also can be cleaved by *Dpn*I. A 12-fold overdigestion with *Dpn*I results in hemimethylated DNA digestion that could be interpreted as suggesting replication failure, where in fact significant replication actually took place. Three- or 6-fold overdigestion digested the fully methylated DNA but preserved approximately half of hemimethylated product (Figure 2). The differential *Dpn*I digestion of fully methylated DNA compared to hemimethylated DNA is not present when the methyl insensitive *Sau*3AI is used on the two substrates. Comparing the *Dpn*I digestion intensity to undigested DNA, the 3–6-fold overdigestions cleave roughly half of the hemimethylated form while completely eliminating the fully methylated form (Figure 3).

Discriminating replicated plasmid from plasmid with radionucleotide incorporation due to repair or short primer extensions is critical for interpreting in vitro replication studies. Underdigestion with *Dpn*I will result in bands that have not been fully replicated, and overdigestion will result in loss of signal from fully replicated plasmid. Rao and Martin (10) have suggested that only the supercoiled band of *Dpn*I-resistant plasmid represents actual replication, while other non-supercoiled bands may not represent true replication. This observation also could be compatible with an underdigestion by *Dpn*I, or the digestion was sufficient to cleave the hemimethylated strand, leading to only cleaved and open circular molecules, though the concise nature of the manuscript precludes a definite conclusion on these possibilities.

The SV40 replication system has illuminated a great deal of information about the role of the large T antigen and human replication protein A in replication (5,6). However, this is a specific viral system and is not shared across all eukaryotes. Unlike the yeast origin of replication, which contains a recognizable consensus sequence, mammals appear to have a more complex mechanism of origin recognition and initiation of replication (4). Clarifying the digestion requirements for *Dpn*I detection of replicated DNA may simplify the analysis of potential mammalian origins of replication using an in vitro system. By working out precise parameters of the interaction of *Dpn*I with hemimethylated DNA, such a technique may be used as a screen for origin-like activity before going to more arduous techniques such as 2-D replication gels (1,14).

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Optimized Sample Preparation for Tandem Capillary Electrophoresis Single-Stranded Conformational Polymorphism/ Heteroduplex Analysis

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ABSTRACT

Here we describe DNA sample preparation methods that allow the rapid, simultaneous generation of both single-stranded conformational polymorphism (SSCP) and heteroduplex DNA elements from a single sample in a single tube, which are suitable for direct injection into a capillary electrophoresis (CE) instrument with excellent sensitivity of genetic mutation detection. The p53 gene was used as a model DNA region for this study, which was performed on a high-throughput MegaBACE™ 96-capillary array electrophoresis instrument. We found that, contrary to the practice common in slab-gel SSCP analysis, denaturants such as formamide are incompatible with this novel technique because they result in homo- and heteroduplex peak broadening in

CE (possibly as a result of incomplete dsDNA re-hybridization) that reduces the peak resolution and hence the sensitivity of mutation detection. We also have found that PCR buffers, which are typically used to suspend samples for slab-gel heteroduplex analysis (HA), but which are less suitable for CE because of the presence of extra salt that reduces the efficiency of electrokinetic injection, may be substituted with a 10 mM Tris-HCl buffer (pH 8.5). The use of this Tris-HCl buffer for sample preparation provides both a high sensitivity of mutation detection by tandem SSCP/HA and high efficiency of electrokinetic injection by CE. In a related study (published elsewhere), we have applied this optimized protocol to the screening of a set of 32 mutant DNA samples from p53 exons 7 and 8 and recorded 100% sensitivity of mutation detection for tandem CE-SSCP/HA, whereas each individual method yielded lower sensitivity on its own (93% for SSCP and 75% for HA).

INTRODUCTION

In the past 10 years, single-stranded conformational polymorphism (SSCP) and heteroduplex analysis (HA) have rapidly gained popularity as two of the most straightforward and versatile methods to screen for DNA alterations (12). Traditionally, SSCP and HA are performed separately and have involved time-consuming (12–14 h), labor-intensive, and cumbersome electrophoresis of radioactively labeled DNA samples under nondenaturing conditions in a highly resolving cross-linked polyacrylamide slab gel and afforded relatively low sensitivity of mutation detection (60%–90%) (12). More recently, however, the advent of automated capillary electrophoresis (CE), in which electrophoretic separation of fluorescently labeled DNA is carried out within a microbore fused-silica capillary filled with an entangled polymer solution to serve as the "gel", has offered a viable alternative to slab-gel SSCP and slab-gel HA and allowed for the potential of much higher throughput, automation, sensitivity (reportedly >90%), and reproducibility of analysis (3,6).

Because of its nature, CE has a much lower tolerance than slab-gel electrophoresis for common sample impurities such as oligonucleotide primers, proteins, salt, and additives such as formamide and NaOH. Yet, to our knowledge, there have been no reports on the development of optimized DNA sample preparation protocols for CE-SSCP or CE-HA. Instead, published reports have mostly focused on the effects of temperature, pH, and DNA chain length on CE-SSCP performance (8,15), while using sample preparation protocols common in slab-gel electrophoresis, including routine

addition of formamide and NaOH to the sample. Studies of CE-HA are more rare in the literature.

We were motivated to carry out the following study of sample preparation protocols for CE-SSCP/HA when we found that these "standard" protocols for slab gels consistently yielded poor CE results (low signal-to-noise ratios, failed injections, and poor resolution of SSCP and HA DNA species). In the course of our studies, we have discovered what seems to be an optimal set of conditions that allow SSCP and HA to be carried out in tandem by CE, a method that involves simultaneous thermal generation and analysis of SSCP and HA conformers from a single sample (10,11,14). This novel technique requires both partial and complete re-annealing of dsDNA to form SSCP and HA conformers, respectively, which places even more stringent requirements on the amount of salt and denaturant contained in the DNA sample, since these additives affect dsDNA hybridization as well as the CE injection and separation performance. To date, there has been only one report on the use of tandem SSCP/HA in CE mode (9), and these critical sample preparation issues have not been discussed there or anywhere else.

MATERIALS AND METHODS

Tandem SSCP/HA

Two wild-type and two mutant DNA sample templates of exons 7 and 8 of the p53 gene (codon 248, CGG→GGG, and codon 273, CGT→AAT) were provided by the National Institute of Standards and Technology (Gaithersburg, MD, USA). PCR amplifications were done on a PTC-150 Minicycler™ (MJ Research, Waltham, MA, USA) using 10 pmol template DNA and a thermal cycling protocol and primers reported elsewhere (13). Crude PCR products, fluorescently labeled with FAM on the forward strand and with JOE on the reverse strand, were purified using either a Microcon®-PCR filtration device (Millipore, Bedford, MA, USA) or a QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. Two microliters of the DNA stock solutions were diluted with 10–40 µL of a 10 mM Tris-HCl buffer (pH 8.5) containing NaCl (0, 10, 20, 50 mM; Aldrich Chemical, Milwaukee, WI, USA) and formamide (0%, 50%, 95%; Aldrich Chemical) to make an SSCP/HA mixture. Formamide was deionized before use with mixed-bed ion exchange resin (Bio-Rad Laboratories, Hercules, CA, USA) according to the procedure recommended by the resin supplier. A 96-

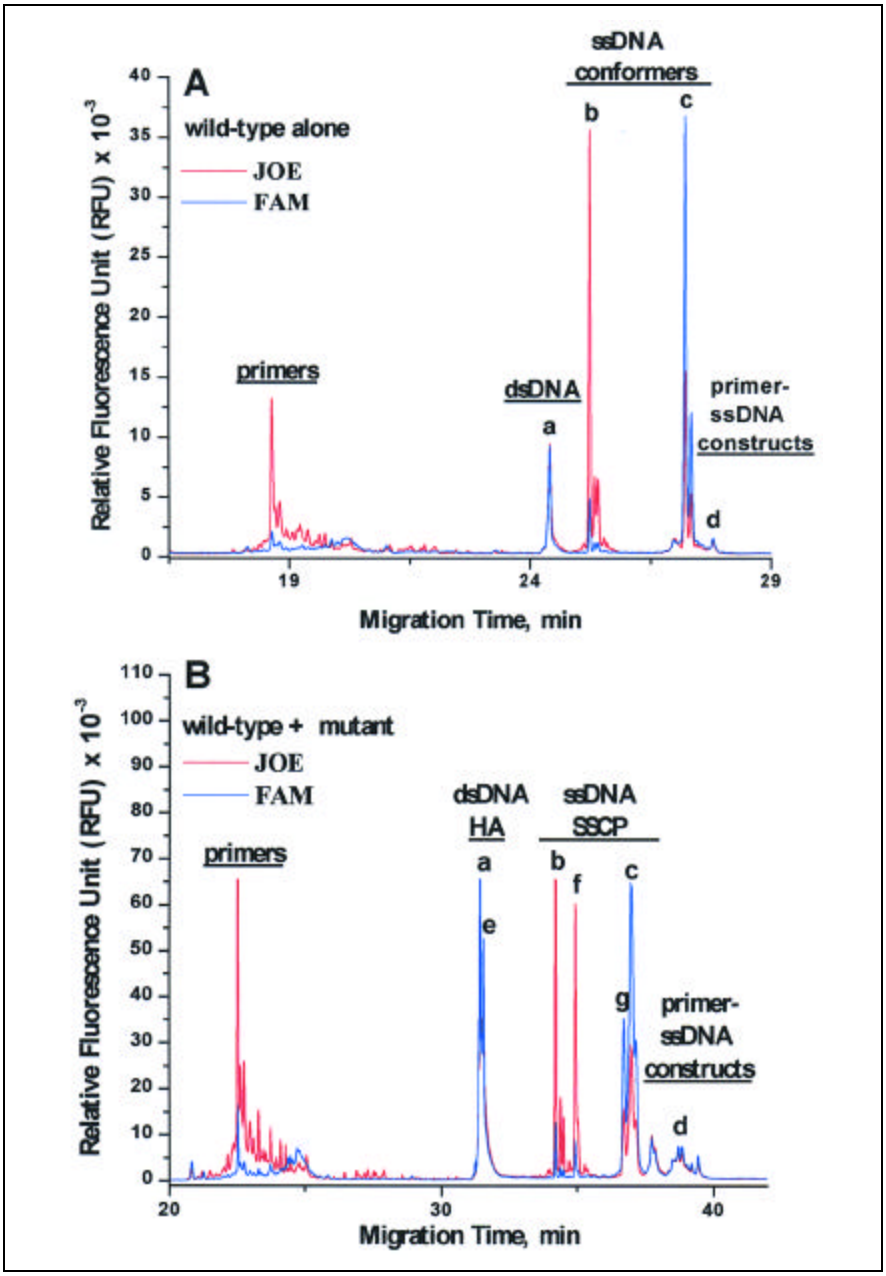

Figure 1. An example of mutation detection by tandem CE-SSCP/HA using a crude DNA sample prepared according to the optimized protocols. (A) p53 gene, exon 7, wild-type alone. (B) p53 gene, exon 7 wild-type + mutant (codon 248 CGG to GGG). a, homoduplexes (FAM + JOE); b, wild-type DNA reverse-strand SSCP (JOE); c, wild-type DNA forward-strand SSCP (FAM); d, primer-ssDNA constructs (FAM + JOE); e, heteroduplexes (FAM + JOE); f, mutant DNA reverse-strand SSCP (JOE); g, mutant DNA forward-strand SSCP (FAM). Electrophoresis conditions: injection 240 V/cm, 25–40 s, 27°C; CE 190 V/cm, 12 µA, 25–40 min, 27°C; bare fused-silica capillaries dynamically coated with polyDuramide (1) and filled with an optimized DNA separation matrix [a 4% (w/v) solution of linear polyacrylamide (M_r, 600 kDa) in a TBE buffer] (7).

well plate (Amersham Biosciences, Piscataway, NJ, USA) was loaded with SSCP/HA mixtures (three repeats for each sample) containing 5 µL DNA and denatured at 95°C for 3 min using a PTC-200™ thermal cycler (MJ Research). After denaturation, the plate was cooled, loaded into a 96-capillary MegaBACE™ (Amersham Biosciences) automated capillary electrophoresis instrument, and analyzed by CE with laser-induced fluorescence detection at 27°C. Raw data were converted to text files and processed using ORIGIN (Microcal Software, Northampton, MA, USA). Although only two MegaBACE arrays were used in this study, all six arrays could be used to screen 96 DNA specimens simultaneously for studies involving larger number of samples. The DNA samples could be kept at 4°C for at least 24 h and analyzed with no detrimental effects on the sensitivity of mutation detection.

RESULTS AND DISCUSSION

The tandem CE-SSCP/HA technique described here first involves the CE analysis of the wild-type p53 DNA (exon 7, 139 bp), as shown in Figure 1A. In the two-color detection mode, dsDNA (homo- and heteroduplexes) exhibits two peaks in the two fluorescence detection channels that are perfectly overlapping with respect to both position and amplitude, while ssDNA (SSCP conformers) shows just one major peak of one color, accompanied by a minor peak of the second color. The minor peak, which is due to some minimal overlap of FAM and JOE dye emission spectra, is centered in the same region as the major one, but the two peaks do not overlap in amplitude. Forward-strand ssDNA labeled with FAM is easily distinguished from reverse-strand ssDNA labeled with JOE. Based on these considerations, we are able to make the following unambiguous assignments of the major peaks seen in Figure 1A: peak labeled a, dsDNA (unresolved homoduplexes); b, reverse-strand SSCPs; c, forward-strand SSCPs (peaks labeled d will be discussed below). In the next phase of mutation detection, we perform a CE analysis of the mixture of the wild-type and the unknown (patient) DNA of interest, as shown in Figure 1B. A genetic sample is scored as containing mutant DNA if the electropherogram of a mixture of the wild-type and mutant DNA exhibits additional peaks associated with either dsDNA (heteroduplexes; Figure 1B, peaks labeled e), reverse-strand SSCPs (Figure 1B, peaks labeled f), or forward-strand SSCPs (Figure 1B, peaks labeled g), which are not found in the electropherogram of the separately analyzed wild-type DNA (Figure 1A, peaks labeled a, b, and c). Note that these peak patterns were highly reproducible from run to run.

Although salt concentrations of greater than 50 mM NaCl or KCl are recommended for the determination of the T_m of dsDNA to screen charge repulsion of phosphate groups in the DNA backbone and ensure its correct re-annealing, no strict guidelines exist for salt content in homo/heteroduplex DNA samples analyzed by CE-HA, or a combined CE-SSCP/HA analysis, despite the fact that these methods involve rapid DNA sample cooling and may require a substantial salt content to effect correct DNA folding or hybridization. At the same time, high salt content reduces the efficiency of electrokinetic injection into capillaries and is hence undesirable for CE analyses. Moreover, for SSCP analysis, PCR products are commonly diluted 10–100× with 50%–95% aqueous formamide, a potent denaturant, which further alters DNA hybridization kinetics. For example, high formamide concentrations (i.e., 95%) will depress the T_m of dsDNA by as much as 60ºC, while an increase in salt concentration from 20–50 to 100 mM will increase the T_m by 5ºC–10ºC (4,5). These observations clearly warrant a more thorough study of the effects of these additives on the sensitivity of CE mutation detection by SSCP, HA, and tandem SSCP/HA.

We have investigated the combined effects of additives such as NaCl and formamide on the sensitivity of tandem CE-SSCP/HA (data not shown). Two wild-type and two mutant p53 gene specimens were used to prepare 12 sample solutions in 10 mM Tris-HCl buffer (pH 8.5) with varying ratios of NaCl and formamide. Our earlier experiments revealed that SSCP samples diluted with pure water in the absence of salt—conditions that are highly desirable in CE because of the efficient electrokinetic injection they allow—yield extremely broad peaks for poorly folded DNA (>20 s width at half-heights) that are unsuitable for mutation detection, confirming similar observations made earlier by Tian et al. (16). Significantly, we later found that 10 mM Tris-HCl (pH 8.5), a common DNA analysis buffer, used as a diluent does not require additional salt for the production of sharp (2–5 s width at half-heights) and reproducible (<2% standard deviation on peak positions) SSCP and HA peaks from a single sample. Therefore, this buffer was selected as a starting point in our optimization of the additives (formamide and NaCl). While these additives seemed to have little effect on SSCP patterns, reproducibility, or peak width, their presence reduced the efficiency of electrokinetic injection. Although the addition of formamide may perhaps be necessary in the preparation of DNA samples with high GC content to ensure complete denaturing, we did not find it useful in routine SSCP analysis of exons 7 and 8 of the p53 gene. Moreover, it reduced the intensity of the peaks for homo- and heteroduplexes and also made them broader, and hence less easily resolved. Therefore, we conclude that 10 mM Tris-HCl buffer (pH 8.5) without added salt or formamide is an excellent sample medium for tandem CE-SSCP/HA.

Typically, samples designated for SSCP analysis are denatured at 95°C and then either snap-cooled or allowed to cool naturally at room temperature, while those analyzed by HA are denatured and cooled slowly over a period of 30–60 min to ensure complete re-annealing. Although it was previously observed that some homo- and heteroduplexes can be formed during snap-cooling, doubts remained until recently about whether the fast kinetics of annealing allow for a perfect re-formation of the duplexes (2). To evaluate the effects of the cooling rate on the preparation of samples for tandem SSCP/HA, we denatured a DNA sample in a 10 mM Tris-HCl buffer (pH 8.5) and then either snap-cooled it, allowed it to cool naturally at room temperature, or gradually cooled it at a linear rate over

10, 20, 30, or 40 min. We observed that the first two modes of cooling yielded very similar electropherograms, in which SSCPs comprised 60%–80% and homo- and heteroduplexes comprised 20%–40% of the injected DNA (data not shown). However, snap-cooling produced less of the unwanted primer-ssDNA constructs. Apparently, during snap-cooling, fewer primers have sufficient time to find their complementary ssDNA and anneal to them. More gradual cooling of the denatured DNA samples was also attempted to balance the relative concentrations of homo- and heteroduplexes and SSCP conformers but led in all cases to the formation of homo- and heteroduplexes exclusively and a complete disappearance of the SSCP peaks.

The impact of the method of post-PCR DNA purification has also been investigated. Salt or oligonucleotides (DNA primers) present in the crude sample interfere with the electrokinetic injection of larger biomolecules, since these low-molecular weight charged species have higher electrophoretic mobilities than the SSCP/HA analytes and hence are preferentially injected. Also, as mentioned above, free primers can anneal to SSCP conformers during the cooling stage before CE analysis to form primer-ssDNA constructs. The peaks associated with these constructs showed two perfectly overlapping signals of the two different colors and had migration times similar to those of the SSCP conformers (Figure 1, A and B, peaks labeled d). The presence of these peaks correlated with a decrease in the intensity of the SSCP peaks and varied from mutant DNA sample to mutant DNA sample. Hence, these are spurious peaks that are not useful for the analysis. This peak assignment was confirmed by experiments in which SSCP/HA was done on a sample of p53 exon 8 that had been purified by preparative slab-gel electrophoresis and subsequently titrated with varying amounts of either the forward or reverse primer. It was observed that addition of the reverse primer led to the appearance of the extra peaks and concomitant disappearance of the forward-strand SSCP conformer. The formation of these primer-ssDNA constructs, which complicates the analysis and peak assignment of electropherograms, is most likely ubiquitous in the literature. We have found that this problem can be minimized by reducing concentrations of the stock solutions of primers from 20 pmol/μL (the standard PCR protocol) to 2–4 pmol/μL.

In summary, we have developed optimal sample preparation protocols for a tandem CE-SSCP/HA mutation detection of the p53 gene (exons 7 and 8) and can conclude that the best ap-

proach is PCR amplification of a template with a reduced primer content (2–4 pmol/μL), followed by 20–40× dilution with 10 mM Tris-HCl (pH 8.5) without additives (salt or formamide), denaturation at 95ºC for 3 min, and snap-cooling on ice. In the interpretation of the resulting electropherograms, a two-color fluorescent labeling scheme was key to high-sensitivity, unambiguous mutation detection. We have used this optimized sample preparation protocol, along with an optimized linear polyacrylamide separation matrix, for the screening of a set of 32 mutant DNA samples derived from p53 exons 7 and 8, selected to include three different DNA sizes (139, 200, and 300 bp), both single- and two-base substitutions, and several different mutation positions (codons 242, 245, 248, 249, 273, and 282) (7). For this larger set of samples, we observed 100% sensitivity of mutation detection by a tandem CE-SSCP/HA approach (all 32 mutations were detected), whereas each individual method yielded lower sensitivity on its own (7). In particular, 30 of 32 mutations (93%) were detected by CE-SSCP, while 24 of 32 mutations (75%) were detected by CE-HA. For clinical implementation of a mutation detection method, sensitivity greater than 97% is desired. It seems reasonable to assume that these protocols will work well for any DNA sample. We are currently testing these sample preparation methods for the mutation analysis of the PTEN and β-catenin genes derived from human prostate tumors to test their broader generality.

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