FEBS Letters 571 (2004) 112–118 FEBS 28617

Inhibition of DNA synthesis by K^+-stabilised G-quadruplex promotes allelic preferential amplification

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Received 5 May 2004; revised 28 June 2004; accepted 28 June 2004

Available online 6 July 2004

Edited by Ned Mantei

Abstract PCR preferential amplification consists of the inefficient amplification of one allele in a heterozygous sample. Here, we report the isolation of a GC-rich human minisatellite, MsH43, that undergoes allelic preferential amplification during PCR. This effect requires the existence of a $(TGGGGC)_4$ motif that is able to form a G-quadruplex in the presence of K^+. This structure interferes with the DNA synthesis of the alleles harbouring this motif during PCR The present results are the first demonstration that the formation of G-quadruplex can be one of the mechanisms involved in some kinds of preferential amplification.

Keywords: Minisatellite MsH43; K^+-dependent G-quadruplex; Stalling of DNA polymerization; Preferential amplification

1. Introduction

The analysis of the variation in the number of tandem repeats of several minisatellites has been employed for genotyping of the individuals in population genetics, molecular anthropology, forensics, diagnostics and paternity tests, as well as in other genetic studies [1]. Currently, the analysis of minisatellites is performed by PCR amplification. However, with this technique there may occur artefacts such as slippage products [2], heteroduplexes [3], allelic drop-out and preferential amplification [4]. The latter artefact consists of a weak amplification of one allele in a heterozygous individual. This effect may lead to poor reliability and misdiagnosis in genetic analysis. The causes of this artefact are not fully understood, but some that have been proposed include differences in allele length or GC richness, small amount of template producing stochastic fluctuations in the number of copies for each allele, and the existence of polymorphisms producing mismatches between the primer and its target sequence [4]. More complex mechanisms such as reannealing of complementary template (complete or truncated) and subsequent PCR clamping were also proposed to be involved in preferential amplification [5].

Motifs for the formation of a G-quadruplex are widely dispersed throughout eukaryotic genomes, and can be found in telomeres, promoters and at recombination hotspots [6]. G-rich DNA can fold up into highly stable G-quadruplex structures by establishing cyclic Hoogsteen bonding to form a series of guanine tetrads [7]. K^+ is a cation that fits well in the cavity between two planes of these tetrads, coordinating the adjacent guanine residues and consequently stabilizing the G-quadruplex [8,9]. Recently, this cation was observed in the crystal structure of quadruplex from human telomeric DNA [10] and it has been found that the K^+-stabilized quadruplexes can form a barrier to polymerase progression [11]. On the other hand, the stability of these structures strongly depends on the repetitive motif sequences. Thus, although the human telomere repeat sequence can fold into a quadruplex structure, it forms a duplex in the presence of the complementary C-rich strand at physiological conditions [12]. However, other repetitive sequences adopt K^+-dependent quadruplex in the presence of an excess of their complementary strand [12]. Moreover, a G-quadruplex could also be formed from duplex DNA under appropriate ionic and pH conditions [13,14] or in the presence of specific ligands [15].

In a previous work, we have reported the isolation of several GC-rich recombinant clones from a human foetal liver DNA library [16], using a small GC-rich DNA fragment (*Sma*IA) derived from the *Herpes simplex* virus type 1 genome as probe [17]. One of these clones contained the low-polymorphic minisatellite sequence MsH42 [18], which originated early in the evolution of the primate lineage [19]. This minisatellite region is able to enhance in vitro intramolecular homologous recombination [20,21]. Here, we report the isolation and characterization of a new human minisatellite, termed MsH43, that shows preferential amplification caused by the formation of a K^+-stabilized G-quadruplex.

2. Materials and methods

2.1. Isolation, sequence and PCR analysis of the human minisatellite MsH43 alleles

The screening of the human foetal liver DNA library [16] with a *Sma*IA fragment derived from the *Herpes simplex* type I viral DNA was carried out as described elsewhere [17]. One of the isolated clones, termed λ43, was digested with several restriction enzymes and the DNA fragment that hybridized with the probe was subcloned and sequenced using the Sequenase system. This allele was named MsH43 79.1. The MsH43 sequence delivered from the human genome databases corresponds to the allele MsH43 78.1 and its chromosome localization was obtained by searching in the ENSEMBL genome server of the Wellcome Trust Sanger Institute/EBI (http://www.ensembl.org). Other alleles (MsH43 80.1 and MsH43 73.1) were isolated by PCR

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.062

from the individual 01 of the CEPH (Centre d'Etude du Polymorphisme Humain) family 1347.

PCR amplification of the MsH43 locus was performed using genomic DNAs from two CEPH reference pedigrees (families 1347 and 1362). The reactions were carried out with 50 ng of genomic DNA, 0.3 μM of the primers P02.1 and P02.2 (Fig. 1), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 units of *Taq* polymerase (Roche) and buffer I (50 mM KCl and 10 mM Tris–HCl, pH 8.3) or buffer II (16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, and 67 mM Tris–HCl, pH 8.8) in a total volume of 25 μl. Cycling conditions were 29 cycles of 95 °C for 1 min, 56 °C for 30 s and 72 °C for 40 s, and a final cycle with an extension of 5 min. The PCR products were cloned in the pGemT-easy vector (Promega) and sequenced using the 377 DNA Automated Sequencer (Applied Biosystems). Several experiments were carried out varying the pH as well as the concentrations of Tris–HCl, KCl, $(NH_4)_2SO_4$ and Tween 20, as indicated in Fig. 3. PCR products were analysed by electrophoresis in 2% agarose gels.

2.2. DMS methylation protection assay

The synthetic oligonucleotides (Sigma Genosys) used in G-quadruplex analysis were F_1, F_2 and F_3 (Fig. 4A). Oligonucleotides were end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega) and purified with the Nucleotide Removal Kit (Qiagen). The specific activities of the end-labelled DNAs were approximately 5×10^8 cpm/μg. For G-quadruplex formation, 280 fmol of each end-labelled oligonucleotide was incubated at 95 °C for 5 min in 200 μl of TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA) with 50 or 100 mM KCl. Incubations were also performed in TE buffer with 100 mM NaCl, LiCl or NH_4Cl instead of KCl. After cooling down to room temperature, the DNAs were held for 60 min at 37 °C during 60 min prior to dimethylsulfate (DMS) treatment. For the methylation protection assay, the end-labelled oligonucleotides were methylated by addition of 1 μl of 1:4 ethanol-diluted DMS and incubated for 5 or 15 min. The reactions were stopped with 50 μl of stop buffer (1 M β-mercaptoethanol and 1.5 M sodium acetate, pH 7.0) and ethanol precipitated using 1 μg of calf thymus DNA as carrier. The methylated products were resuspended in 10 μl of TE buffer and treated with piperidine. Finally, the cleaved products were ethanol precipitated and resolved on an 18% polyacrylamide denaturing sequencing gel.

2.3. DNA polymerase interference assay

To analyse the inhibition of DNA synthesis provoked by the $(TGGGGC)_4$ motif, we carried out a DNA sequence assay with and without KCl. The sequencing reactions were performed following the protocol of the ABI PRISM sequencing kit (Applied Biosystems). We employed 0.16 pmol of plasmid DNA, 1.6 pmol of primer PQ2.2 and *Taq* DNA polymerase in a total volume of 10 μl. In the experiments where KCl is present, this salt was added to the reaction mixture to a final concentration of 50 and 62.5 mM. The sequencing reactions were subjected to 24 cycles of 96 °C for 10 s and 54 °C for 2 min, followed by ethanol precipitation. Samples were resuspended in 2.5 μl of loading buffer and after heating at 95 °C during 1 min, they were sequenced with a 377 DNA automated sequencer (Applied Biosystems).

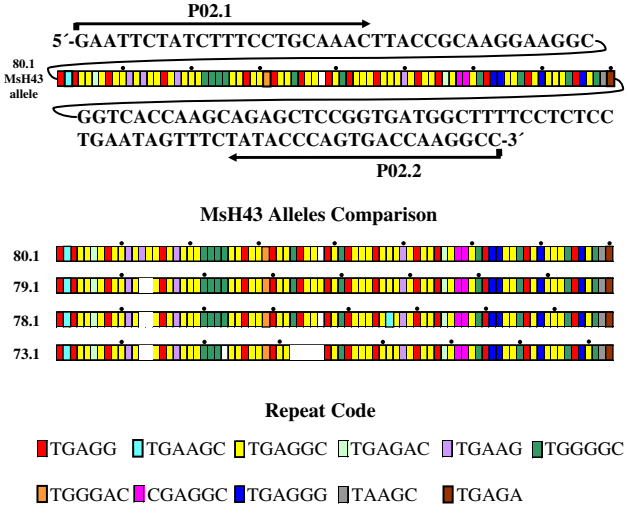

Fig. 1. Schematic representation of the human minisatellite MsH43 alleles. The alignment of the 80.1, 79.1, 78,1 and 73.1 repeat arrays is shown to facilitate their comparison. Minisatellite repeats are symbolized in a colour code showed at the bottom of the Figure. Blanks indicate arbitrary deletions used for the alignment of the alleles. Nomenclature of the MsH43 alleles is based on their number of repetitive units. Arrows signal the position of the PCR primers P02.1 and P02.2 at the MsH43 flanking sequences. Dots mark stretches of 10 repeats.

3. Results

3.1. Characterization of the human minisatellite MsH43

In a search for human repetitive GC-rich sequences, a genomic DNA library was hybridized with a viral *Sma*IA DNA fragment of 75% GC content. From this library we isolated λ43, a clone that contains the minisatellite MsH43 which is formed by 79 short repeats of hexamers and pentamers (allele 79.1 in Fig. 1). Furthermore, searching the human genome databases revealed the existence of another allele of the minisatellite MsH43 comprising 78 repeat units (allele 78.1 in Fig. 1). The sequence of this allele shows the deletion of repeat 37 and a transition A to G in repeat 46, with respect to the allele 79.1. The PCR products of the alleles 79.1 and 78.1 using the primers P02.1 and P02.2 (Fig. 1) were 560 and 554 bp, respectively. Other alleles were isolated from genomic DNAs of one CEPH reference family (see below). The MsH43 locus was localized in chromosome 2, at 2q37.1, within an intergenic area at the subtelomeric region.

3.2. Preferential PCR amplification occurs at the MsH43 locus

To analyse the inheritance of the MsH43 locus, the genomic DNA from two CEPH reference families (1347 and 1362) was amplified using the primers P02.1 and P02.2 and the buffer I described in Section 2.1. The results of these amplifications are shown in Fig. 2. Interestingly, in the individuals 01, 03, 04, 06, 09, 11, 12 and 16, the amplification of the shortest allele (allele 73.1) was stronger than that observed for the longer alleles (78.1, 79.1 and 80.1), indicating the existence of preferential amplification in the heterozygous individuals. Similar results were obtained with the CEPH family 1362 (data not shown). In contrast, no preferential amplification was detected with the buffer II (Fig. 2B). Since the enzyme used in both PCR assays was *Taq* DNA polymerase, we concluded that a component of buffer I should be responsible for the observed preferential amplification.

3.3. The allelic preferential amplification of MsH43 is mediated by K^+

Several PCR buffers were prepared by the combination of components from buffers I and II (Fig. 3A). When DNA from the individual 01 of the 1347 family was amplified with these buffers, we observed normal amplification in the reactions containing $(NH_4)_2SO_4$ instead of KCl (lanes 4, 7–9 and 11). This indicates that KCl is the component responsible for the MsH43 allelic preferential amplification. When both salts (KCl and $(NH_4)_2SO_4$) were present, poor amplification (lane 6) or none (lane 12) was obtained, probably by an inactivation of *Taq* polymerase as a consequence of the high ionic strength in these reactions.

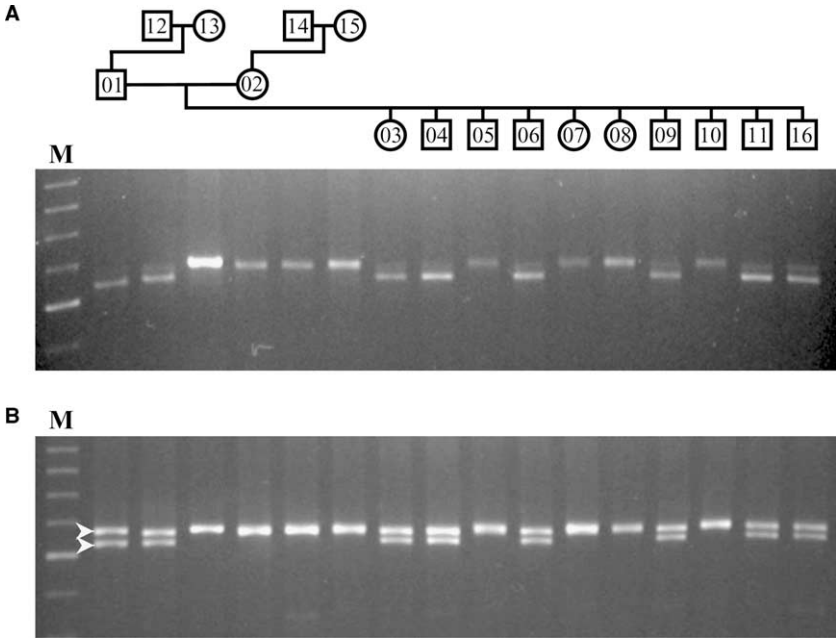

Fig. 2. Electrophoretic analyses of the MsH43 PCR products from the CEPH family 1347. (A) PCR products obtained with buffer I. Note that the products from the individuals 01, 03, 04, 06, 09, 11, 12 and 16 present preferential amplification for the allele 73.1 (short allele). (B) PCR products obtained with buffer II. The products of these reactions do not show the allelic preferential amplification. Arrowheads indicate the bands corresponding to the amplification of the MsH43 alleles 80.1 (upper) and 73.1 (lower) from the individual 01. M, 100 bp ladder.

To corroborate that KCl provokes the preferential amplification, we prepared a series of buffers (based on buffer I) with increasing concentrations of KCl (Fig. 3B). Thus, KCl concentrations in the range of 50–75 mM promoted preferential amplification, whereas KCl concentrations below 50 mM diminished the effect and higher than 87.5 mM produced *Taq* inactivation. The low ionic strength in reactions below 12.5 mM KCl also inactivated the DNA polymerase. Likewise, when KCl was progressively substituted by $(NH_4)_2SO_4$, keeping the ionic strength constant, the long allele increases its amplification gradually, whereas the shortest one remains unaltered (Fig. 3). Altogether, these data demonstrate that KCl is responsible for the preferential amplification observed in this locus.

3.4. K^+ stabilizes a G-quadruplex structure in the alleles containing the $(TGGGGC)_4$ motif

Why is the longest allele disfavoured during PCR amplification in the presence of K^+? To address this question, we sequenced both alleles of the individual 01. As shown in Fig. 1, the long allele (80.1) has 565 bp organized in an 80 repeat array, whereas the short allele (73.1) has 525 bp with 73 repetitive units. There are other differences between both alleles: (i) the allele 80.1 contains a duplication of the repeats 11 and 12, which is not present in the allele 73.1, (ii) in the allele 80.1 as well as in the other two (78.1 and 79.1), there are four contiguous TGGGGC repeats, the $(TGGGGC)_4$ motif, whereas in the allele 73.1 only three of these repeats are present, and (iii) the stretch comprising the repeats 33–36 in 80.1 is absent in the allele 73.1. Interestingly, the sequence TGGGGC is similar to the repeat unit TTGGGG of the *Tetrahymena* telomeric sequence, which has been described to acquire G-quadruplex structures [22] that could interfere with the DNA synthesis [23]. Accordingly, the $(TGGGGC)_4$ motif in the allele 80.1 would constitute a putative intrastrand G-quadruplex motif, which could participate in the K^+-dependent preferential amplification in MsH43.

K^+ is the most effective monovalent cation for stabilizing DNA G-quadruplexes [6], provoking the arrest of DNA synthesis [11]. Thus, it is plausible that this cation is interacting with the $(TGGGGC)_4$ motif present in several MsH43 alleles (78.1, 79.1 and 80.1), stabilizing a G-quadruplex structure which, in turn, would provoke the defective amplification of such alleles. Therefore, the preferential amplification of the allele 73.1 could be a consequence of its incapacity to form such a secondary structure due to the absence of the $(TGGGGC)_4$ motif in this allele.

To verify this hypothesis, we carried out a DMS protection assay. Since DMS methylates the N^7 guanine [24], protection from this methylation can be used as a diagnostic for non-Watson–Crick base pairing, characteristic of the guanine tetraplexes forming G-quadruplexes [25]. For this purpose, we designed the oligonucleotides F1, F2 and F3 (Fig. 4A). F1 contains eight repeats including the $(TGGGGC)_4$ present in the alleles 78.1, 79.1 and 80.1, F2 lacks one TGGGGC repeat, representing the allele 73.1, and F3 contains nine repeats of the minisatellite central portion and was used as a control sequence. The experiments with F1 in the presence of K^+ showed that the guanines included in the $(TGGGGC)_4$ were protected from the DMS modification. In contrast, this protection was not found in the absence of this cation (Fig. 4B). When the DMS incubations were carried out with F1 in the presence of Na^+, Li^+ or NH_4^+, no protection was detected (data not shown), indicating that these cations do not stabilize the G-quadruplex in MsH43. This result is consistent with a tetraplex formation whereby the guanines of the $(TGGGGC)_4$ act as N^7 donors, revealing that this motif is able to form G-quadruplexes in the presence of K^+. In contrast, no protection was

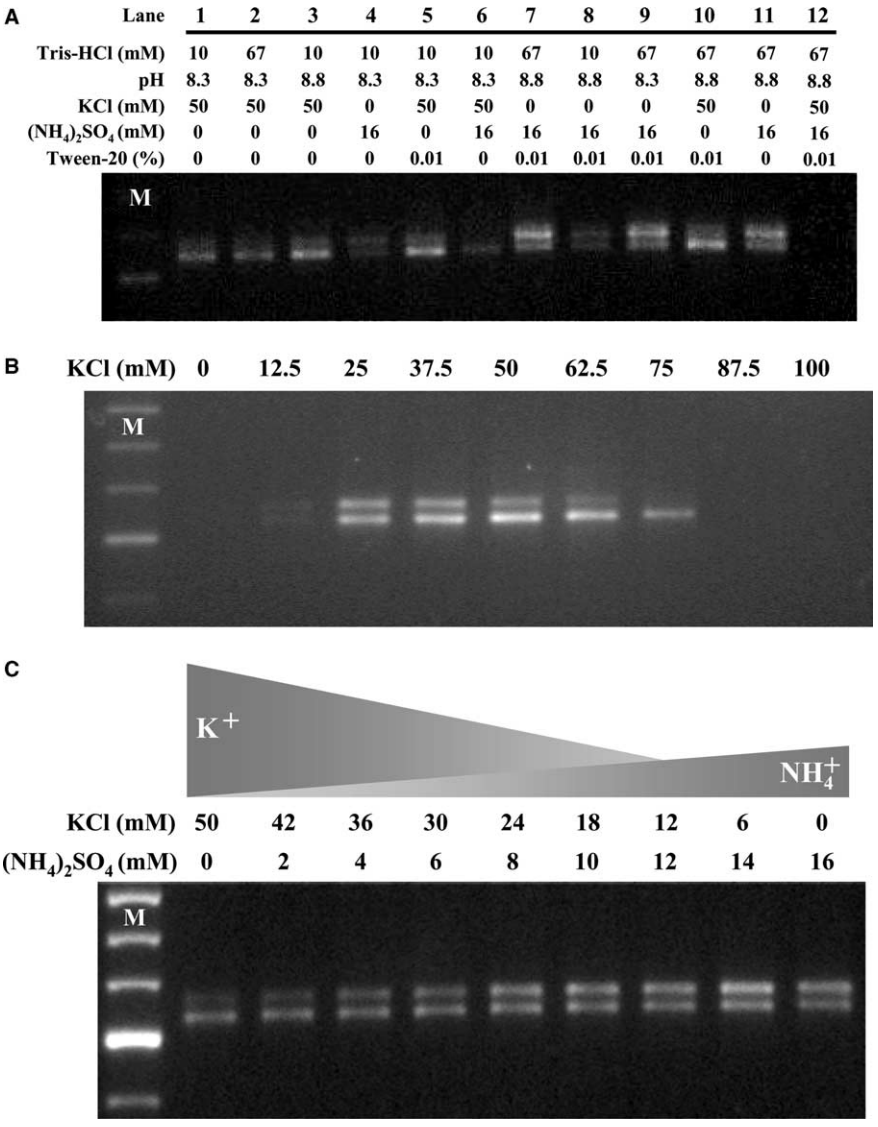

Fig. 3. Electrophoretic analyses of MsH43 amplification products employing different PCR buffers. The template DNA for these experiments was from individual 01 of the CEPH family 1347. (A) PCR products obtained with different combinations of the components from buffers I and II. Lanes 1 and 7 correspond to reactions employing buffers I and II, respectively. (B) PCR products with variants of buffer I at the indicated KCl concentration. (C) PCR products with variants of buffer I at the indicated concentrations of KCl and $(NH_4)_2SO_4$. M, 100 bp ladder.

observed in reactions using F2 or F3 oligonucleotides (Fig. 4B). Note that the guanines G_1, G_5, G_9 and G_{13} are partially reactive with DMS (Fig. 4B), suggesting that these residues are located in the loops of the G-quadruplex rather than in its stem and hence are more accessible to the reagent. Taking into account that neither F2 nor F3 have the $(TGGGGC)_4$ sequence, it is clear that this motif is absolutely necessary to form the G-quadruplex structure. Taken together, these findings suggest that MsH43 alleles containing the $(TGGGGC)_4$ motif are poorly amplified in reactions with K^+ because they can form stable G-quadruplexes that hinder the polymerase activity.

3.5. The presence of G-quadruplex provokes the stalling of DNA polymerase

The inhibition of DNA synthesis provoked by the $(TGGGGC)_4$ motif was investigated using a modified PCR sequencing reaction. In these experiments, the plasmids harbouring the 80.1 and 73.1 alleles were sequenced using the primer P02.2 with and without KCl. In the 80.1 allele, DNA synthesis was seen to stall at the $(TGGGGC)_4$ motif in the presence of 50 and 62.5 mM of K^+ (Fig. 4C). It is worth noting that this effect was detected even in the absence of K^+, but it was less prominent than in its presence. In contrast, this blockage of DNA polymerization did not occur in the 73.1 allele (Fig. 4C). These results clearly demonstrate the stalling of DNA polymerization at the location of the $(TGGGGC)_4$ motif, indicating its direct correlation with the preferential amplification detected at the MsH43 locus.

4. Discussion

Preferential amplification is provoked by a different efficiency in the amplification of the alleles in a heterozygote sample [4], entailing an important factor of misdiagnosis in

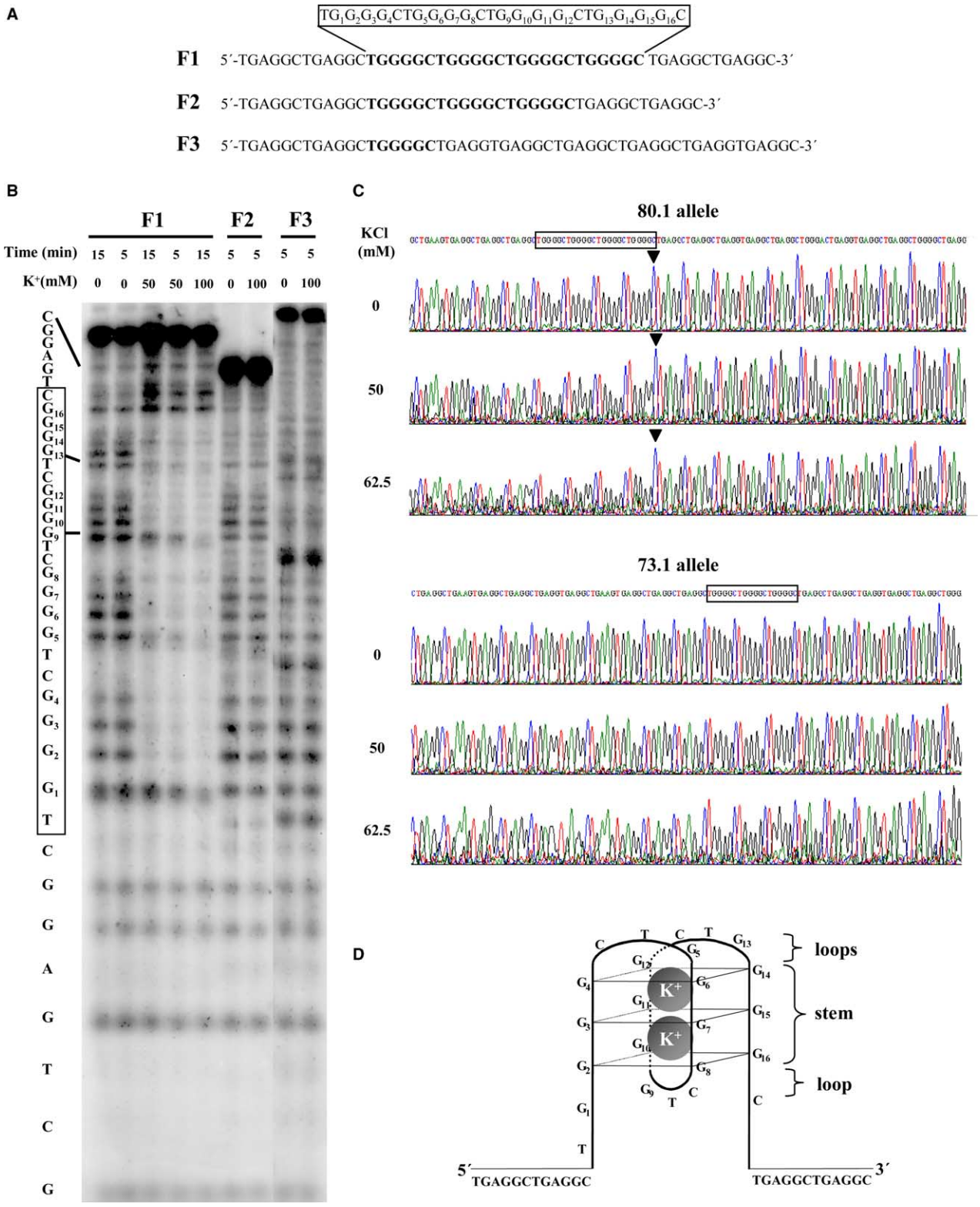

Fig. 4. G-quadruplex analysis. (A) Sequences of the oligonucleotides F1, F2 and F3 employed in the DMS methylation protection assays. The TGGGGC repeat is represented in bold face. The $(TGGGGC)_4$ motif is shown within a box and the guanine residues involved in the MsH43 G-quadruplex are numbered. (B) Autoradiogram of the denaturing PAGE gel showing the DMS methylation pattern. The DMS incubation time and the K^+ concentration in each reaction are also indicated. The oligonucleotide F1 sequence is represented on the left side. (C) Electropherograms of the DNA polymerase interference assays carried out with the MsH43 80.1 and 73.1 alleles at the indicated KCl concentrations. Boxes indicate the TGGGGC sequence in both alleles. Arrowhead shows the beginning of the DNA synthesis inhibition at the motif present in the 80.1 allele. (D) Schematic representation of a possible conformation for the G-quadruplex detected in the MsH43 locus.

many genetic analyses. For instance, this effect has been detected in DNA amplifications from both single cells and human blastomeres [26,27] and it was recently reported in the amplification of the CEB205 minisatellite locus [28]. Here, we have shown that PCR buffers containing 50 mM KC1 can lead to preferential amplification of the allele 73.1, but not of 80.1. Both alleles do not present any of the differences previously proposed to favour preferential amplification [4]. Thus, they show a similar GC content (66.2% for 80.1 and 66.0% for 73.1) and length (565 bp for 80.1 and 525 bp for 73.1). In addition, the amount of template (50 ng of genomic DNA, ~15 400 molecules) is enough to ensure that stochastic fluctuation in the number of DNA molecules in the first cycles of PCR is avoided. Moreover, since the substitution of KCl by $(NH_4)_2SO_4$ restores the efficient amplification of both alleles, mechanisms such as reannealing of complementary template [5] can be discarded as the main cause of preferential amplification in the MsH43 locus. Therefore, the preferential amplification detected in the MsH43 locus must be provoked by a mechanism different from those described previously to explain this effect. It has been demonstrated that the use of K^+-free buffers eliminates a common cause of premature chain termination in PCR and PCR sequencing [29]. Removal of K^+ is effective since it presumably eliminates or greatly reduces the stability of alternate DNA structures that can form in G-rich regions. The G-rich strand has shown the capability to form K^+-dependent intramolecular G-quadruplex in some minisatellites [30–32]. In this sense, the K^+ dependence of the preferential amplification in the MsH43 locus points towards an interaction between this cation and the DNA substrate, favouring conformational changes in the allele 80.1 that could interfere with *Taq* DNA polymerase.

The $(TGGGGC)_4$ motif in the MsH43 alleles 78.1, 79.1 and 80.1 is similar to those described to form G-quadruplexes [33]. Then, the formation of such a structure in these alleles during PCR could favour the preferential amplification of the allele 73.1, which lacks that motif. The results obtained in the DMS protection experiments, demonstrating that the $(TGGGGC)_4$ generates a G-quadruplex in the presence of 50 mM of K^+, support the hypothesis that the allelic preferential amplification is mediated by this structure. Confirming this hypothesis are the results obtained in the polymerase interference assays which show a decay of the polymerase activity coincident with the presence of the $(TGGGGC)_4$.

A possible model for the G-quadruplex structure detected in the MsH43 locus is depicted in Fig. 4D. This model is based on the observation that the guanine residues G_1, G_5, G_9 and G_{13} are partially reactive with DMS, suggesting that the N^7 groups of these guanines are not involved in the hydrogen bonds of the G-tetrads necessary to build the G-quadruplex. G_1 is probably located in the 5′-overhang region of the G-quadruplex, whereas G_5, G_9 and G_{13} would be in the loops rather than in the stem of this secondary structure, being more accessible to the DMS methylation. We propose a predominant structure for the G-quadruplex of the MsH43 locus consisting of three G-tetrads stabilized by K^+ with a diagonal loop presenting crossed strands. Similar models have been proposed for other G-quadruplex motifs [23,33,34].

The existence of proteins that promote the formation of G-quadruplex or bind to these structures for their stabilization suggested the presence of these DNA secondary structures in vivo [24]. Recently, the unfolding of the G-quadruplex formed in the mouse minisatellite Pc-1 by the binding protein UP1 was reported, indicating that this unfolding is required for DNA synthesis processes [35]. Moreover, the characterization of nucleases specific for G-quadruplexes from yeast [36] and humans [37] suggests that these structures may act as intermediates in processes such as recombination at G-rich sequences. Direct evidence for a G-quadruplex in the P1 promoter region of c-*MYC* oncogene has been described [34]. Since we have demonstrated that the MsH43 G-quadruplex was stabilized by 50 mM K^+, it is likely that this secondary structure may exist at physiological K^+ concentrations, which are approximately 150 mM in mammalian cells [38].

Acknowledgements: This work was supported by a grant of the Spanish Ministerio de Ciencia y Tecnología (BMC2001-3242). M.G.B. is recipient of a FPU grant from the Spanish Ministerio de Educación, Cultura y Deporte.

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