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Chemical mismatch cleavage combined with capillary electrophoresis: detection of mutations in exon 8 of the cystathionine β-synthase gene

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Mutation detection by chemical mismatch cleavage (CMC) is based on the chemical modification and cleavage at the site of mismatched C or T in heteroduplexes, using hydroxylamine or osmium tetroxide (OsO_4) as chemical probes. In the present study, we evaluated CMC in combination with capillary electrophoresis (CE) by determining the common T833C and G919A mutations in exon 8 of the cystathionine β-synthase gene in heterozygous and homozygous samples. A 186-bp fragment encompassing exon 8 was amplified by PCR with both primers labeled with 5′-fluorescein. Labeled single strands of 40 and 61 nucleotides (nt) were formed from the coding strand of the T833C sample and non-coding strand from the G919A sample, respectively. These single-stranded DNA (ssDNA) products were analyzed under denaturing conditions by CE with short-chain linear polyacrylamide as the sieving matrix and were detected by laser-induced fluorescence (LIF), using a sensitive, one-channel sheath-flow detector. The CE-LIF format afforded relatively high resolution of ssDNA (down to 1 nt), precise size assessment of CMC products, sensitive detection with small sample requirements, and fast analysis. Thus, CMC combined with CE-LIF is suitable for screening of known mutations, giving expected CMC products, but will also detect unknown mutations, the locations of which are indicated by the fragment sizes.

The method based on chemical mismatch cleavage (CMC)[1] for mutation analysis was developed by Cotton et al. *(1)* and has been used successfully for the detection and identification of mutations in several genes *(2–5)*. When compared with denaturing gradient gel electrophoresis *(6)* and single-stranded conformation polymorphism *(7)*, CMC has a higher diagnostic sensitivity and can analyze larger DNA fragment lengths *(8)*.

The principle of the CMC method is the formation of heteroduplex double-stranded DNA (dsDNA) by annealing single-stranded DNA (ssDNA) from the wild-type and mutant alleles. The two alleles can either be derived from a heterozygous DNA sample or by combining two samples, one with wild-type DNA and one containing the mutant allele. The base at the mismatch reacts either with hydroxylamine or osmium tetroxide (OsO_4), which modify unpaired cytosine or thymine residues, respectively. DNA is then cleaved at the modified base by piperidine, and the products are separated by denaturing gel electrophoresis and detected by autoradiography *(1, 3)* or by fluorescence *(5, 9, 10)*.

Capillary electrophoresis (CE) in entangled polymers has become an attractive alternative to gel electrophoresis techniques for the analysis of DNA fragments. CE can be automated and is characterized by short analysis times, small sample requirements, high resolution and separation efficiency, and when coupled to a laser-induced fluorescence (LIF) detector, unsurpassed sensitivity *(11–15)*. Low viscosity sieving media like short-chain linear polyacrylamide (SLPA) have the additional advantage of efficiently filling small diameter (<75 μm) capillaries with sieving medium. Small diameters ensure efficient heat dissipation and thereby fast analysis without loss of resolution *(16–18)*.

There is extensive documentation of efficient analysis of dsDNA by CE. Data on the separation of ssDNA are sparse *(15)*, however, and analysis of CMC cleavage products by CE has been suggested but not investigated *(8)*.

We have previously described single-stranded conformational polymorphism analysis by CE, demonstrating high resolution of ssDNA by CE using SLPA as the sieving matrix *(18)*. Notably, a recent study compared CE analysis of ssDNA and dsDNA and showed the enhanced resolution and selectivity of ssDNA migrating in 50-μm

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[1] Nonstandard abbreviations: CMC, chemical mismatch cleavage; ds, double-stranded; ss, single-stranded; CE, capillary electrophoresis; LIF, laser-induced fluorescence; SLPA, short-chain linear polyacrylamide; CBS, cystathionine β-synthase; and nt, nucleotide.

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capillaries filled with linear polyacrylamide *(19)*. In the present study, we transferred CMC mutation analysis to the capillary format by analyzing the fluorescein-tagged cleavage product by CE-LIF, using SLPA as the sieving matrix. The expected fragment lengths for T833C and G919A mutations in exon 8 of the cystathionine β-synthase (*CBS*) gene *(20–23)* were obtained, demonstrating the detection of both known and unknown mutations by CMC combined with CE-LIF.

Materials and Methods

MATERIALS

The acrylamide, N,N,N′,N′-tetramethylenediamine, and ammonium peroxydisulfate were purchased from Bio-Rad Laboratories. The 3-methacryloxypropyl-trimethoxysilan and fluorescein-labeled dsDNA markers (fragment sizes of 50–500 bp) were from Pharmacia LKB Biotechnology AB. The hydroxylamine, diethylamine, 40 g/L OsO_4 solution, HEPES, and tRNA were from Sigma Chemical Co. The hydroxylamine stock solution (6 mol/L) was aliquoted into 1-mL Eppendorf tubes and stored at −70 °C; it was stable for at least 3 months under these conditions. The OsO_4 solution was stored at 4 °C and was stable for at least 3 weeks. The pyridine was from Chemical Limited Walkerburn. The piperidine was a product of Merck. The reaction tubes (thin-walled, Gene Amp) for PCR reactions were from Perkin-Elmer. The fused capillaries (50 μm i.d., 192 μm o.d.) were products of Polymicro Technologies Inc. The QIAquick PCR Purification Kit and QIAamp Blood Kit were products of QIAGEN Co. The 5′-fluorescein-labeled primers were synthesized by Eurogentec. Water, doubly distilled and purified on a MilliQ Plus Water Purification System (Millipore), was used for preparation of all aqueous solutions. The short-chain linear polyacrylamide (SLPA) was synthesized according to a slight modification *(18)* of the procedure described by Grossman *(16)*.

OsO_4 and piperidine are toxic chemicals, and skin and eye contact must be avoided. The handling and chemical cleavage reactions were performed under a fume hood. The supernatants of the ethanol precipitation after OsO_4 modification were collected for safe disposal *(9)*.

DNA SAMPLES AND PCR AMPLIFICATION OF EXON 8

Blood from four different subjects was used. One subject had a presumably wild-type *CBS* genotype, one was heterozygous for the T833C mutation, one heterozygous for the G919A mutation, and one was homozygous for the G919A mutation. The *CBS* mutations in these samples have been determined by DNA sequencing *(24)*.

Template DNA used in PCR reaction was extracted from whole blood, using the QIAamp Blood Kit according to the instructions from the manufacturer. The PCR reaction mixture contained 10 mmol/L Tris-HCl, pH 9.0, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 g/L gelatin, 1 mL/L Triton X-100, 125 μmol/L each dNTP, 0.2 μmol/L each primer, 0.2 U of Taq polymerase (Super Taq, HT Biotechnology Ltd.), and ~100 ng of template DNA in a final volume of 100 μL. The PCR reaction was performed on a Perkin-Elmer 480 thermocycler, using a three-step thermocycling profile with 35 cycles of 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 20 s, preceded by 94 °C for 3 min and followed by 72 °C for 5 min.

The primers used were 5′-fluorescein-ACTGGCCTT-GAGCCCTGAA-3′ (F1, forward) derived from intron 7 and 5′-fluorescein-AGGCCGGGCTCTGGACTC3′(F2, reverse) from intron 8 of the *CBS* gene *(21–23)*. The primers define a 186-bp PCR product that includes both the T833C and G919A mutations, as depicted in Fig. 1.

HETERODUPLEX FORMATION AND CMC

The generation and cleavage of heteroduplex DNA have been described before *(1, 2, 5)*. Briefly, heteroduplex DNA was formed by mixing 100 μL of PCR product (containing both the wild-type and mutated alleles) with 60 μL of 3 mmol/L Tris-HCl, pH 7.7, containing 1.2 mol/L NaCl and 3.5 mmol/L $MgCl_2$, followed by heating at 96 °C for 6 min and annealing at 42 °C for 1.5 h. The DNA was then precipitated with ethanol and purified with the QIAquick PCR Purification Kit, according to the instructions from the manufacturer. This purification step was included to remove fluorescent material and fluorescent primers, which interfered with the analysis of the CMC cleavage products by CE-LIF. The final volume of the purified DNA sample was 50 μL.

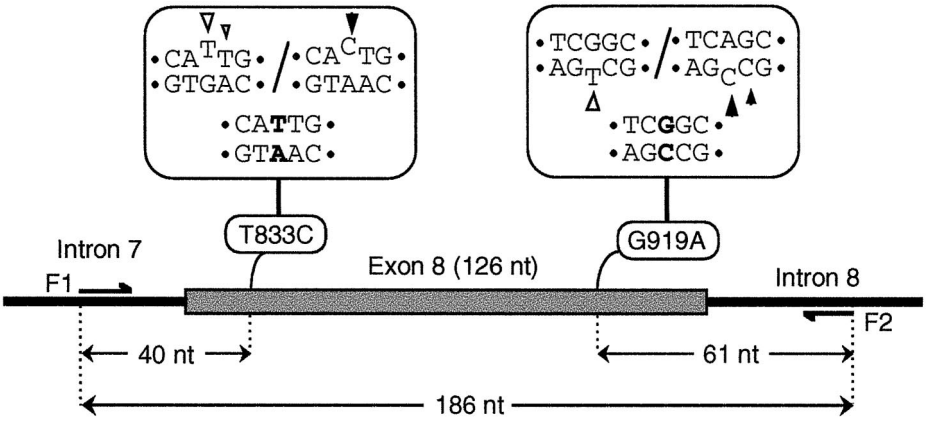

Fig. 1. Sizes and positions of the PCR and fluorescent cleavage products used for the detection of the T833C and G919A mutations in exon 8 of the cystathionine β-synthase gene by CMC.

F1 and *F2* indicate the forward and reverse primers, respectively. The *upper two boxes* show the site of mutation (*bold-faced type*) in a 5-bp context and the corresponding heteroduplexes. The sites of modification of unpaired cytosine by hydroxylamine or thymine are indicated by *solid arrows* and *open arrows*, respectively.

Specific modification of unpaired C and T residues was obtained with hydroxylamine and OsO_4, respectively. Five microliters of DNA was mixed with a solution of 20 μL of hydroxylamine adjusted by diethylamine to pH 6.0 (final concentration, 2.0–4.0 mol/L) or OsO_4 containing 20 mL/L pyridine, 0.5 mmol/L Na_2EDTA, and 5 mmol/L HEPES, pH 8.0 (final concentration, 0.8–5.6 g/L), and then incubated for 5–105 min at 20–45 °C. The reaction conditions are given in the figure legends. The reaction was stopped by transferring the solution to ice and adding 200 μL of 0.3 mol/L sodium acetate buffer with 0.1 mmol/L Na_2EDTA and 25 mg/L tRNA, pH 5.2. The DNA was precipitated with ethanol, and the DNA pellet was washed once with 700 mL/L ethanol. The DNA was then cleaved at the modified base by dissolving the DNA pellet in 50 μL of 1 mol/L piperidine, followed by incubation at 90 °C for 30 min. To minimize the loss of DNA, the sample was finally lyophilized. Before electrophoresis, the lyophilized DNA was dissolved in 8 μL of 800 mL/L formamide, incubated at 95 °C for 5 min, and then cooled in ice water. Fluorescein-labeled dsDNA markers and PCR products, dissolved in 800 mL/L formamide, were converted into ssDNA using the same procedure.

CE instrumentation

CE was performed on a commercial CE instrument (Prince Technologies). The LIF detector was built in-house with a sheath-flow cuvette constructed essentially as described by Dovichi et al. *(25)*. An argon ion laser (Uniphase Ltd.) with 488 nm emission (20 mW) was focused on the sheath-flow cuvette 30 μm below the capillary outlet. A fluorescence emission signal was collected at 90° with a microscope objective and amplified by a photomultiplier (Hamamatsu); the signal was then transferred to a computer. We used Prince software (Ver. 1.14) and Caesar software (Ver. 4.0), both from Prince Technologies, for instrument control and data collection, respectively.

CE procedure

The capillary (length, 40 cm), coated with linear polyacrylamide *(18)*, was rinsed with Tris-borate-EDTA buffer (89 mmol/L Tris-borate buffer containing 1 mmol/L EDTA, pH 8.3) for 5 min and then filled with sieving medium consisting of 60 g/L SLPA in Tris-borate-EDTA buffer containing 7 mol/L urea (pH 8.3). Tris-borate-EDTA was used as the electrophoresis buffer. Samples were introduced by electrokinetic injection at −30 kV for 18 s. Electrophoresis was performed at reverse polarity mode under the conditions specified in the figure legends. The sieving medium in the capillary was replaced between each electrophoretic run.

Calculation of cleavage efficiency

After heteroduplex DNA formation, a heterozygous sample contains four dsDNAs, i.e., two homoduplexes and two heteroduplexes. For each mutation, only one out of the two heteroduplexes is a target for the site-directed probe, i.e., either hydroxylamine or OsO_4. Notably, only one of the two strands is cleaved, which implies that the maximal efficiency of the cleavage is one out of eight fluorescein-labeled single strands. The cleavage yield (Y) in percentage of the theoretical maximum is given by the equation:

$$Y(\%) = C \cdot 8 \cdot 100 / \Sigma C \quad (1)$$

where C refers to the concentration of the cleaved product, and ΣC is the sum of the cleaved and uncleaved products.

In CE, the mass (M) injected is the product of the injection volume (V) and the analyte concentration (C), which in turn are proportional to the corrected peak area (A):

$$M = V \cdot C = k \cdot A \Leftrightarrow C = k \cdot A / V \quad (2)$$

where k is constant for all analytes labeled with the same fluorescent reagent.

When samples are injected electrokinetically, the injection volume V of a given analyte depends on its injection velocity v_i:

$$V = \pi \cdot r^2 \cdot l = \pi \cdot r^2 \cdot v_i \cdot t_i \quad (3)$$

where r denotes the radius of the capillary, t_i is the injection time, and l is the length of the injected sample plug.

The injection velocity, v_i, in turn is a function of the ionic mobility of the analyte, μ, which is proportional to the electric field strength E_i applied during the injection *(26, 27)*:

$$v_i = \mu \cdot E_i \quad (4)$$

The ionic mobility μ of the analyte can be determined using the following equations:

$$v_s = \mu \cdot E_s = L / t_s \Leftrightarrow \mu = L / (E_s \cdot t_s) \quad (5)$$

where v_s is the migration velocity, E_s refers to the electric field strength during the separation, L is the length of the capillary from the inlet to the detection window, and t_s is the migration time.

By combining Eqs. 3, 4, and 5, we can rearrange Eq. 2:

$$C = k \cdot E_s \cdot t_s \cdot A / (\pi \cdot r^2 \cdot t_i \cdot E_i \cdot L) \quad (6)$$

Combining Eqs. 1 and 6 gives an expression of the percentage of yield:

$$Y(\%) = 8 \cdot A \cdot t_s \cdot 100 / (\Sigma A \cdot t_s) \quad (7)$$

Results and Discussion

Genetic defects in CBS are the most common cause of the inborn error homocystinuria *(28)*. However, the number of pathogenic mutations in this gene now number ~30 *(29)*, which makes mutation detection in these patients a demanding task. CMC is a useful technique for screening under conditions of genetic heterogeneity because it not only detects new mutations but also indicates their location *(8)*. In the present work, we demonstrate the use of CMC coupled to CE-LIF for the rapid detection of the

T833C and G919A substitutions that are the most prevalent pathogenic mutations in the *CBS* gene.

IDENTIFICATION OF T833C AND G919A MUTATIONS

The principles of the CMC method for detection of the T833C and G919A mutations in the *CBS* gene are shown in Fig. 1. Hydroxylamine selectively modifies the mismatched cytosine residues, probably by binding to its 5–6 double bond, whereas OsO_4 catalyzes the dihydroxylation of thymine residues. After such chemical modification, the polynucleotide chain becomes susceptible to cleavage by piperidine *(30)*. In our present study of the *CBS* gene, the amplified DNA strand is labeled only at the 5′ end. Hence, the chemical cleavage yields a fluorescent fragment at the 5′ end, whereas the other cleavage product (3′ end) is not detectable by CE-LIF.

A DNA sample that is heterozygous for the G919A or the T833C mutation will contain one heteroduplex with an A:C mismatch, which can be modified by hydroxylamine, whereas the other heteroduplex has a T:G mismatch, which can be modified with OsO_4. The uncleaved amplified DNA strand is 186 bp. In samples with the T833C mutation, the fluorescent cleavage product is from the coding strand. The length from the mutation site to the 5′ end of this strand is 40 nucleotides (nt). The cleavage in the G919A mutation occurs in the non-coding strand, and produces a 61-nt fluorescent fragment (Fig. 1).

The electropherograms from chemically modified samples with wild-type *CBS*, the T833C mutation, or the G919A mutation are shown in Figs. 2 and 3. In samples with wild-type *CBS*, heteroduplexes are not formed, and as expected, there was no cleavage product. In samples containing two different alleles, hydroxylamine treatment produced distinct peaks of the size expected for both mutations. In contrast, cleavage products after OsO_4 treatment were only observed for the T833C mutation. This is in accordance with a previous observation *(31)* that the extent of cleavage is influenced by the base context of the mutation site. We also observed that the G919A exposed to hydroxylamine and the T833C mutation exposed to OsO_4 produced double peaks in the electropherogram. This phenomenon can probably be explained by adjacent mismatch cleavage *(5, 9)*.

DETECTION OF HOMOZYGOUS MUTATION

We used the CMC technique for the detection of the G919A mutation in a homozygous sample. Heteroduplex formation was obtained by mixing the patient's DNA sample with a wild-type DNA sample. The highest yield of the cleavage product (61 nt) was observed when these samples were mixed in a 1:1 ratio. The electropherogram was identical to that obtained with the heterozygous G919A sample (Fig. 2D)

CHEMICAL CLEAVAGE EFFICIENCY AND OPTIMIZATION

The cleavage efficiency with both hydroxylamine and OsO_4 was much higher for the T833C mutation than for the G919A mutation, although both create the same mismatch (Fig. 1). This may be related to differences in the base context of these two mutations. The T883C site is located between two T:A pairs, whereas the G919A mutation is located between two C:G pairs, and a mutation in the latter context is more resistant to chemical cleavage *(31)*. In addition, there have been consistent reports that

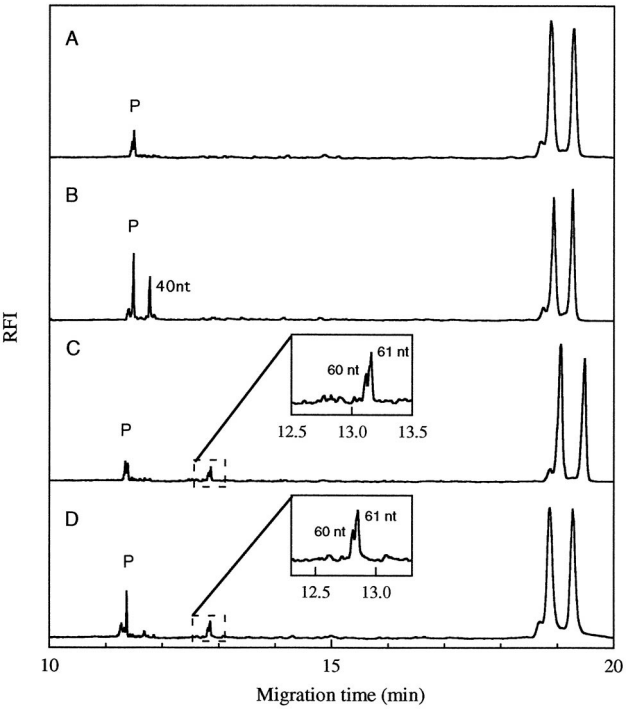

Fig. 2. Electropherograms of CMC cleavage products obtained using hydroxylamine as chemical probe.

PCR products were treated for 60 min at 37 °C with 3.8 mol/L hydroxylamine. (*A*) Wild-type DNA sample; (*B*) T833C mutant heterozygous sample; (*C*) G919A mutant heterozygous sample; (*D*) mixture (at ratio 1:1) of wild-type DNA sample and G919A mutant homozygous sample. Electrophoresis was carried out under denaturing conditions at 25 °C, and the applied voltage was −20 kV. *P*, peaks derived from residual primers and degradation products; *RFI*, relative fluorescent intensity.

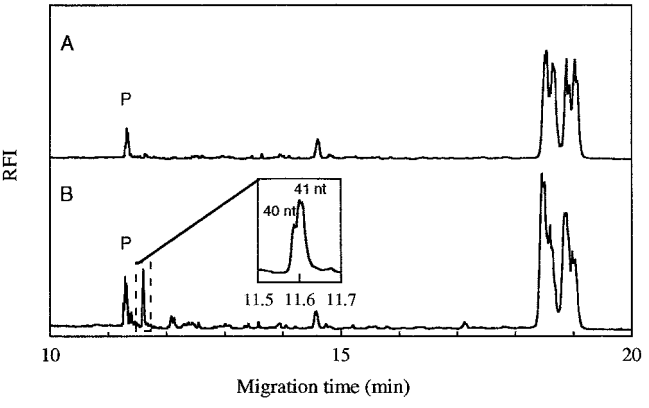

Fig. 3. Electropherograms of CMC cleavage products obtained using OsO_4 as chemical probe.

PCR products were treated for 20 min at 25 °C with 3.2 g/L OsO_4. (*A*) Wild-type DNA sample; (*B*) T833C mutant heterozygous sample. Electrophoretic condition and abbreviations are given in the legend for *Fig. 2*.

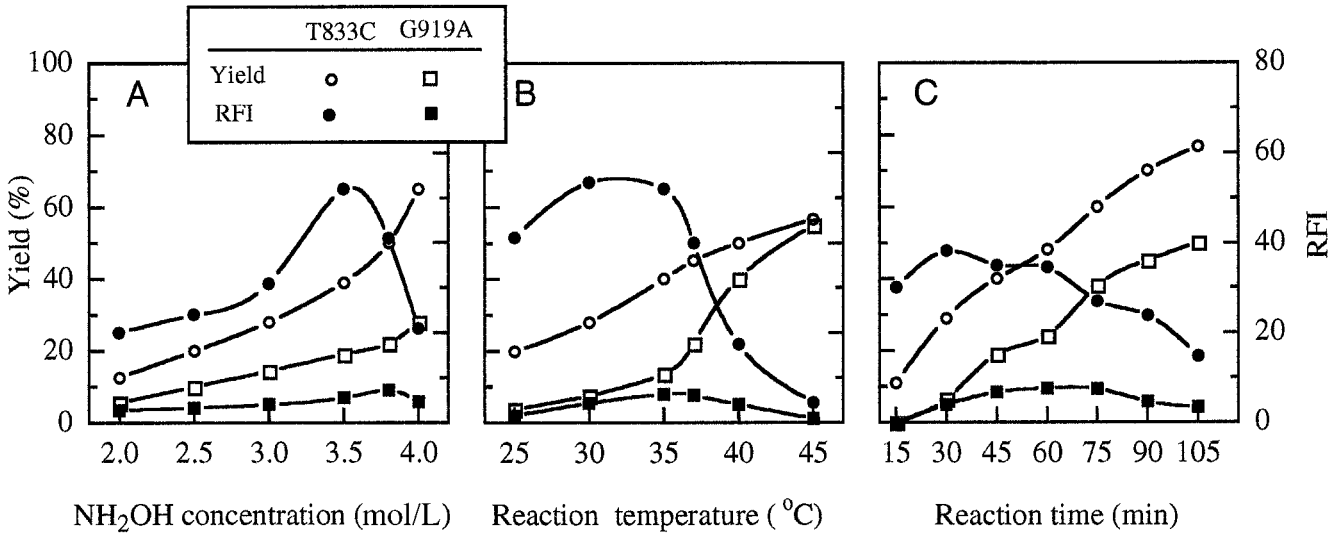

Fig. 4. Cleavage efficiency (*Yield*) of the hydroxylamine reaction and relative fluorescence intensity (*RFI*) of the cleavage products.

Panels *A–C* show the effect of hydroxylamine concentration (at 37 °C for 60 min), reaction temperature (at 3.8 mol/L hydroxylamine for 60 min), and reaction time (at 3.8 mol/L hydroxylamine and 37 °C), respectively. Electrophoretic conditions were as described in the legend for *Fig. 2*.

the T:G mismatch is only weakly reactive towards OsO_4 *(9, 10, 31)*, which may explain why the G919A mutation created no cleavage product with OsO_4 (data not shown).

Hydroxylamine and OsO_4 are known to modify their respective base targets (C and T) in a concentration-, time-, and temperature-dependent manner *(1)*. This was confirmed for both probes in our study (Figs. 4 and 5). However, the relative fluorescence intensity declined when optimal cleavage conditions were obtained. This reduction of relative fluorescence intensity during high probe concentration, high temperature, or long incubation time (Figs. 4 and 5) may be related to loss of selectivity for mismatched residues, degradation of dsDNA, and degradation or oxidation of the fluorescent group *(1)*. Hence, optimal cleavage conditions should be balanced against an acceptable signal-to-noise ratio, including small degradation peaks (Fig. 5) possibly derived from the cleaved fragment.

For hydroxylamine, the optimal relative fluorescence intensity relative to yield was obtained at a concentration of 3.0–3.8 mol/L, a temperature of 35–37 °C, and with a reaction time of 45–75 min (Fig. 4). The corresponding parameters for OsO_4 were 2.0–4.0 g/L OsO_4, 25–30 °C, and 15–25 min (Fig. 5).

ASSESSMENT OF CLEAVAGE PRODUCT SIZE

We used the denatured fluorescein-labeled DNA calibrator (50–500 nt) as the size calibrator. The calibrator was mixed and analyzed together with the intact PCR product

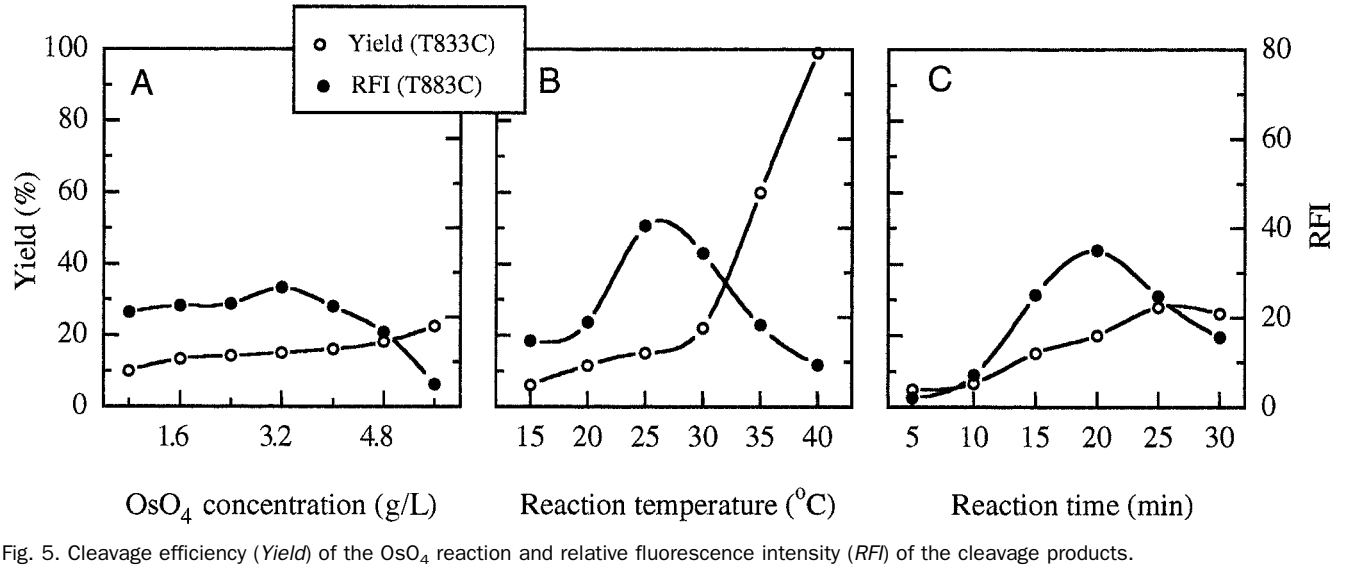

Fig. 5. Cleavage efficiency (*Yield*) of the OsO_4 reaction and relative fluorescence intensity (*RFI*) of the cleavage products.

Panels *A–C* show the effect of OsO_4 concentration (at 25 °C for 20 min), reaction temperature (at 3.2 g/L OsO_4 for 20 min), and reaction time (at 3.2 g/L OsO_4 and 25 °C), respectively. Electrophoretic conditions were as described in the legend for *Fig. 2*.

(186 nt), which eluted as a double peak, presumably because of the different mobilities of the two strands (Fig. 6). Sufficiently high resolution of ssDNA was obtained to resolve a 1-nt difference in size (Fig. 2).

For the subsequent analyses of the cleavage products, we used the first component of this doublet as an internal standard. The sizes of the products were then estimated on the basis of their relative migration times, using the equation for the calibration curve (size M vs relative migration time t_r) for the size makers (Fig. 6, inset). Table 1 compares the estimated and real sizes, and small but important differences were observed. Furthermore, the estimates were characterized by high precision (relative standard deviation <0.12%, Table 1).

The small difference between estimated and real size of the cleavage products (Table 1) as well as the separation of the two opposing strands of the intact PCR product (Fig. 6) may be attributed to sequence-specific migration, as has previously been demonstrated by Guttman et al. *(32)* for oligonucleotides separated by CE under denaturing conditions. Likewise, separation of opposing strands has also been observed during slab gel electrophoresis of CMC products *(5, 9)*. This anomalous migration impedes the exact localization of the mutation site by CMC, which should be performed by subsequent DNA sequencing.

Table 1. The real and estimated sizes of the original PCR product and cleaved products after CMC (n = 8).

Genotype	Real size, nt	Estimated size, nt	RSD,[a] %
1st peak (PCR)	186	176	0.16
2nd peak (PCR)	186	184	0.16
T833C	40	36	0.12
G919A	61	60	0.11

[a] RSD, relative standard deviation.

Summary and Conclusion

The present work demonstrates the precise estimation of molecular size and yield of CMC cleavage products by CE-LIF and exploits the efficient separation of ssDNA, demonstrating the fast analysis, the quantitative detection, and the small sample requirements of this technology. Both primers were labeled with the same fluorophore, and the products were detected by a single-channel detector. This precludes differentiation between cleavage of the coding or non-coding strand, which requires labeling with different fluorophores and dual-channel detection. An alternative and less expensive approach is repeated analysis using only one labeled primer. Thus, this technology is suitable for screening of known mutations giving expected CMC products. The main advantage compared with mutation assays based on restriction cleavage or primer extension is that unknown mutations are also detected and distinguished from known mutations, making this technology suitable for newborn screening for homocystinuria. However, because sequence-specific electrophoretic migration may prevent accurate assessment of fragment size, the exact localization of new mutations would require subsequent DNA sequencing.

The main disadvantage of the CMC technique is the use of toxic reagents, which could be avoided by using enzyme mismatch cleavage. However, not all mutations are detected by the latter technique, more than one enzyme is required, and unspecific cleavage of homodu-

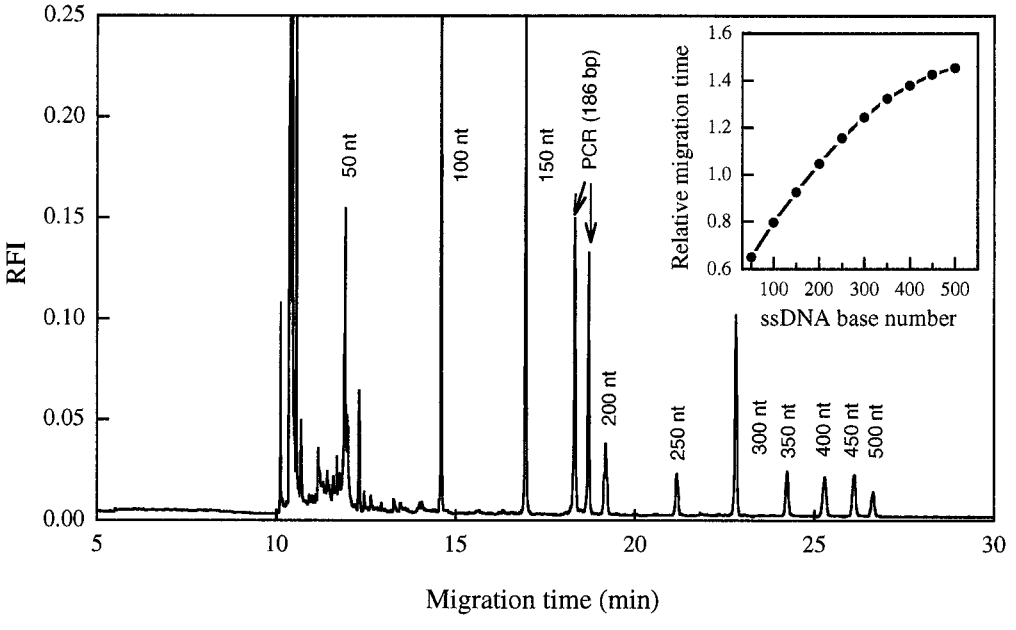

Fig. 6. Capillary electrophoresis of DNA size markers and PCR product migrating as single strands under denaturing conditions.

The two peaks (*arrows*) are derived from coding strand and non-coding strand DNA of the 186-bp PCR product. The first peak of this doublet is used as an internal standard to calculate the relative migration time. The *inset* shows a plot of DNA size vs relative migration time. Electrophoretic conditions were as described in the legend for *Fig. 2*.

plexes has been observed *(33, 34)*. When these problems are solved, the combination of enzyme mismatch cleavage and CE-IF detection should be feasible.

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