Experimenting with Trinucleotide Repeats: Facts and Technical Issues

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Abstract	Trinucleotide repeats are a peculiar class of microsatellites involved in many neurological as well as developmental disorders. Their propensity to generate very large expansions over time is supposedly due to their capacity to form specific secondary structures, such as imperfect hairpins, triple helices, or G-quadruplexes. These unusual structures were proposed to trigger expansions in vivo. Here, I review known technical issues linked to these structures, such as slippage during polymerase chain reaction and aberrant migration of long trinucleotide repeats during agarose gel electrophoresis. Our current understanding of interactions between trinucleotide repeat secondary structures and the mismatch-repair machinery is also quickly reviewed, and critical questions relevant to these interactions are addressed.	
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Chapter 1

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Abstract

Trinucleotide repeats are a peculiar class of microsatellites involved in many neurological as well as developmental disorders. Their propensity to generate very large expansions over time is supposedly due to their capacity to form specific secondary structures, such as imperfect hairpins, triple helices, or G-quadruplexes. These unusual structures were proposed to trigger expansions in vivo. Here, I review known technical issues linked to these structures, such as slippage during polymerase chain reaction and aberrant migration of long trinucleotide repeats during agarose gel electrophoresis. Our current understanding of interactions between trinucleotide repeat secondary structures and the mismatch-repair machinery is also quickly reviewed, and critical questions relevant to these interactions are addressed.

Key words Trinucleotide repeat, Secondary structure, PCR, Agarose gel electrophoresis, Mismatch repair

1 Introduction

Trinucleotide repeats are a peculiar class of microsatellites, extremely frequent in all eukaryotic genomes sequenced so far (reviewed in [1]). They became more famous almost 30 years ago when it was demonstrated that the large expansion of trinucleotide repeat tracts was linked to some neurological human pathologies [2–4] at the present time, trinucleotide repeat expansions are involved in more than two dozen human neurological disorders, including Huntington disease, Steinert disease (myotonic dystrophy type 1), fragile X syndrome, and Friedreich's ataxia. These disorders are monogenic, one single locus is responsible for the disease in association studies, although *cis*- and *trans*-acting genetic factors are known to modulate trinucleotide repeat instability in human cells as well as in model systems (reviewed in [1, 5–9]). Therefore, each disease is associated to the expansion of one single trinucleotide repeat tract at one single genomic location; for example, CTG triplets are expanded in Steinert disease, GAA in

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Friedreich's ataxia, CGG in fragile X syndrome, etc. (Fig. 1). Interestingly, other microsatellites besides trinucleotide repeats were also found to be responsible for several disorders: CCTG tetranucleotide repeats in myotonic dystrophy type 2, ATTCT and TGGAA pentanucleotides or GGCCTG hexanucleotides in spinocerebellar ataxias type 10, 31, and 36, respectively, and GGGGCC hexanucleotide repeat in amyotrophic lateral sclerosis (ALS) (Fig. 1). Note also that the expansion of a 12-bp GC-rich minisatellite is associated to progressive myoclonic epilepsy, a rare epileptic syndrome including seizures together with progressive neurological decline.

The molecular mechanism responsible for these large expansions is not totally understood, but past experiments in model systems (bacteria, yeast, mouse, and human cells) showed that de novo repeat-templated DNA synthesis was prone to generate contractions and expansions of the repeat tract: S-phase replication, double-strand break repair, nucleotide excision repair, and base

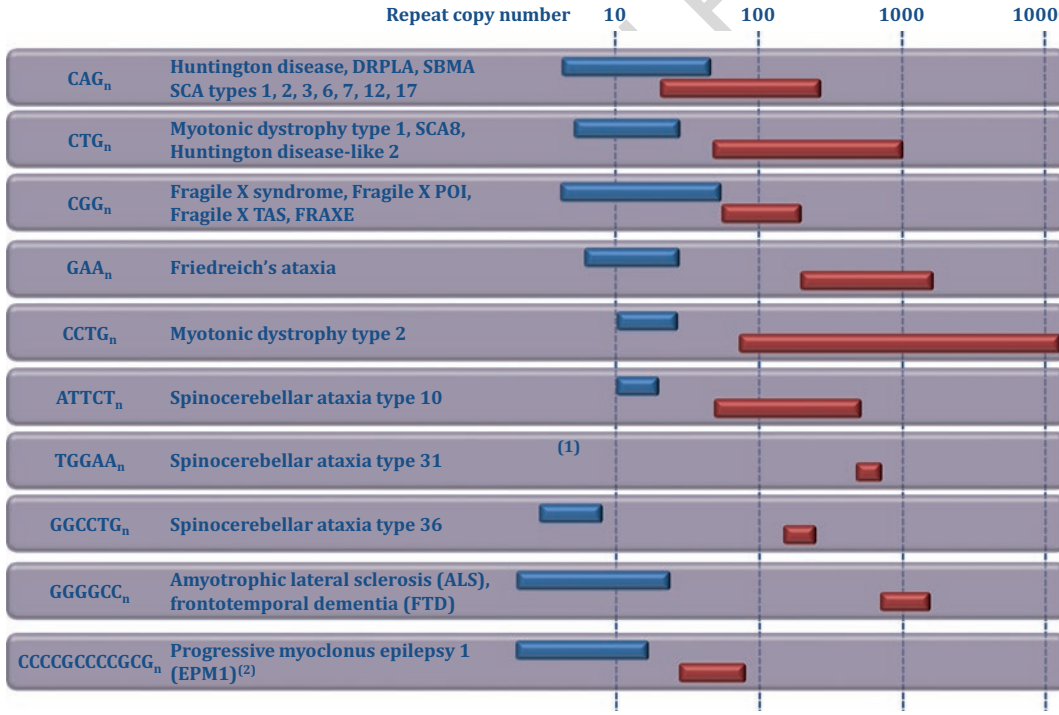

Fig. 1 Microsatellite expansion disorders. For each microsatellite, the corresponding disease(s) are indicated, as well as normal (in blue) and pathological allele lengths (in red), on a logarithmic scale. Abbreviations used: *DRPLA* dentatorubral-pallidoluysian atrophy, *SBMA* spinal and bulbar muscular atrophy, *SCA* spinocerebellar ataxia, *Fragile X POI* Fragile X-associated primary ovarian insufficiency, *Fragile X TAS* Fragile X-tremor/ataxia syndrome, *FRAXE* Fragile X mental retardation syndrome. (1) The TGGAA pentanucleotide repeat is inserted within a low complexity (TAGAA)n (TAAAATAGAA)n repeat. (2) The expanded sequence in EPM1 is not a microsatellite but is technically considered to be a minisatellite (base motif >10 bp)

excision repair all induce trinucleotide repeat instability in various experimental systems. It is not the purpose of the present chapter to describe molecular mechanisms involved in trinucleotide repeat instability; those have been thoughtfully reviewed in many places [1, 5–8]. I will rather try to focus on the peculiar properties of such sequences and on the technical issues raised by their study.

2 Trinucleotide Repeats Form Secondary Structures In Vitro

In a seminal article by the McMurray laboratory, it was early demonstrated that CAG, CTG, and CGG DNA repeats prone to expansions exhibited the property to form stable imperfect hairpins in vitro [10] (Fig. 2a, b). This work was rapidly followed by others studying hairpin properties of single-stranded CTG [11], CAG and GAC [12], GTC [13], and CGG [14] trinucleotide repeats. It must be noted that RNA molecules containing CAG, CCG, CGG, or CUG triplet repeats also fold into stable hairpins in a test tube [15, 16]. Subsequent studies showed that GAA/TTC repeats, involved in Friedreich's ataxia, were shown to form a triple helix, containing both Watson–Crick and Hoogsteen bonds (Fig. 2c) [17, 18], a specific structure common to all polypurine-polypyrimidine tracts, as demonstrated by Sergei Mirkin more than 30 years ago [19]. In addition, CGG as well as GCC triplet repeats are able to fold into DNA tetraplex (or G4, or quadruplex), similar to structures formed at the end of human telomeres [20, 21]. Formation of a CAG or CTG hairpin on one DNA strand leads to a slipped-stranded structure in which one or more mung bean nuclease-sensitive hairpin(s) are visible as bulges by electron microscopy [22]. More recently, atomic force microscopy showed that very long CAG trinucleotide repeats of various lengths (111–415 triplets) exhibited unusual structural features such as convolutions as well as single and multiple protrusions, suggesting that these structures were most probably multiple hairpins [23]. In all the above cases, the stability of the repeat-containing secondary structure increased with repeat length, making long trinucleotide repeats more prone to fold than shorter ones. Since the propensity to expansion was known to be tightly correlated to repeat size (the formerly called "Sherman paradox" [3]), soon emerged the idea that secondary structures could be triggering the expansion process [24].

3 Secondary DNA Structures and Mismatch Repair

The mismatch-repair system (hereafter abbreviated MMR), is conserved from bacteria to man [25] and is involved in detecting synthesis mistakes made by polymerases and signaling them to the repair machinery. In its absence, microsatellite instability exhibits

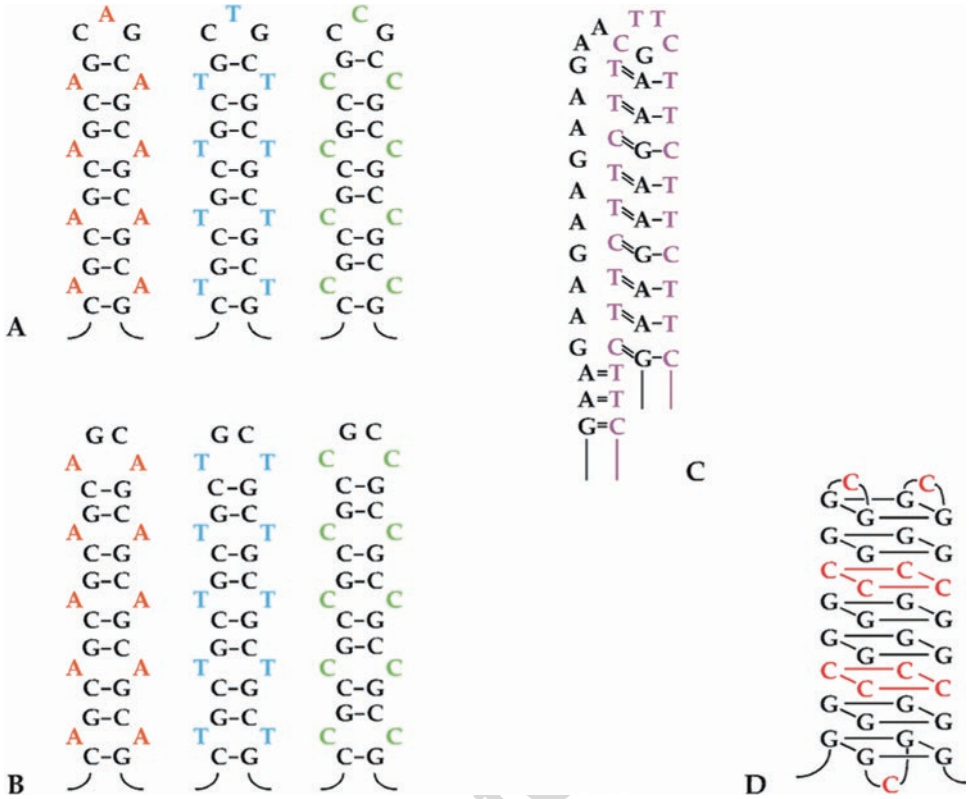

Fig. 2 Secondary structures formed by different trinucleotide repeats. (**a**) CAG, CTG, and CCG hairpins formed by odd number triplets. Unpaired bases are colored. (**b**) CAG, CTG, and CGG hairpins formed by an even number of triplets. (**c**) Triple helix formed a GAA repeat tract. Watson–Crick pairings are shown by double lines, Hoogsteen pairings by single lines. (**d**) Tetraplex structure formed by CCG repeats. Guanosine quartets are shown in black. (Figure originally published by G.-F. Richard and reprinted with permission from the American Society for Microbiology [1])

several hundredfold to thousandfold increases in *Saccharomyces cerevisiae* [26–28] and *Schizosaccharomyces pombe* [29]. Two kinds of damage are recognized by the MMR: base substitution and insertions or deletions (indels). They are processed by two slightly different machineries. The MSH2-MSH6 heterodimeric complex (MutSα) recognizes single base mismatches and small indels of 1–2 nucleotides, whereas the MSH2-MSH3 heterodimer (MutSβ) recognizes larger indels. Bound MutS complexes subsequently recruit MutL complexes, the EXO1 exonuclease and the DNA synthesis machinery to repair the error (reviewed in [30]). When the first trinucleotide repeat disorders were discovered, mismatch repair became the first obvious candidate for a possible role in large repeat expansions. Soon, experimental assays were designed in yeast to detect large trinucleotide repeat length alterations. Very surprisingly, it was shown that short CAG or CTG repeat tracts (25 triplets) were not significantly more expanded or contracted in an

msh2 mutant as compared to wild type [31]. When slightly longer CAG repeat tracts were assayed, a modest twofold increase in contractions was observed in an *msh2* strain, far from the thousandfold destabilization of microsatellites in the absence of MMR [32, 33]. It must be noted here that trinucleotide repeats, like all microsatellites, exhibit small length changes in MMR mutants, mainly +1 or −1 triplet [34], but larger expansions or contractions did not seem to be significantly more elevated in MMR-deficient backgrounds. However, when it comes to trinucleotide repeats, things are often more complicated than they seemed at first. In 1997, Christopher Pearson and Richard Sinden published a remarkable article in which they showed that purified MSH2 protein bound in vitro to CAG/CTG slipped-stranded structured DNA [35]. It was subsequently established that the MSH2-MSH3 complex bound CAG hairpins [36, 37], and that CAG and CTG repeats when more prone to contractions in transgenic mice deficient for the MSH2 protein [38, 39]. It was later demonstrated that the MSH2 ATPase activity was essential to generate repeat expansions, strongly suggesting that a functional MMR was indeed required to promote them [40]. Reinvestigation of CAG/CTG repeat tract dynamics in yeast showed that these sequences accumulated small incremental expansions over time, that were suppressed in an *msh3*Δ mutant background [41]. Finally, Msh2p was shown to be enriched in yeast cells at a CAG or CTG long repeat tract, in an *MSH3*-dependent manner [42]. All these data point to a role for the MutSβ complex in CAG/CTG repeat expansions. Similar results were observed for CGG/CCG repeats in a fragile X premutation mouse model [43] and for GGA/TTC repeats in a Friedreich's ataxia mouse model [44], but several questions remain open. Does MutSβ hairpin binding plays a stabilizing role, protecting them from degradation or repair by the cellular machinery? Is the MMR also involved in repeat expansion for other microsatellites? How do MutSβ-bound secondary structures interact with histones and with replication, recombination, and repair machineries? Is the expansion mechanism promoted by MutSβ iterative or saltatory? In other words, does it happen once or more than once in a short period of time? In any case, it seems that trinucleotide repeat-forming secondary structures activate the mismatch-repair machinery in such a way that it promotes their expansion, by a mechanism that still needs to be clarified.

4 Technical Issues to Be Considered When Trying to Amplify CAG/CTG Trinucleotide Repeats by PCR

A convenient way to analyze trinucleotide repeat length is by using two flanking primers to amplify the repeat tract by PCR. However, this approach is might be laborious due to possible slippage of the

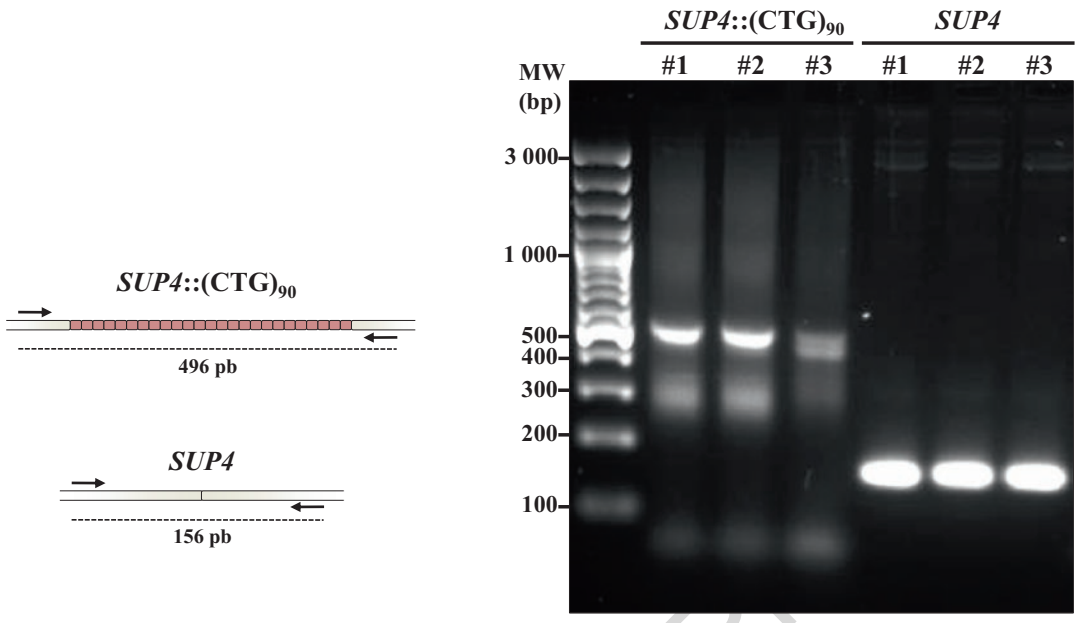

Fig. 3 PCR amplification of wild-type and CTG repeat-containing locus. The same genomic DNA was used as a template in triplicate PCR reactions (labeled 1, 2, and 3). A sharp unique band was obtained when the locus that does not contain a CTG repeat was amplified (*SUP4*). Nonreproducible bands and a smear were obtained when the CTG repeat-containing locus was amplified (*SUP4*::CTG_{90}). Similar results were obtained with other primer sets located close to the repeat tract

newly synthesized strand over its template during the PCR reaction itself, leading to amplification products of aberrant apparent sizes. One such example is shown in (Fig. 3). A $(CTG)_{90}$ repeat was integrated into a yeast chromosome, at the *SUP4* locus, and amplified by PCR using primers located upstream and downstream, close to the repeat tract. The PCR reaction was run in triplicate using the same genomic DNA as a template. In the three reactions, shorter products around 300 bp were detected as a smear migrating below the expected size of 496 bp. In addition, in reaction #3 an additional band was visible below the main product, suggesting that slippage of a few repeat units occurred in one of the earliest PCR cycles (Fig. 3). This very frequent problem could be reduced by designing primers further away from the repeat tract, but in that case the total length of the final PCR product would become the limiting factor. With very large expansions, such as those observed in DM1, DM2, Friedreich's ataxia, or ALS, the repeat tract length itself may be too large to be amplified by PCR, independently of the position of flanking primers and one has to use alternative methods such as Southern blot [42] or exotic PCR reactions [45] to estimate tract length. An example of a Southern blot used to analyzed trinucleotide repeat length is shown in (Fig. 4).

Another caveat of PCR amplification of trinucleotide repeat tracts results from allele length heterogeneity in template genomic

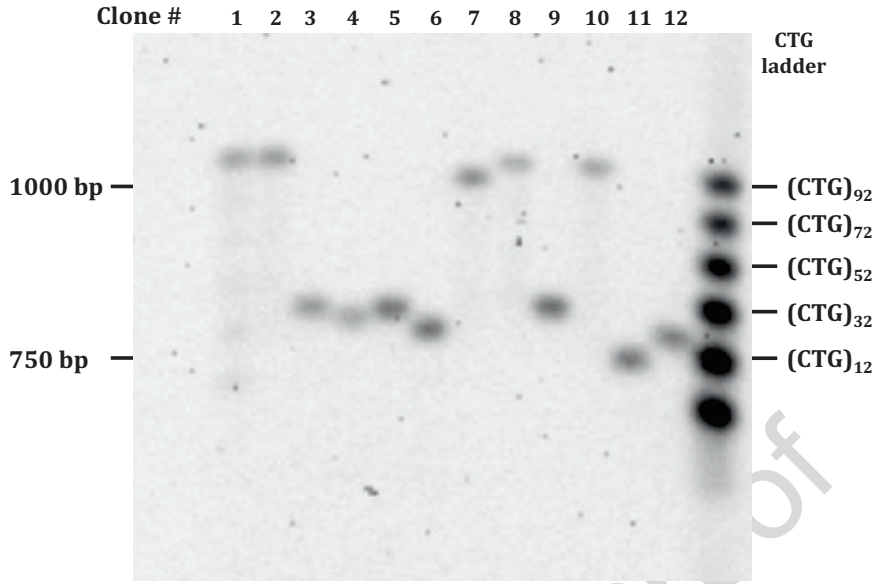

Fig. 4 Southern blot to determine CTG repeat tract length. DNA was prepared and the gel was run, transferred, and hybridized as previously described [42]. The repeat tract length of 12 independent yeast clones was analyzed (#1 to 12). To the right is a molecular weight ladder corresponding to the number of expected triplets. In this strain, the CTG repeat tract should normally be around 100 triplets. This is the case for clones #1, 2, 8, and 10, all other clones exhibiting contractions of variable lengths. Note that clone #1 shows a number of faint discrete bands of lower molecular weight, indicating repeat tract mosaicism

DNA. This is specially an issue if DNA was extracted from a tissue or a mix of tissues, and was not amplified clonally from a single cell (or a very small number of cells). In that case, one particular allele may be preferentially amplified, usually but not necessarily the shortest one, most often the most frequent allele in the population considered. This tends to underrepresent rare alleles present in the population and therefore gives a biased picture of trinucleotide repeat tract instability in the corresponding tissue or culture. To palliate this problem, small-pool PCR protocols were designed and optimized to amplify trinucleotide repeat tracts. In short, template DNA is diluted to a small number of genomes per reaction (typically ~5–10) and PCR is carried out on many individual reactions at the same time. This allows to determine more precisely the amount of trinucleotide repeat length variability within a DNA sample [46, 47].

5 Aberrant Trinucleotide Repeat Tract Mobility on Agarose Gels

In addition to artifacts due to PCR slippage, trinucleotide repeats may show abnormal mobility in agarose gels. This has been known for a long time, since more than 20 years ago it was shown that migration of an expanded CGG allele from a fragile X patient

(>200 CGG triplets) in an agarose gel was aberrant when the gel and running buffer were supplemented with 5 μg/ml ethidium bromide during electrophoresis. When the gel was stained after migration the CGG-containing band migrated at the expected position according to its molecular weight. However, when staining occurred during gel electrophoresis, the expected band migrated as a diffuse smear of molecules [48]. The author concluded that when precise CGG repeat tract length determination was important, staining of agarose gels should be performed after the migration, for unambiguous results.

Quite surprisingly, the opposite observation was made with CTG repeats. Recently, Gomes-Pereira and Monckton showed that PCR products amplified from CTG-containing templates migrated in agarose gels as discrete bands when electrophoresis was performed in the presence of 0.2 μg/ml ethidium bromide. This held true for repeat lengths ranging from five to 200 CTG triplets. On the contrary, when electrophoresis was performed without ethidium bromide and the gel was stained afterward, smeary additional bands of higher molecular weights were detected above the expected repeat size [49]. Additional experiments supported the hypothesis that these smears corresponded to slipped-stranded molecules formed during sequential cycles of denaturation-polymerization performed during the PCR reaction. It was not completely clear, though, how the presence of ethidium bromide during electrophoresis completely suppressed the detection of such molecules. Nevertheless, the authors concluded that precise repeat tract length determination by electrophoresis in agarose gels of CTG trinucleotide repeats should always be performed in the presence of ethidium bromide, to avoid such artifacts.

GAA trinucleotide repeats form triple helices (Fig. 2c) and plasmids carrying such repeats were shown to exhibit aberrant mobility in agarose gels, a retarded band of high molecular weight being detected at different ethidium bromide concentrations [50]. At the present time, it is unclear whether other microsatellites expanded in human disorders also exhibit similar properties, but it should be kept in mind that precise size determination of trinucleotide repeat tract length by agarose gel electrophoresis should be considered with extreme care.

In conclusion, all studies on trinucleotide repeats had to face these technically challenging features: frequent repeat size changes, stable secondary structure formation, and aberrant mobility in agarose gels. Most of the analytical molecular methods developed to study trinucleotide repeats aimed at circumventing these obstacles. Several of them are extensively described in the present book and will hopefully be useful to researchers in the many areas of this fascinating field.

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