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Journal of Chromatography A, 1014 (2003) 21–27
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of site-directed mutagenesis constructs by capillary electrophoresis using linear polymer sieving matrices

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Abstract

Site-directed mutagenesis is a novel molecular biology tool, which introduces mutations into DNA fragments of interest in a well-defined manner. Sequences with designed mutations can be generated in this way to express altered protein sequences for structure–function relationship studies. However, prior to gene expression, it is important to analyze the DNA construct to see whether the introduction of the mutation was indeed successful. Currently DNA sequencing is the method of choice for this verification. This paper introduces the combination of primer extension and capillary electrophoresis using linear polymer sieving matrices as an efficient alternative for this type of mutation analysis. The site-directed mutagenesis construct served as template in the primer extension reaction that employed a fluorophore labeled primer in close proximity to the mutation. Appropriate ddNTP was used to block the extension when the mutation was present, while the other three dNTPs enabled elongation of the primer. Alternatively, non-labeled primers can be used with the proper fluorophore labeled ddNTPs to block the reaction. Rapid analysis of the labeled primer extension products (mutant or wild type) was obtained by capillary electrophoresis using denaturing sieving matrix and laser-induced fluorescence detection.

Keywords: DNA

1. Introduction

With the advent of modern molecular biology techniques, a variety of chemical and enzymatic techniques have been developed for DNA modification to introduce mutations in genes that determine protein structure or DNA sequences that function as genetic regulatory elements. The two most commonly used techniques for introducing mutations are alteration of single bases and deletion of a short DNA sequence section [1]. Early day approaches generally required single-stranded DNA as template and were quite labor intensive and technically challenging [2,3]. During the past couple of years various novel strategies were developed to simplify and increase the efficiency of site-directed mutagenesis [4]. Most methods are based on the use of a synthesized primer, which anneals to the desired site of mutation. The primer is then extended and ligated through the use of appropriate enzymes and thermocycling. After sufficient amplification, a special selection process breaks down the original non-mutated strand. For example, the *Dpn* I restriction enzyme efficiently digests Gm6 DNA [5], resulting in complete breakdown of the original plasmid DNA, since DNA from nearly all *Escherichia coli* strains is

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00851-3

6-methylated on G nucleotides. Once the selection protocol is accomplished, the DNA is simply transformed into *E. coli* cells, which will now carry and express the mutant plasmid. However, prior to gene expression study, successful introduction of the mutation into the DNA construct should be verified. There are currently a plethora of methods available for detecting known mutations including allele specific amplification, ligase chain reaction, denaturing gradient gel electrophoresis, single-stranded conformational polymorphism analysis, DNA sequencing and primer extension [6]. This later method is simple, rapid and readily accommodated by capillary electrophoresis [7,8].

The most promising advances in electrophoretic separation of nucleic acids arose from the exploration of novel separation matrices. Linear polymer networks became very popular and widely used these days in capillary electrophoresis of biologically important polymers [9,10]. These non-cross-linked matrices are not attached to the inside wall of the capillary and feature very flexible dynamic pore structure accommodating separation of biomolecules in a wide molecular mass range. The actual pore size of these polymer solutions are defined by dynamic interactions between the polymer chains, and can be varied at any time by changing such variables as temperature, separation voltage, salt concentration or pH. Due to their lower viscosity non-crosslinked polymer solutions can be easily replaced in the capillary providing fresh separation medium for each analysis, also preventing contamination from previously injected samples. Most importantly, linear polymer sieving matrices support both electrokinetic and pressure injection methods, enabling sample stacking and excellent run-to-run peak area reproducibility supporting routine quantitative analysis.

Similar to DNA sequencing, capillary electrophoresis using denaturing linear polymer solutions is frequently employed for size separation of relatively short single stranded oligonucleotides [11]. The most commonly used denaturing agents are urea and formamide. Capillary electrophoresis with laser-induced fluorescence (LIF) detection has been reported as an efficient tool to analyze known point mutations using single-nucleotide extension [7]. Three different point mutations in human mitochondrial DNA associated with Leber's hereditary optic neuropathy (LHON) were detected by annealing a primer immediately 5′ to the mutation on the template and extending by one fluorescently labeled dideoxy terminator complementary to the mutation. In another approach, rapid molecular diagnosis of 21-hydroxylase deficiency was accomplished by detecting the most common mutation in the 21-hydroxylase gene using regular primer extension and capillary electrophoresis with high-molecular mass ($1.3\cdot 10^6$) polyvinylpyrrolidone sieving matrix [8]. The advantages of using capillary electrophoresis with linear polymer sieving matrices and LIF detection to analyze primer extension reaction products include speed, ease of analysis, absence of radioactivity, and potential for automation.

In this paper we demonstrate rapid analysis of site-directed mutagenesis products by primer extension in conjunction with capillary electrophoresis using linear polymer matrices. Sieving efficiencies of non-denaturing polyvinylpyrrolidone and denaturing linear polyacrylamide solutions are compared in separating both primer labeled and dideoxy terminator labeled primer extension products.

2. Materials and methods

2.1. Chemicals

Tris base, boric acid, $EDTANa_2$, ammonium persulfate, urea and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Sigma (St. Louis, MO, USA). The high-molecular mass polyvinylpyrrolidone (PVP, M_r $1.3\cdot 10^6$) was from Aldrich (Milwaukee, WI, USA). Deoxyribose nucleotide triphosphates (dNTPs) were from Life Technologies (Rockville, MD, USA), ddCTP was from Sigma, fluorescein-ddCTP was from Applied Biosystems (Foster City, CA, USA), Thermo Sequenase and Sequenase reaction buffer was from Amersham (Cleveland, OH, USA). Both fluorophore labeled (fluorescein and Cy5) and unlabeled oligonucleotide primers were synthetised and high-performance liquid chromatography (HPLC) purified by Sigma Genosys (The Woodlands, TX, USA). Linear polyacrylamide (LPA) sieving matrix containing 7 *M* urea was synthesized in house. The polymerization

process involved dissolving the sufficient amount of acrylamide monomer (4%) in an aqueous solution of 89 m*M* Tris 89 m*M* boric acid, 2 m*M* EDTA Na_2 (pH 8.3) containing 7 *M* urea, followed by the addition of 4 μl/ml reaction mixture of 10% ammonium persulfate (initiator) and 4 μl/ml reaction mixture TEMED (catalyst). Vacuum degassing of the polymerization reaction mixture assured removal of excess oxygen that otherwise inhibits polymerization. The matrix was used for separation after overnight polymerization. All buffer solutions were filtered through a 0.2 μm filter before use (Schleicher and Schuell, Keene, NH, USA).

2.2. Site-directed mutagenesis

pUC18 with Protein G domain III insert plasmid isolated from JM101 *E. coli* was used as a template for the Stratagene Quickchange in vitro site-directed mutagenesis kit (Stratagene, San Diego, CA, USA). After the initial primer annealing step the DNA polymerase extended the oligomers followed by ligation to form closed circular DNA (ccDNA). The product was then treated with *Dpn* I restriction enzyme for selection, since DNA isolated from almost all *E. coli* strains is G6-methylated and therefore susceptible to *Dpn* I digestion [12].

2.3. Primer extension reaction

Subsequently, 2–200 ng of pUC18 plasmid (3 kilo base pairs, kb), as well as the wild type, served as a template for primer extension using either fluorophore labeled dideoxy terminator (FL-ddCTP) or fluorophore labeled primer: 5′ FL-GAC GAA ATC ATC AAC AAA GC 3′. The reaction mixture contained 250 n*M* primer, 0.2 U Thermo Sequenase, 0.2 m*M* of the three dNTPs (dATP, dGTP and dTTP) and 0.01 m*M* to 0.1 m*M* of unlabeled or labeled ddCTP in 1×Thermosequenase buffer in a final volume of 10 μl. The template–primer ratio was optimized to yield the maximum amount of extension products [7]. Thermocycling conditions: 3 min initial denaturation at 94 °C and 40 cycles of 30 s 94 °C, 30 s 48 °C, 30 s 72 °C, followed by a 2 min final extension at 72 °C.

2.4. Capillary electrophoresis

For all the separations a P/ACE-MDQ (Beckman Coulter, Fullerton, CA, USA) instrument was used in reversed polarity separation mode (anode at the detection side). The separations were monitored on-column by LIF using both a green Ar-ion laser (excitation: 488 nm; emission filter 520 nm) and red diode laser (excitation: 635 nm; emission filter 670 nm). The temperature of the cartridge holding the separation capillary column was thermostated at 25±0.1 °C by the active liquid cooling of the device. A 50 cm effective (60 cm total) separation length coated capillary with (100 μm I.D.) was used for the analysis (eCAP DNA Capillary, Beckman Coulter) of the denatured primer extension products. Then, 10% polyvinylpyrrolidone or 4% linear polyacrylamide/7 *M* urea solutions were used as sieving matrices in 1×TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA Na_2, pH 8.4). The separation medium was replaced in the capillary after each run. The primer extension samples were diluted 20-fold with deionized water (18 MW) and electrokinetically injected at 3–7 kV for 2–5 s, and separated at 30 kV. The data were acquired and evaluated by the P/ACE System MDQ 32 karat software package (Beckman Coulter).

3. Results and discussion

In vitro site-directed mutagenesis is an invaluable technique for carrying out vector modifications and for studying protein structure-function relationships in gene expression. Fig. 1 depicts the general outline of site-directed mutagenesis. Plasmid isolated from *E. coli* cells was used as template. The procedure utilized a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation (Fig. 2).

3.1. Step 1, annealing

The original plasmid was denatured in the thermocycling process and the primers (both forward and reverse) annealed to their designed sites.

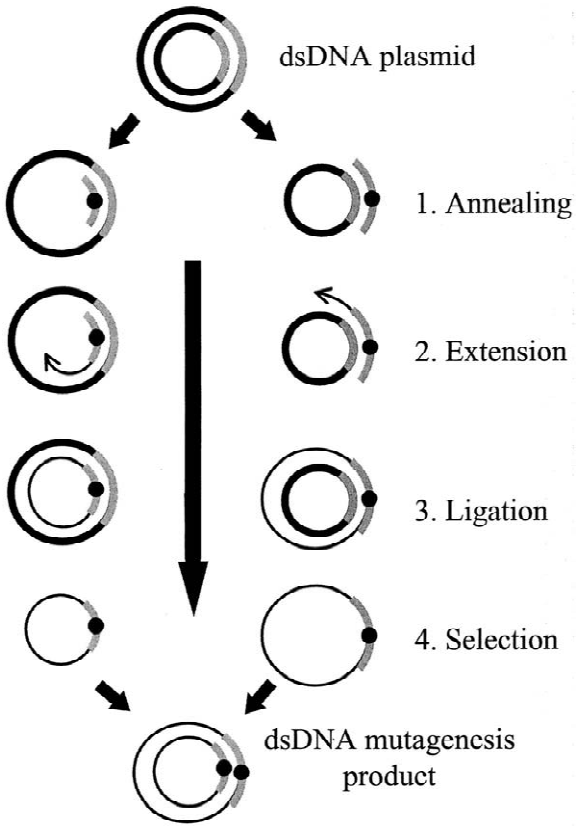

Fig. 1. Schematic representation of a four step site-directed mutagenesis protocol. Carefully designed primers anneal to the desired site of mutation followed by extension and ligation through the use of appropriate enzymes and thermocycling. After sufficient amplification a special enzymatic selection process breaks down the original non-mutated strand, leaving the mutant ready for transformation.

3.2. Step 2, elongation

The oligonucleotide primers, each complementary to the opposite strand of the vector, were extended during temperature cycling with DNA polymerase.

3.3. Step 3, ligation

DNA polymerase replicated both strands of the plasmid with high fidelity and without displacing the mutant primers. Incorporation of the oligonucleotide primers generated mutated plasmid containing staggered nicks. The ligation step was coupled with the elongation, forming a new closed circular DNA (ccDNA).

3.4. Step 4, selection

After temperature cycling, the original wild type DNA was digested using *Dpn* I restriction enzyme which was specific for methylated and hemimethylated DNA with a target sequence of 5′-Gm6ATC-3′. It was used to digest the parental DNA template in order to select the mutation-containing synthesized DNA [12].

3.5. Primer extension

Once the selection procedure was completed the two mutated strands annealed and the complete dsDNA product was ready to be transformed into *E. coli* for expression. To verify the presence or absence of the mutation, the resulting plasmid was used as a template in primer extension reaction. Fig. 3 depicts the relevant sequence sections of the wild type and mutant templates, as well as the primers used in the extension reaction. The extension primer was designed to be 20 bases long with its 3′ end annealing immediately upstream of the mutation site. The extension reaction mixture contained dATP, dTTP, dGTP and ddCTP, this later assured stopping the extension reaction at the very next G on the template strand, thus, to create a length difference between the mutant and wild type extension products. For the wild type the elongation can go past the

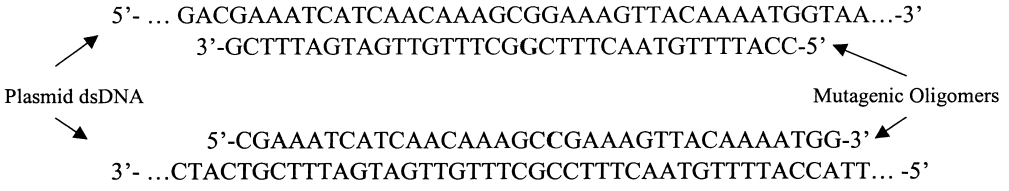

Fig. 2. Site-directed mutagenesis primers and relevant template sequences.

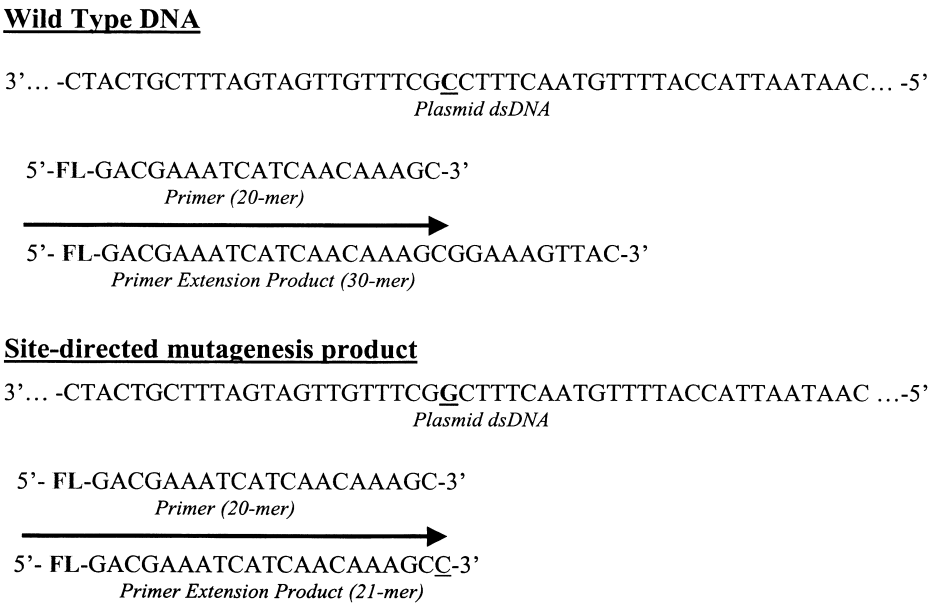

Fig. 3. Sequences of the wild type and mutant templates, the primers and the extension reaction products.

first nucleotide (C) all the way until the next G, which is 10 bases downstream. In the mutant, the first base following the complimentary primer sequence is G, so the extension stops at that point by the incorporation of ddCTP, since the DNA polymerase is unable add onto dideoxy-nucleotides. From this extension reaction design, the wild type and mutated templates resulted in 30mer and 21mer extension products, respectively, which were subject of capillary electrophoresis analysis.

3.6. Capillary electrophoresis

Based on previous findings [8] we first attempted capillary electrophoresis analysis of the primer extension products by using 10% polyvinylpyrrolidone sieving matrix in an uncoated, bare fused-silica capillary column. In this instance the primer extension reaction contained unlabeled primer and 0.01 n*M* fluorescein labeled ddCTP. Fig. 4 shows the electrophoretic traces of the mutant (upper trace) and wild type (lower trace) reaction mixtures. We assume that peak tailing was originated form the use of uncoated bare fused-silica column that was not perfectly covered by the polyvinylpyrrolidone sieving matrix. The upper trace clearly delineates that the

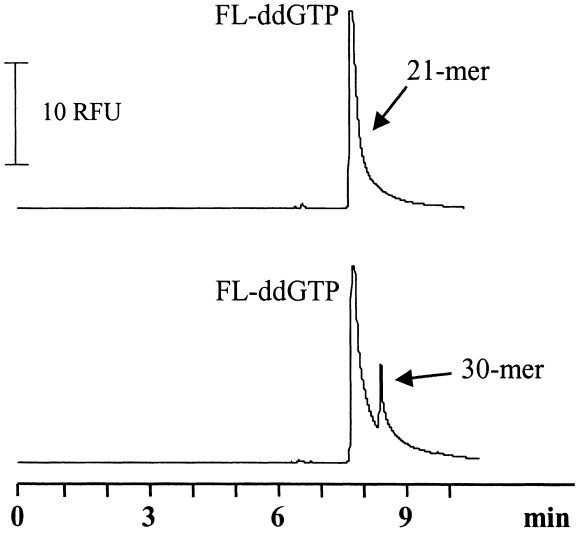

Fig. 4. Capillary electrophoresis analysis of primer extension products using polyvinylpyrrolidone sieving matrix. Upper panel: electropherogram of the primer extension reaction with the mutant template. Lower panel: electropherogram of the primer extension reaction with the wild type template. Conditions: capillary: 50 cm effective (60 cm total, uncoated) length, I.D. 100 μm. Separation matrix and running buffer: 10% polyvinylpyrrolidone in 1×TBE, applied voltage: 30 kV, injection: 2 s/3 kV, temperature: 30 °C.

separation power of this particular system was not appropriate to accomplish the task, since the tailing section of the excess fluorophore-labeled ddCTP peak apparently covered the mutant extension product (21mer). However, having the wild type probe as control, one can always be sure that the extension reaction worked, therefore, the lack of the peak corresponding of the 30-mer could indirectly indicate the success of mutagenesis.

Next we run the primer extension reaction with fluorescein labeled primer and analyzed the product by capillary electrophoresis with 4% linear polyacrylamide/7 *M* urea matrix using a coated column. Fig. 5 shows the successful separation of the mutant (upper trace) and the wild type (lower trace) reaction products from the labeled primer. Please note, that the use of this higher resolving power denaturing separation matrix along with a quite long separation distance of 50 cm, the 20mer (primer) and the mutant reaction product (21mer) are adequately separated.

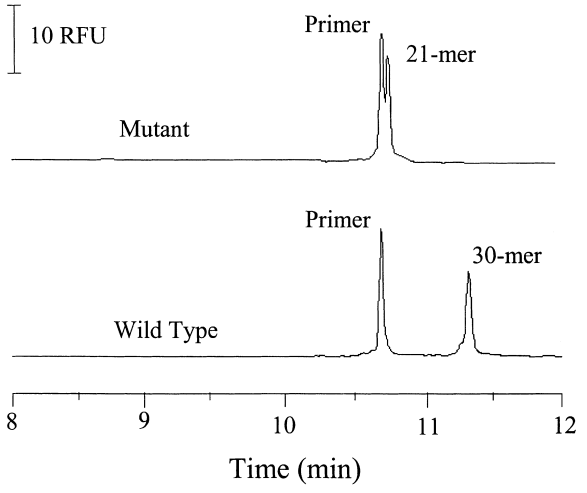

Fig. 5. Analysis of site-directed mutagenesis constructs using primer extension and capillary electrophoresis with denaturing linear polyacrylamide sieving matrix. Upper panel: electropherogram of the primer extension reaction with the mutant template. Lower panel: electropherogram of the primer extension reaction with the wild type template. Conditions: capillary: 50 cm effective (60 cm total, coated) length, I.D. 100 μm. Separation matrix and running buffer: 4% LPA in 1×TBE containing 7 *M* urea, applied voltage: 30 kV, injection: 3 s/5 kV, temperature: 30 °C.

3.7. Two color detection

To evaluate the usefulness of the two spectral channel setting of our capillary electrophoresis system, we attempted a scouting experiment to combine different color labeling for the primer and dideoxy terminator in the extension reaction for the analysis of the wild type template. Cy5 labeled primer (detection with the red diode laser) and fluorescein labeled ddCTP (detection with the blue Ar-ion laser) were chosen. Fig. 6 depicts the electrophoresis traces in the blue and the red channels. The upper trace delineates the signal from the blue channel exhibiting a large overloaded peak at 9.6 min corresponding to the excess fluorophore-labeled ddCTP and a single peak at 10.2 min corresponding to the primer extension product. The small peak on the upper trace at 9.1 min is an impurity originating form the labeled dideoxy terminator. In this instance, the reaction mixture contained 0.1 m*M* fluorophore labeled ddCTP. The lower trace depicts the signal from the red channel, and one can observe a nice peak at 10.2 min corresponding the primer extension product. The small peak at 9.6 min is the result of some spectral overlap from the blue channel. Please also note that

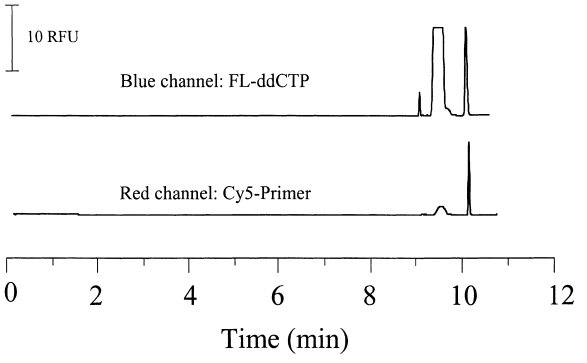

Fig. 6. Multispectral capillary electrophoresis analysis of a double-labeled primer extension product. Upper trace: Blue channel showing the separation of the fluorescein labeled ddCTP termination product. Lower trace: Red channel depicting the separation of the Cy5 labeled primer based product. Conditions: capillary: 50 cm effective (60 cm total) length, I.D. 100 μm, coated. Separation matrix and running buffer: 4% LPA in 1×TBE containing 7 *M* urea, applied voltage: 30 kV, injection: 5 s/7 kV, temperature: 30 °C.

the double fluorescent labeling (Cy5 and fluorescein) of the reaction product shifted the mass to charge ratio and consequently the migration time of the extension product compared to the single labeled product. The Cy5 labeled primer (20mer) was not detectable because given its relatively low concentration and high number of cycles, apparently all primer molecules were used up in the extension reaction.

4. Conclusions

Our study has demonstrated that primer extension in conjunction with capillary electrophoresis using linear polymer sieving matrices is a good alternative to sequencing for the analysis of site-directed mutagenesis products, provided that the downstream CE method can adequately separate the resulting fragments, which in the worst case scenario may differ by only one nucleotide. In this latter case with a single nucleotide extension product, the 10% polyvinylpyrrolidone sieving matrix did not provide appropriate separation power, however application of 4% LPA with 7 *M* urea proved to be an adequate sieving network in conjunction with a coated capillary column. This matrix enabled rapid analysis of short single stranded primer extension products and distinguished the extension product from the primer peak. Please note again, that our template sequence was actually most unfavorable for regular primer extension protocol since the difference in lengths of the primer and the extension product was miniscule, i.e. only one base. For sequences where the expected size difference between the primers and the extension products is longer, this method could result much faster analysis since shorter capillary and consequently higher applied electric field can be employed. Based on our encouraging preliminary results with the two-color detection system, we plan to use single primer extension with one color ddNTP for the wild type DNA (e.g. fluroescein) and another color ddNTP for the mutant DNA (e.g. Cy5) in a single reaction, therefore the presence or absence of the introduced mutation could be reliably assessed from a single capillary electrophoresis run.

Acknowledgements

The author wishes to thank the Nowick research group of the Department of Chemistry, University of Irvine for the plasmid templates and helpful support, as well as Drs Zsolt Ronai and Julia Kandurina for their helpful insight.

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