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RESEARCH ARTICLE

Temperature and pH Effects on Single-Strand Conformation Polymorphism Analysis by Capillary Electrophoresis

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We investigated the effects of temperature and pH on single strand-conformation polymorphism (SSCP) analyzed by capillary electrophoresis (CE) using short-chain linear polyacrylamide as the sieving medium. Nine different mutations (in factor V, cystathionine β-synthase, and methylenetetrahydrofolate reductase genes), including both transitions and transversions, were investigated. We confirmed that low temperature in general increased the number of detectable single-strand conformations and thereby the sensitivity of the analysis. The pH effects of the separation matrix on the migration pattern, and thus the assay sensitivity, varied markedly between the different DNA fragments. Seven of nine single point mutations were detected at the ordinary pH of 8.3, whereas the CBS T833C mutation was discriminated at the extreme pH values of 9.0 and 6.4, and the CBS G797A mutation could not be detected at any pH value within the range 6.4–9.0. These data emphasize the importance of the pH of the separation matrix in detecting certain mutations by SSCP. Hum Mutat 13:458–463, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: **SSCP; CE-LIF; MTHFR gene; factor V gene; CBS gene; mutation detection**

INTRODUCTION

Among the various techniques developed for mutation detection [Nollau and Wagener, 1997], single-strand conformation polymorphism (SSCP) analysis has become the most popular method for screening of unknown mutation in small stretches of DNA [Orita et al., 1989; Glavac and Dean, 1993; Hayashi, 1993; Sekiya, 1993]. Its widespread use is related to its simplicity and low cost. However, variable sensitivity (range: 30% to >90%) has been reported, reflecting different size and sequence characteristics of the DNA fragment, as well as different electrophoretic conditions [Jordanova et al., 1997].

SSCP analysis is based on the principle that altered nucleotide sequence caused by a mutation affects single-strand DNA (ssDNA) conformation, hence the electrophoretic mobility of DNA fragments in a nondenaturant gel [Orita et al., 1989]. The mobility is conventionally analyzed by slab gel electrophoresis, which is a simple and robust technique, but handling and disposal of radioactive or toxic waste represent drawbacks.

Capillary electrophoresis (CE) in entangled polymers has become an attractive alternative to slab gel electrophoresis techniques for analysis of DNA fragments [Grossman and Soane, 1991; Chang and Yeung, 1995; Ren et al., 1996]. CE can be automated and is characterized by short analysis time, small sample and reagent requirements, high resolution and separation efficiency, and, when coupled to laser-induced fluorescence (LIF) detector, unsurpassed detection sensitivity. CE-LIF has recently been used instead of slab gel electrophoresis for the separation of DNA fragments as part of SSCP analysis [Kuypers et al., 1993, 1996; Hebenbrock et al., 1995; Katsuragi et al., 1996; Ren et al., 1997; Inazuka et al., 1997; Atha et al., 1998; Wenz et al., 1998; Iwahana et al., 1994].

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Abbreviations: CE, capillary electrophoresis; dsDNA, double-stranded DNA; ssDNA, single-strand DNA; SSCP, single-strand conformation polymorphism; PA, polyacrylamide; MTHFR, methylenetetrahydrofolate reductase; FV, factor V; CBS, cystathionine β-synthase; LIF, laser-induced fluorescence; SLPA, short-chain linear polyacrylamide; i.d., internal diameter; o.d., outer diameter; PCR, polymerase chain reaction.

TABLE 1. Composition of Various Buffers

pH value						
9.0	8.7	8.4	8.3	8.2	7.2	6.4
167 mM Tris–33 mM boric acid	125 mM Tris–75 mM boric acid	89 mM Tris–89 mM boric acid–1 mM EDTA	89 mM Tris–89 mM boric acid–2 mM EDTA	89 mM Tris–89 mM boric acid–2 mM EDTA + HCl	29 mM Tris–68 mM HEPES	15 mM Tris–27 mM MES–0.5 mM EDTA

In a previous study [Ren et al., 1997], we found that short-chain and linear polyacrylamide (SLPA) provided efficient high-resolution separation of ssDNA fragments in SSCP analyses. Moreover, the low viscosity of this matrix ensured efficient filling of thin capillaries (i.d < 75 μm). In a recent paper, Kukita et al. [1997] found that the sensitivity of SSCP analysis based on slab gel electrophoresis of long DNA stretches can be increased if low pH buffers are used. In the present work, we investigated the separations of a spectrum of SSCP fragments by CE at various pH values ranging from 6.4 to 9.0, using SLPA as the sieving medium. The experiments were conducted at a temperature below ambient, provided by a liquid cooling device that was part of some commercial CE systems.

MATERIALS AND METHODS

Materials

Acrylamide, N,N,N′,N′-tetramethylenediamine (TEMED), and ammonium peroxydisulfate (APS) were purchased from Bio-Rad Laboratories (Hercules, CA). Hydroxypropylmethylcellulose (HPMC), 4,000 cP, 2% aqueous solution, 25°C, 2-(N-morpholino)ethanesulfonic acid (MES), and N-(2-hydroxyethyl)piperazine-N′(2-ethanesulfonic acid (HEPES, Molecular Biology grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Reaction tubes (thin-walled, Gene Amp) for polymerase chain reaction (PCR) reactions were from Perkin-Elmer (Norwalk, CT). Fused capillaries (50 μm i.d., 192 μm o.d.) were products of Polymicro Technologies (Phoenix, AZ) and DB-17-coated capillaries (100 μm i.d., 375 μm o.d.) were from J & W Scientific (Folsom, CA). QIAquick PCR Purification kit and QIAamp Blood Kit were products of QIAGEN (Hilden, Germany). 5-Fluorescein-labeled primers were synthesized by Eurogentec (Seraing, Belgium). Water, double distilled and purified on a Milli-Q plus Water Purification System (Millipore, Bedford, MA), was used for preparation of all aqueous solutions. SLPA was synthesized with a slight modification [Ren et al., 1997] of the procedure described by Grossman [1994]. Buffers are described in Table 1.

DNA Extraction and PCR Amplification

Blood samples were collected from subjects who were genotyped with respect to variants in the CBS gene (MIM# 236200) [Kim et al., 1997], the MTHFR gene (MIM# 236250) [Ren et al., 1997; Ulvik et al., 1996] and factor V gene (FV_5 or F5; MIM# 227400) [Ulvik et al., 1998; Ren et al., 1998], according to published methods. The nine genetic variants detected are listed in Table 2.

Template DNA used in the PCR reaction were extracted from whole blood using QIAamp Blood Kit according to the manufacturer's instructions. The PCR reaction mixture contained 10 mM Tris-

TABLE 2. Sequence of Primers, PCR Conditions, and Size of PCR Products

Gene	Mutation	Sequence of primer (5′–3′)	PCR condition	Size of product (bp)
MTHFR	C 677T	F-GGAGCTTTGAGGCTGACCTGAA F-GACGATGGGGCAAGTGAT	94°C 30 sec, 58°C 30 sec 72°C 10 sec, 36 cycles	146
	A1298C	F-CTTTGGGGAGCTGAAGGACTACTAC F-CACTTTGTGACCATTCCGGTTTG	94°C 30 sec, 62°C 30 sec 72°C 20 sec, 36 cycles	163
Factor V	G1691A	F-GGCAGGAACAACACCATGAT F-TCAAGGACAAAATACCTGTATTC	94°C 30 sec, 55°C 30 sec 72°C 30 sec, 38 cycles	232
CBS	C785T G797A (exon 7)	F-CCAGGCAGGGACCCAAGAAT F-CCACTCCGCACTGTCCCTCT	94°C 30 sec, 58°C 30 sec 72°C 30 sec, 36 cycles	170
	T833C G919A (exon 8)	F-ACTGGCCTTGAGCCCTGAA F-AGGCCGGGCTCTGGACTC	94°C 30 sec, 60°C 30 sec 72°C 20 sec, 35 cycles	186
	T959C (exon 9)	F-ACGGGCTGTGGTGGGGTC F-CGCACAGCAGCCCCTCTTG	94°C 30 sec, 58°C 30 sec 72°C 10 sec, 34 cycles	111
	C1105T (exon 10)	F-GCACGTGCACAATTCATGCATA F-GCTGCCGGTTCTCAGGTGA	94°C 35 sec, 58°C 40 sec 72°C 30 sec, 38 cycles	277

PCR, polymerase chain reaction; MTHFR, methylenetetrahydrofolate reductase; CBS, cystathionine β-synthase.

HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% Triton X-100, 125 μM of each dNTP, 0.2 μM of each primer, 0.5 U *Taq* polymerase (Super *Taq*, HT Biotechnology Ltd., UK), and approximately 100 ng template DNA in a final volume of 100 μl. The PCR reaction was performed on a Perkin-Elmer 480 thermocycler. The sequences of primers, the size of PCR products and the PCR procedures are depicted in Table 2.

The PCR products were purified with the QIAquick PCR Purification Kit according to the manufacturer's instructions. This purification step was included to remove fluorescent materials, which can interfere with the SSCP analysis. The final volume of the purified DNA sample was 50 μl. The samples were stored at –20°C until analysis. Immediately before CE, the PCR products were diluted 1:10 in water, heated to 95°C for 5 min, and then cooled in ice water for 10 min.

Capillary Electrophoresis Instrumentation

The Beckman P/ACE System 2210 (Beckman Instruments, Palo, CA) was used in this study. It was equipped with a P/ACE LIF detector in which an argon ion laser source provided excitation light (4 mW) at 488 nm. A 520-nm bandpass filter was used as an emission cutoff filter. Beckman System gold software (version 8.10) was used for system control, data collection, and processing.

Capillary Electrophoresis Procedure

A DB-17-coated capillary (total length 37 cm, effective length 30 cm) was mounted in the capillary cartridge of Beckman CE system. Before use, the new DB-17-coated capillary was rinsed with pH 8.4 buffer (TBE), and then filled with 0.5% HPMC; electrophoresis voltage of –12 kV was applied for 15 min. This pretreatment was observed empirically to increase the stability of these columns. Before each injection, the DB-17-coated capillary was rinsed with a buffer and filled with SLPA sieving medium. Samples were introduced by electrokinetic injection at –2 to –5 kV for 6–15 sec. Electrophoresis was performed at reverse polarity under the conditions specified in the figure legends.

RESULTS AND DISCUSSION

The purpose of the present study was to investigate the influence of parameters such as temperature and pH of the CE sieving matrix on the migration and resolution of SSCP analysis of nine single point mutation/polymorphisms. The genotypes were the C677T and A1298C polymorphisms [Frosst et al., 1995; van der Put et al., 1998] of the MTHFR gene, the G1691 A (Leiden) mutation of factor V gene [Dahlback, 1995], and six mutations in exon 7 (C785T and G797A), exon 8 (T833C and G919A), exon 9 (T959C), and exon 10 (C1105T) of the CBS gene [Kraus, 1998]. For all genotypes, except for the T833C and T959C CBS, homozygous mutant (++), heterozygous (+–) and homozygous wild type (––) were obtained, as verified by reference techniques [Ren et al., 1997, 1998; Kim et al., 1997; Ulvik et al., 1996, 1998; van der Put et al., 1998].

Temperature

The effects of the temperature on SSCP analysis based on slab gel electrophoresis and CE have been investigated previously [Sekiya, 1993; Ren et al., 1997; Atha et al., 1998; Hongyo et al., 1993; Arakawa et al., 1996]. Temperature-specific migration profiles have been observed for each mutation, demonstrating that temperature control is paramount.

In the present work, we used a Beckman CE instrument with a liquid cooling system that provided adequate temperature control within the range 15–50°C. We investigated the migration and resolution of the ssDNA conformations at 15, 20, and 25°C. The pH value of the separation matrix was 8.3. For all nine mutations/polymorphisms studied, improved resolution and detection sensitivity were obtained at 15 and 20°C, as compared with 25°C. Electropherograms obtained at various temperatures for the factor V Leiden mutation (which could not be detected at 25°C) and the A1298C MTHFR polymorphism are presented in Figures 1 and 2.

The effect of temperature on SSCP is probably related to altered conformation of the ssDNA fragments, which attain a less folded structure at higher temperature. At high temperature, strands of equal size obtain similar migration time, as demonstrated by Wenz et al. [1998]. In addition, elevation of temperature may affect the entanglement and viscosity of the sieving matrix and thereby influence DNA fragment migration.

pH of the Separation Matrix

Some [Glavac and Dean, 1993; Hayashi, 1993] but not all [Ren et al., 1997; Teschauer et al., 1996] studies have demonstrated that glycerol affect the migration profile in a way that increases the sensitivity of SSCP analysis based on slab gel electro-

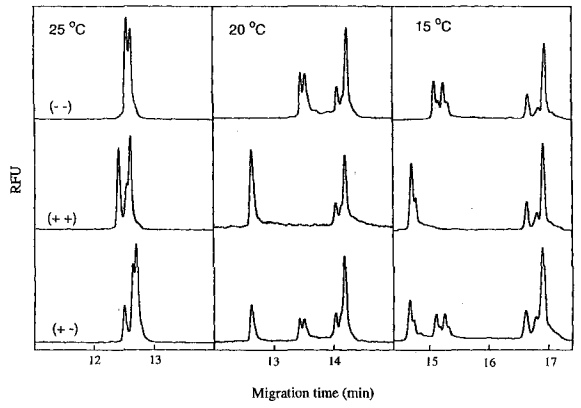

FIGURE 1. **Temperature effect on single-strand conformation polymorphism (SSCP) analysis of the factor V G1691A gene mutation. Electropherograms of wild-type (--), homozygous mutant (++), and heterozygous (+-) samples, obtained at 15°C, 20°C, and 25°C are shown. The electrophoresis medium is 89 mM Tris-borate pH 8.3 containing 2 mM EDTA, and 6% short-chain linear polyacrylamide (SLPA). The applied voltage is -12 kV. RFU, relative fluorescence units.**

phoresis. Recently, Kukita et al. [1997] reported that the effect of glycerol is attributable to reduction of pH of the TBE buffer caused by the reaction of glycerol with borate ion. Previous to this report, pH and buffer components in relation to the performance of the SSCP mutation detection have not been systematically investigated.

We investigated the CE migration profiles of ssDNA fragment during SSCP analysis at 15°C over a wide pH range of 6.4–9. Notably, the effects of pH on the ssDNA resolution and migration profiles were specifically related to the

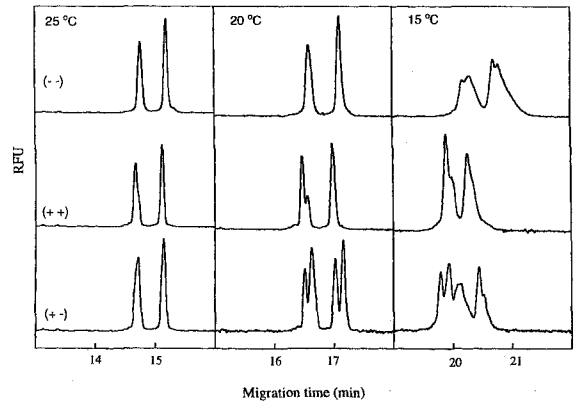

FIGURE 2. **Temperature effect on the single-strand conformation polymorphism (SSCP) analysis of the A1298C methylenetetrahydrofolate reductase (MTHFR) gene mutation. Electropherograms of wild-type (--), homozygous mutant (++), and heterozygous mutant (+-) samples, obtained at 15°C, 20°C, and 25°C are shown. The electrophoretic conditions are as described in legend to Figure 1. RFU, relative fluorescence units.**

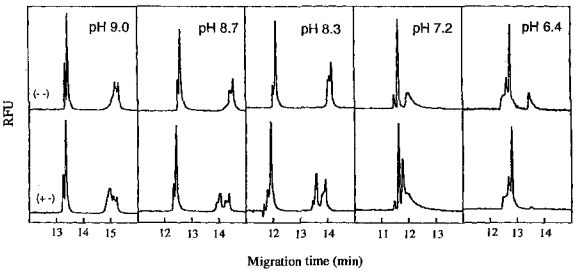

FIGURE 3. **pH effect on the single-strand conformation polymorphism (SSCP) analysis of the T959C cystathionine β-synthase (CBS) gene mutation. Electropherograms of wild-type (--) and heterozygous mutant (+-) samples, obtained at five different pH values ranging from 6.4 to 9.0 are shown. The composition of the electrophoresis buffers are given in Table 1. Temperature is 15°C, applied voltage -12 kV and 6% short-chain linear polyacrylamide (SLPA) is used as sieving matrix. RFU, relative fluorescence units.**

mutation/polymorphism analyzed. For example, the CBS T959C mutation was discriminated at high pH (≥8.2) (Fig. 3), whereas the CBS T833C mutation gave discriminating profiles only at the extreme pH (of 6.4 and 9.0) (Fig. 4). Both mutations are T-C substitutions. The MTHFR A1298C polymorphism was detected over the whole pH range (Fig. 5). Moreover, discriminative profiles were obtained for the CBS C785T mutation at all and for the G797A mutation at no pH values tested (Fig. 6). The latter observation illustrates the mutation specific effect of matrix pH, as analysis of both mutations is based on the same PCR fragment (170 bp) and the sites are located only 12 bp apart.

Kukita et al. [1997] reported improved mutation detection by SSCP based on gel electrophoresis at low pH, whereas we found that the optimal pH for mutation discrimination were observed at both high and low values, depending on the spe-

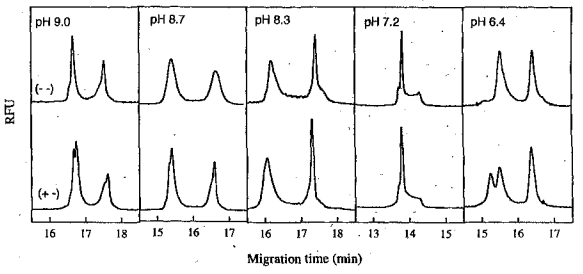

FIGURE 4. **pH effect on the single-strand conformation polymorphism (SSCP) analysis of the T833C cystathionine β-synthase (CBS) gene mutation. Electropherograms of wild-type (--) and heterozygous (+-) samples obtained at five different pH values ranging from 6.4 to 9.0 are shown. The electrophoretic conditions are as described in legend to Figure 3. RFU, relative fluorescence units.**

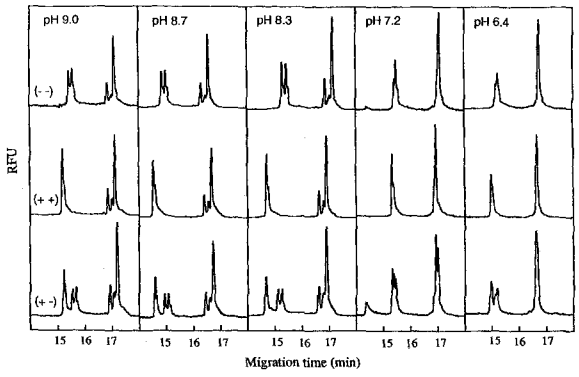

FIGURE 5. **pH effect on the single-strand conformation polymorphism (SSCP) analysis of the A1298C methylenetetrahydrofolate reductase (MTHFR) gene mutation. Electropherograms of wild-type (– –), homozygous mutant (+ +), and heterozygous mutant (+ –) samples obtained at five different pH ranging from 6.4 to 9.0 are shown. The electrophoretic conditions are as described in legend to Figure 3. RFU, relative fluorescence units.**

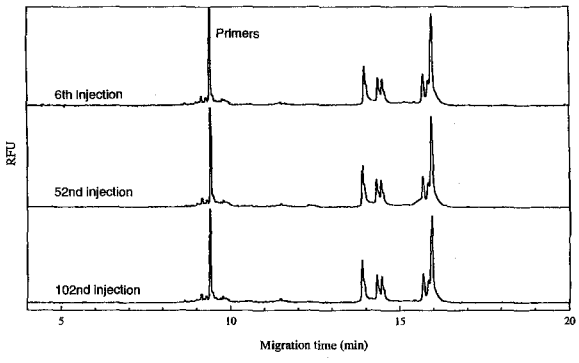

FIGURE 7. **Reproducibility of the single-strand conformation polymorphism (SSCP) pattern through 102 injections. A heterozygous A1298C methylenetetrahydrofolate reductase (MTHFR) sample is injected repeatedly and consecutively for about 17 hr. Temperature is 15°C. The electrophoretic conditions are as described in legend to Figure 1. RFU, relative fluorescence units.**

cific mutation investigated (Fig. 6). Kukita and colleagues explained the effect of low pH by suppression of the charge of the phosphate backbone, leading to reduced intermolecular repulsion and increased involvement of base interactions in stabilization of the ternary structure. The mutation specificity suggests additional mechanisms related to the differential dissociation constant of the A, G, C, and T bases [Perego et al., 1997]. The enolate forms in the hetero aromatic rings of G and T dissociate within the pH range from 6.4 to 9, which may affect the conformation and the charge of ssDNA. This explains how a single base substitution may influence the electrophoretic mobility of a ssDNA fragments and is in accordance with the observation made by Perego et al. [1997] on the migration of oligonucleotides at low pH.

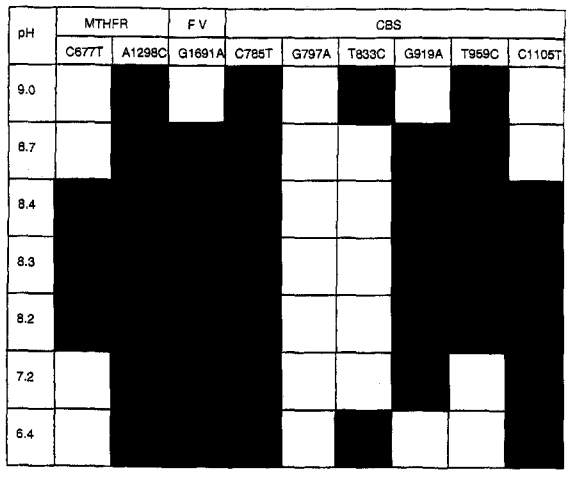

FIGURE 6. **The sensitivity of single-strand conformation polymorphism (SSCP) analysis at different pH. The various mutations investigated are listed in upper horizontal column and the pH at the left row. The electrophoretic conditions are as described in legend to Figure 3. *Shaded area,* SSCP profile permits the identification of the actual genotype.**

Reproducibility

We tested the reproducibility and stability of the SSCP migration patterns during repeated injections. As illustrated for the CE-SSCP pattern of the MTHFR A1298C polymorphism in Figure 7, reproducible profiles were obtained for more than 100 injections.

SUMMARY AND CONCLUSION

The present paper adds to the data demonstrating the suitability of SLPA as sieving matrix in the SSCP analysis based on CE. We also confirmed that temperature has a profound effect on the migration profiles and the sensitivity of the analysis. For the mutations/polymorphisms tested here, a temperature of ≤20°C displayed patterns affording best discrimination. In addition, a strong effect of pH of the capillary sieving matrix was demonstrated for the first time. This effect was highly dependent on the sequence context, but also on the specific mutation/polymorphism to be analyzed. We conclude that temperature and matrix pH should be systematically varied and optimized to increase the sensitivity of SSCP analysis based on CE.

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