTTC)_4$ TTTT TT $(CTTT)_{14}(CTTC)_3$ $(CTTT)_3$ CTCC $(TTCC)_4$	30.2
246	$(TTTC)_4$ TTTT TT $(CTTT)_{18}(CTTC)_3$ $(CTTT)_3$ CTCC $(TTCC)_4$	34.2
294	$(TTTC)_4$ TTTT TT $(CTTT)_{12}(CTGT)_5$ $(CTTT)_{13}(CTTC)_3(CTTT)_3$ CTCC $(TTCC)_4$	46.2

Discussion

The objective of the present study was to sequence the 22 HumFGA alleles which have currently been identified in order to establish their repeat unit structure and assign allelic designations. These alleles were identified in population databasing studies using DNA obtained from British Caucasian and Afro-Caribbean populations. Although the majority of alleles characterised in the present study have been observed in both ethnic groups some alleles have been observed only in individuals of Afro-Caribbean appearance. The allelic size range for HumFGA alleles (168–294 bp) reported in this paper cannot, therefore, be regarded as being representative of each population but merely indicative of the size range which may be encountered in casework samples.

In accordance with the recommendations of the DNA Commission of the International Society of Forensic Haemogenetics alleles of STR loci are designated according to the number of repeat units each allele contains. When an allele does not conform to the standard repeat unit motif it is designated by the number of complete repeats with a suffix showing the number of base pairs present in the partial repeat (ISFH recommendations 1992, 1994). Such a system eliminates problems associated with a nomenclature based on amplified fragment size, which requires very accurate sizing of individual alleles. It also simplifies the presentation of STR evidence in court and facilitates direct comparison of STR typing results obtained in different laboratories. Alleles at the 4 STR loci comprising the multiplex DNA profiling system currently employed in this laboratory have been designated in accordance with this system (Urquhart et al. 1994). It is clearly desirable, therefore, that this nomenclature is also applied to alleles of STR loci comprising the second-generation multiplex system currently under development. This is particularly important, as the two multiplex systems have the 2 STR loci HumvWA and HumTHO1 in common.

The application of such a nomenclature for the designation of HumFGA alleles is problematic in the light of sequence data reported in this paper. Alleles at the lower end of the observed size range (168–221 bp) each contain a simple repeat region based on the tetranucleotide TCTT, and could be designated according to the number of repeats they contain. These alleles are divided into those possessing a TC dinucleotide prior to the 1st repeat unit and those alleles where this dinucleotide is not present. Alleles possessing a TC dinucleotide would therefore be

assigned a suffix (.2) to denote that they contained an incomplete repeat. Thus, the alleles of 182 and 184 bp would be designated as 11 and 11.2 respectively. However, the larger alleles (224–294 bp) display a compound repeat region containing at least 1 non-consensus repeat unit. If these non-consensus repeats are not taken into account then allelic designations will increase disproportionately with allele size at the upper end of the observed size range. An allele may also have a designation which is lower than other alleles of a smaller molecular size. For example, based solely on the number of TCTT repeat units, the designations for the 230 and 212 bp alleles are 17 and 18, respectively. The situation is further complicated by the observation that the 3 largest alleles (230, 246 and 294 bp) have an additional repeat unit inserted within the 5′ flanking sequence and a duplication of 8 bp within the 3′flanking sequence.

We propose that, to create an unambiguous system for the designation of HumFGA alleles,

1. The sequence of the basic tetranucleotide repeat unit be redefined to include the non-consensus repeat sequences in order that an increase in allele size be accompanied by a proportional increase in repeat copy number. This will necessitate the use of ambiguity codes in accordance with the recommendations of the Nomenclature Committee of the International Union of Biochemistry (1985). This system has been used with alleles of other STR loci displaying compound repeat regions. For example, the repeat unit of HumvWA is designated as TCTR where R represents A or G (Kimpton et al. 1992).

2. The repeat region be extended to incorporate the additional repeat unit motif inserted in the 5′ flanking region and the 8 base pair duplication in the 3′ flanking region of the 3 largest HumFGA alleles.

The base sequence and allelic designation of the revised repeat region for each of the 22 HumFGA alleles identified in this study are presented in Fig. 5. The repeat region has been defined as the DNA sequence lying between the first tetranucleotide unit showing variation in copy number in the 5′ flanking region and the last tetranucleotide unit to show variation in the 3′ flanking region. The tetranucleotide repeat unit has been redefined as $(YYBY)_n$ using ambiguity codes in accordance with IUB recommendations. The 8 alleles (182, 190, 198, 202, 206, 230, 246 and 294 bp) that possess an incomplete repeat unit have been designated with a suffix (.2). In the revised nomenclature the number of repeat units range from 15 for the 168 bp allele to 46.2 for the 194 bp allele. However, the possibility that additional alleles may be observed, which are larger or smaller than those reported here, cannot be ruled out.

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