ELSEVIER

Journal of Chromatography A, 677 (1994) 169–177

JOURNAL OF CHROMATOGRAPHY A

Polymerase chain reaction heteroduplex polymorphism analysis by entangled solution capillary electrophoresis

Jing Cheng[a], Takao Kasuga[a], Keith R. Mitchelson[a,*], Eric R.T. Lightly[b], Nigel D. Watson[c], William J. Martin[b], David Atkinson[d]

[a]*Department of Molecular and Cell Biology, University of Aberdeen, Aberdeen AB9 1AS, UK*
[b]*Department of Technology Development, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, UK*
[c]*Forensic Science Unit, Department of Pure and Applied Chemistry, University of Strathclyde, 204 George Street, Glasgow G1 1XW, UK*
[d]*Land Resources Department, Scottish Agriculture College, 581 King Street, Aberdeen AB9 1UD, UK*

First received 7 March 1994; revised manuscript received 5 April 1994

Abstract

Heteroduplex DNA polymorphism analysis (HPA) makes use of conformational polymorphisms to alter electrophoretic mobility of fragments and can be used to detect non-restrictable loci. We have developed a novel application of entangled solution capillary electrophoresis (ESCE) to separate heteroduplex and homoduplex DNA molecules. The addition of ethidium bromide and glycerol to the free solution sieving buffer resulted in the improved peak resolution and good reproducibility. Reannealed polymerase chain reaction products could be used directly for mutation screening and with fully automated ESCE the entire HPA may be completed in less than 30 min including sample handling. This technology could provide a rapid and highly efficient way for screening rare mutations among large numbers of individuals.

1. Introduction

High-performance capillary electrophoresis (HPCE) has been increasingly used for discriminating DNA length polymorphism in many areas of medical science [1,2], agriculture and biological analysis [3]. It has been applied to the rapid analysis of double-stranded DNA (dsDNA) [1,4] and single-stranded DNA (ssDNA) [5,6] where reliability, speed and automated data handling are an advantage.

The great demands for identifying disease-causing genes and characterizing the mutations that disrupt them has enabled substantial progress in the development and application of various mutation-scanning methods. Sequence polymorphisms in polymerase chain reaction (PCR)-amplified DNA fragments can be efficiently detected by techniques such as denaturing gradient gel electrophoresis [7], single-stranded conformation polymorphism analysis [8], RNase A cleavage [9], chemical cleavage of mismatch [10] and heteroduplex polymorphism assay (HPA) [11]. Among these techniques HPA has proved to be one of the most sensitive and simple methods for mutation detection and therefore has found application in many areas, especially in medical research [12–15]. However,

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00368-J

conventionally HPA has used polyacrylamide gel electrophoresis (PAGE). Heteroduplex molecules with single, or low numbers of mismatches are often resolved poorly or not at all by PAGE.

We report here a method for heteroduplex DNA polymorphism analysis using entangled solution capillary electrophoresis (ESCE) and non-denaturing conditions. Using our conditions, heteroduplex DNA of 126 base pairs (bp) with a single-nucleotide mismatch can be differentiated from the homoduplex in less than 30 min. In addition, PCR products were applied directly for the analysis without any pre-treatment. The conditions for this pilot analysis have not been previously reported, although the resolution of heteroduplex with single-base substitution has very recently been achieved using constant denaturant CE [16].

2. Experimental

2.1. DNA

ΦX174 DNA Hae III digest (Sigma) was used as the molecular mass marker and diluted to a concentration of 20 ng/μl before injection.

Separate PCR reactions were performed in a total mixture of 25 μl with 50 m*M* KCl, 10 m*M* Tris–HCl, pH 8.3, 1.5 m*M* $MgCl_2$, 200 μM each of deoxyadenosine 5′-triphosphate, deoxycytidine 5′-triphosphate, deoxyguanosine 5′-triphosphate and thymidine 5′-triphosphate, 25 μg gelatine, 225 n*M* primer oligonucleotides and 0.5 units of Taq DNA polymerase (Boehringer) with 5 ng template DNA from each of four European *Heterobasidion annosum* genotypes [17]. Oligonucleotides were fungal rRNA primers: HA1 (5′-TTAGCGAGACCCTTGTGGTG-3′) and HA2 (5′-GATTTGAGGTCAAGTTTCGA-3′) which amplify part of the intergenic transcribed spacer region flanking the 5.8S rRNA gene (S = Svedberg unit = 10^{-13} s).

Heteroduplex DNAs were prepared as follows: 10 μl of each of the two PCR products were mixed in a 0.5-ml Eppendorf tube, heated to 94°C for 5 min and then allowed to cool slowly to room temperature. The DNAs were then maintained at this temperature until electrokinetic loading onto the capillary.

2.2. Buffer

The stock buffer system for ESCE consisted of 90 m*M* Tris base, 90 m*M* boric acid, 2 m*M* EDTA, pH 8.5 (1 × TBE), to which 0.5% (w/v) hydroxypropylmethylcellulose (HPMC) was added. The viscosity of a 2% aqueous solution of this cellulose derivative (H-7509, Sigma) was 4000 cP at 25°C. The HPMC was dissolved into the 1 × TBE buffer using the method recommended by Ulfelder et al. [2]. Ethidium bromide (3 μM) and 4.8% (v/v) of glycerol (final concentrations) were then added to the above solution. The buffer was filtered using a 0.2-μm filter and kept at 4°C before use.

2.3. Capillary electrophoresis

ESCE was performed on the P/ACE system 2050 (Beckman) in the reversed-polarity mode (negative potential at the injection end of the capillary). The temperature was set at 22°C and UV absorbance was monitored at 260 nm. Post-run analysis of data was performed using the Gold Chromatography Data System (version 7.11). Surface-modified fused-silica capillary, DB-17 (J & W Scientific) was used for all analysis. The capillary (57 cm × 100 μm with effective length of 50 cm) was conditioned daily with five volumes of the above-mentioned buffer and then subjected to voltage equilibration for 15 min until a stable baseline was achieved. Samples were introduced into the capillary by electrokinetic injection at negative polarity of 175 V/cm for 13 s. Separations were performed under constant voltage at 228 V/cm for 30 min. The capillary was rinsed with two column volumes of the buffer after each run prior to the next injection.

3. Results and discussion

In a typical heteroduplex mixture four DNA fragments are present. They include two

homoduplex DNA fragments AB and A'B', and two corresponding heteroduplex DNA fragments AB' and A'B. Electrophoretic migration of mismatched heteroduplex DNAs is retarded relative to homoduplex DNA because of conformational distortion created by the mismatched region(s).

PCR-amplified products from the ITS2 region of *H. annosum* rRNA gene repeat were used in a HPA assay for mutation detection using ESCE. Three intersterility groups (S, F and P) can be distinguished by the sequence variations in the ITS regions [17]. The amplification products formed a series of molecules which differ by a range of mutations from a single-base substitution to three deletions and three-base substitutions (see Table 1). The details of these sequence variations are also shown in Fig. 1. Despite the variety of mismatches present in the heteroduplex molecules, fractionation by conventional PAGE does not clearly resolve all molecular species (data not shown). In addition, heteroduplex bands appear diffuse. This may be due to multiple and changing conformations assumed by the heteroduplex molecules, or may be due to insufficient heat dissipation in conventional PAGE.

To establish the buffer system the separation of ΦX174 DNA Hae III digest marker was examined by CE. Glycerol and ethidium bromide were introduced into the entangled polymer sieving buffer which is basically the 1 × TBE buffer with 0.5% (w/v) HPMC (Fig. 2a). Ethidium bromide has previously been reported to improve the resolution of dsDNA fragments by CE [18]. Ethidium bromide preferentially intercalates into G + C-rich sequence, increasing the chain length and altering the mobility of

S_{Fi}: HA1----T--------XX---A--G--A----C----HA2 (125 bp)

S_{Ha37}: HA1----C--------XX---A--G--A----C----HA2 (125 bp)

F_{It}: HA1----C--------XX---A--G--X----C----HA2 (124 bp)

P_{GB}: HA1----C--------TG---G--A--A----T----HA2 (127 bp)

Fig. 1. Illustration of the base differences among the PCR-amplified products from samples S_{Fi} (S strain), S_{Ha37} (S strain), F_{It} (F strain) and P_{GB} (P strain) of *H. annosum*. HA1/HA2 are *H. annosum* specific oligos (17 bp). X indicates the absence of a nucleotide residue. Homoduplex DNAs are 125 bp for S_{Fi}, 125 bp for S_{Ha37}, 124 bp for F_{It} and 127 bp for P_{GB}.

some DNA fragments. Here it was found that the addition of glycerol improved the resolution and reproducibility in the separation of all DNA fragments especially for these ranging from 200 to about 1200 bp regardless of the G + C content, but also retarded the migration of the DNA (see Fig. 2b and c). The detailed study will be reported elsewhere. The best resolution was achieved by the addition of both glycerol and ethidium bromide (Fig. 2a).

Homoduplex and heteroduplex PCR mixtures from S_{Fi} (S strain) and F_{It} (F strain) were separated by ESCE and shown in Fig. 3. From the electropherograms it can be clearly seen that two PCR-amplified DNA fragments differing by one base deletion and one base substitution (1D + 1S/1B) are well resolved by ESCE in less than 20 min. Pre-treatment of PCR products such as desalting and deproteinising are not necessary. In our hands, ESCE with desalted heteroduplex molecules did not improve resolution between peaks but did increase both the detection and the mobility of all species (results not shown). The gain in run speed and sensitivity

Table 1
Pair-wise comparison of the differences among the PCR products

	S_{Ha37}	S_{Fi}	F_{It}	P_{GB}
S_{Ha37}	0	0D + 1S/0B	1D + 0S/1B	2D + 3S/2B
S_{Fi}		0	1D + 1S/1B	2D + 4S/2B
F_{It}			0	3D + 3S/3B
P_{GB}				0

D = Deletion; S = substitution; B = base pairs.

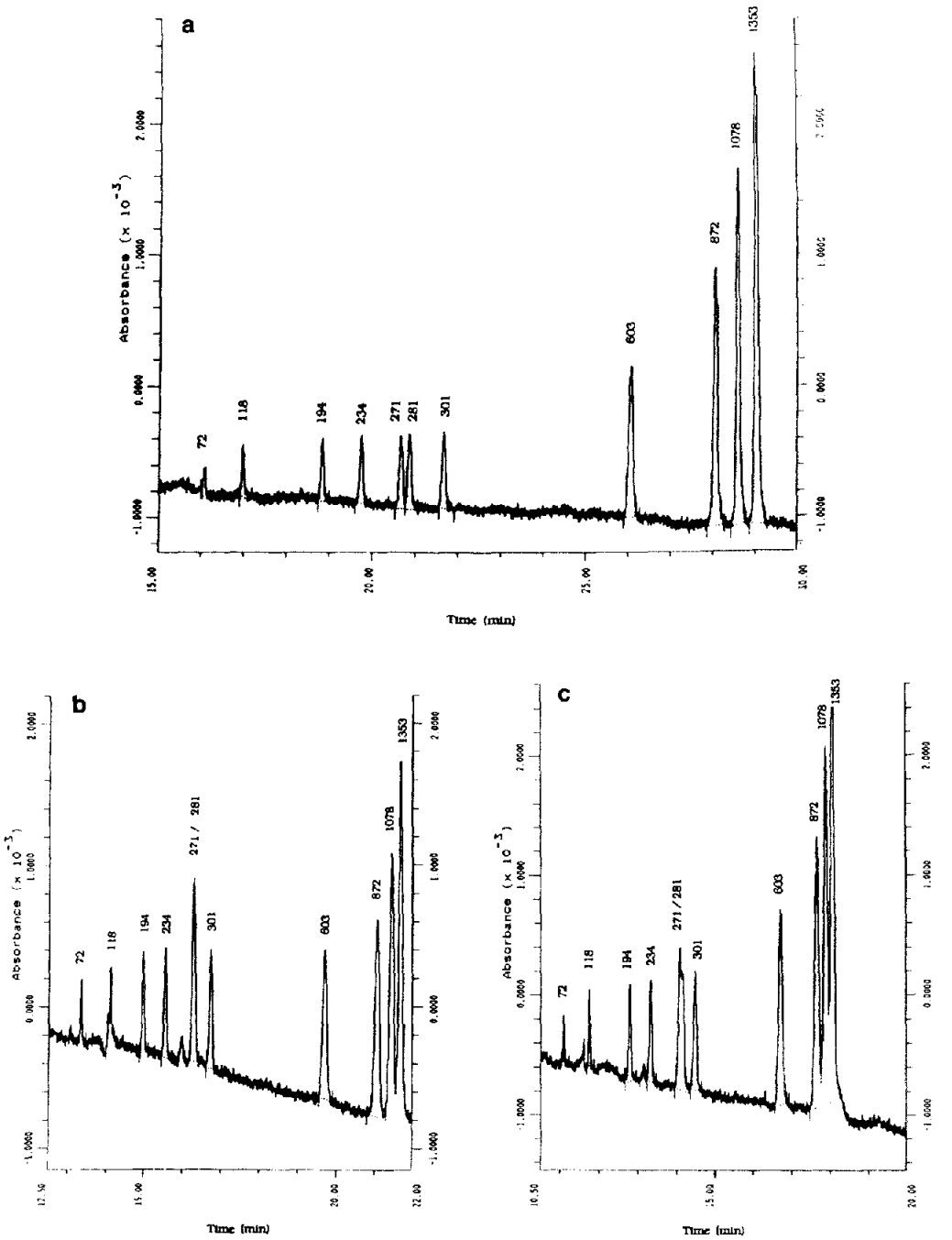

Fig. 2. Electropherograms of ΦX174 DNA Hae III digest marker (diluted to 20 ng/ml with water) obtained using the sieving buffer (a) with both glycerol (4.8%, v/v) and ethidium bromide (3 μM), (b) without ethidium bromide and (c) without glycerol and ethidium bromide. The unit of the DNA fragment length is base pairs. The sample was electrokinetically loaded into the capillary (DB-17, 57cm × 100 μm, the effective length was 50 cm) at 175 V/cm for 10 s. The separation was carried out at 228 V/cm for 30 min. The UV absorbance was monitored at 260 nm. The temperature of the external surface of the capillary was maintained between 23 ± 0.3°C.

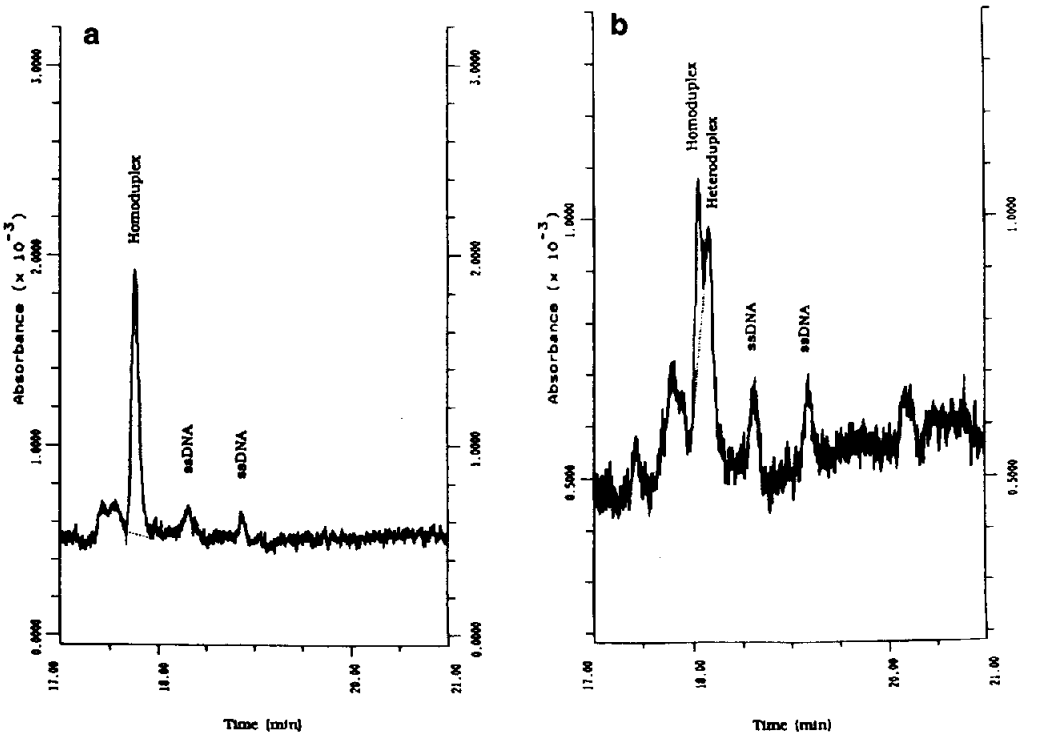

Fig. 3. Electropherograms of PCR mixtures from S_{Fi} (S strain) and F_{It} (F strain) homoduplex (a) and heteroduplex (b). ESCE conditions as in Fig. 2a except the injection lasted for 13 s.

however does not justify the additional processing and handling required to desalt the DNAs.

Homoduplex and heteroduplex PCR mixtures from S_{Fi} (S strain) and P_{GB} (P strain) were separated by ESCE and shown in Fig. 4. From the electropherograms it can be seen that baseline separation between the homoduplex DNA fragments and the heteroduplex molecules containing a two-base deletion and a four-base substitution mismatch (2D + 4S/2B) was obtained.

Homoduplex and heteroduplex PCR mixtures from F_{It} (F strain) and P_{GB} (P strain) were separated by ESCE and shown in Fig. 5. Heteroduplex molecules containing a three deletion and a three-base substitution mismatch (3D + 3S/3B) were further separated at baseline level compared to the results achieved with the (2D + 4S/2B) mismatch heteroduplex (see Fig. 4b). Additionally, partial separation of the two homoduplex fragments differing by three base pairs can also be seen in both Fig. 5a and b.

Homoduplex and heteroduplex PCR mixtures from S_{Fi} (S strain) and S_{Ha37} (S strain) were separated by ESCE and shown in Fig. 6. The results show that the heteroduplex DNA fragments from the same strain containing one base substitution mismatch (0D + 1S/0B) could not be resolved from homoduplex molecules by ESCE.

Homoduplex and heteroduplex PCR mixtures from F_{It} (F strain) and S_{Ha37} (S strain) were separated by ESCE and shown in Fig. 7. The results show that the heteroduplex DNA fragments containing one-base deletion mismatch (1D + 0S/1B) in 124 bp can still be resolved from homoduplex molecules.

Our previous analysis of this sequence by conventional restriction polymorphism analysis [17] showed that European P strain can be

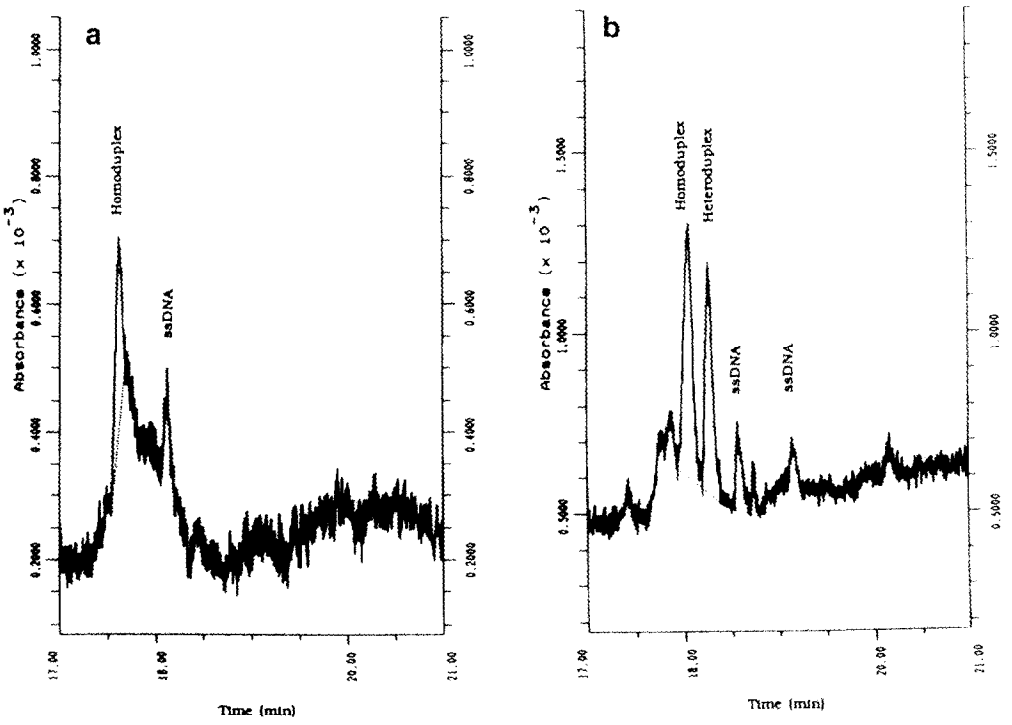

Fig. 4. Electropherograms of homoduplex (a) and heteroduplex (b) PCR mixtures from S_{Fi} (S strain) and P_{GB} (P strain). ESCE conditions as in Fig. 2a except the injection lasted for 13 s.

differentiated from European S and F strains of *H. annosum*. However, discrimination could not be achieved between S strain isolates varying by a single-base pair substitution, or between F and S strains differing by (1D + 0S/1B) or (1D + 1S/1B) (Table 1). Here we demonstrate ESCE of heteroduplex molecules greatly improves strain identification in that F and S strain isolates may now be clearly differentiated. Above all, the method is rapid, reproducible and simple as well as permitting superior resolution to the fractionation achieved by conventional PAGE.

Although a single-base substitution mismatch was not resolved by our system this study clearly demonstrates that the combination of a single-base deletion mismatch *cis*-linked with a single-base substitution mismatch can be resolved from a single-base deletion mismatch by non-denaturing ESCE. The presence of a single-base deletion mismatch amplifies the change of the mobility of heteroduplex caused by the single-base substitution mismatch (Fig. 3). This observation has previously been reported by Van den Akker et al. [19] using a Pharmacia PhastGel electrophoresis system. In our DNA model the single-base substitution mismatch is located 78 bp from the single-base deletion, which are 22 and 27 bp from the fragment termini, respectively. This is comparable to the distance between mismatch regions with which Van den Akker et al. [19] achieved PAGE fractionation using a portion of the *Escherichia coli lac Zα* gene. While clear fractionation of *H. annosum* heteroduplex DNAs occurred, optimisation of our ESCE conditions may be required for particular DNA species as factors such as fragment length, DNA sequence, or the nature of the DNA mismatch can influence the migration of heteroduplex species. However, this present work and the results of Van den Akker et al. [19] suggest that a variety of single-base substitution mutations *cis*-linked to a single deletion mismatch can be resolved electrophoretically from molecules possessing a single deletion mismatch under non-

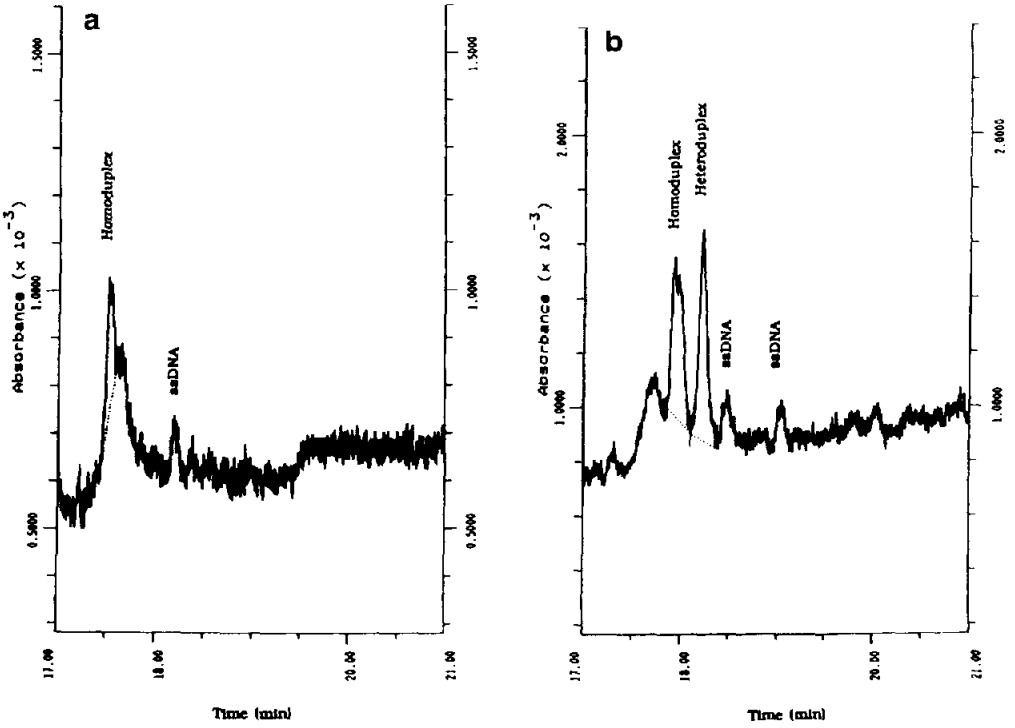

Fig. 5. Electropherograms of homoduplex (a) and heteroduplex (b) PCR mixtures from F_{It} (F strain) and P_{GB} (P strain). ESCE conditions as in Fig. 2a except the injection lasted for 13 s.

denaturing and ambient temperature conditions. Therefore, the technique should be useful for fractionation of a variety of heteroduplex DNA molecules from biological samples.

Khrapko et al. [16] describe an application of HPCE to the analysis of conformational polymorphisms in DNA molecules part-melted in a denaturing (chemical and thermal) environment, termed constant denaturant capillary electrophoresis (CDCE). While the resolution between mismatched heteroduplex and homoduplex molecules achieved by the CDCE technique are impressive, the DNA sequences employed in that study have particular features that allow application of CDCE. The DNA fragment had a CG-rich domain (a CG-clamp) that conferred duplex stability under denaturing conditions necessary for amplification of conformational differences in CG-poor domain containing the mismatch. Notably, small changes in temperature above that required to observe conformational polymorphism could melt the CG-clamp at which discrimination between heteroduplex and homoduplex molecules was no longer detectable. While we do not wish to compare the ESCE system directly to CDCE, analysis using the latter requires the choice of either DNA molecules possing a CG-clamp domain, or the synthesis of PCR products with CG-clamp extensions necessary to provide the duplex stability in heteroduplex molecules in denaturing conditions. The ESCE system does not require the provision of natural or artificial CG-clamp domains and hence may be used on a range of natural PCR species or other DNA fragments. The ESCE system does achieve good resolution of mismatched molecules in each case tested except for the single-base substitution mismatch (Fig. 6). In addition, the migration of homoduplex and heteroduplex molecules during ESCE was not highly sensitive to temperature in the range of 18 to 22°C although resolution between molecular species improved at the lower temperature (results not shown).

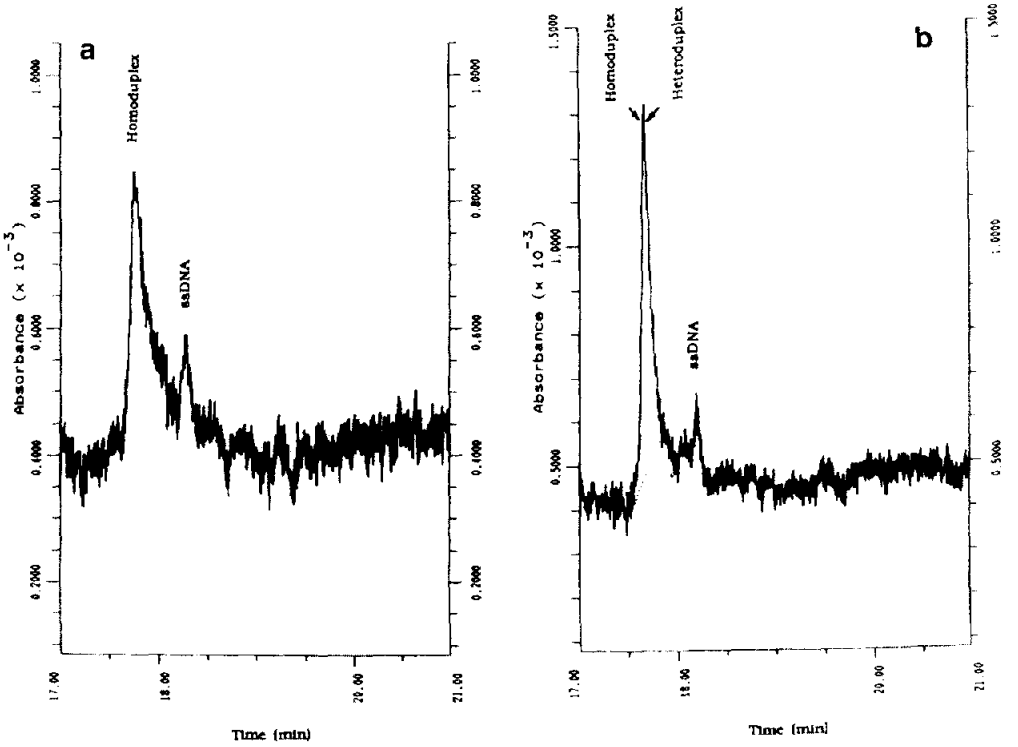

Fig. 6. Electropherograms of homoduplex (a) and heteroduplex (b) PCR mixtures from S_{F1} (S strain) and S_{Ha37} (S strain). ESCE conditions as in Fig. 2a except the injection lasted for 13 s.

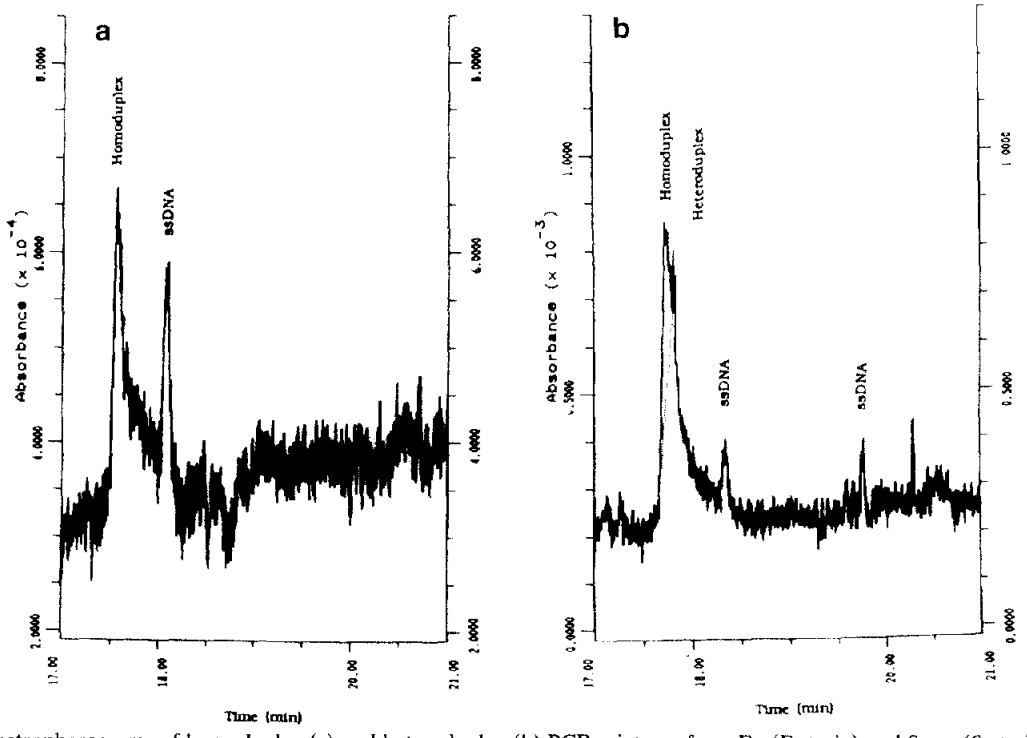

Fig. 7. Electropherograms of homoduplex (a) and heteroduplex (b) PCR mixtures from F_{F1} (F strain) and S_{Ha37} (S strain). ESCE conditions as in Fig. 2a except the injection lasted for 13 s.

The conditions we established in this work are not selective in regard to DNA sequence information and therefore generally applicable to heteroduplex polymorphism analysis using HPCE. The separation between mismatched heteroduplex and homoduplex was observed to increase with each additional substitution mismatch or deletion mismatch. Although we have not explored position effects we feel that ESCE will be able to be used to predict the nature of mismatches in defined PCR fragments. In addition, creation of a series of single-point deletions in a DNA template covering a locus would provide a set of analytical tools for detection of substitution mismatches by ESCE. The deletion series would be used to form mismatch heteroduplex with amplification products from individuals. The position of common substitution mutations could be identified by the extent to which the mutation altered migration in ESCE. Rare, novel substitution mutations would also be identifiable directly by the unusual migration of their heteroduplexes formed with the deletion series standards. It is also believed that use of laser induced fluorescence rather than UV would dramatically improve the sensitivity of detection of heteroduplex conformation isomers resolved by ESCE. More work is in progress to further develop the applications of ESCE for mutation detection.

Acknowledgements

This work was supported by the Scottish Office (SOAFD), the UFC-Biotechnology Initiative and the NERC (GR9/370). The authors wish to thank Beckman Instruments, Inc. for the loan of the P/ACE system 2050 and the capillary column.

References

[1] H.E. Schwartz and K.J. Ulfelder, *Anal. Chem.*, 64 (1992) 1737–1740.
[2] K.J. Ulfelder, H.E. Schwartz, J.M. Hall and F.J. Sunzeri, *Anal. Biochem.*, 200 (1992) 260–267.
[3] F. Martin, D. Vairelles and B. Henrion, *Anal. Biochem.*, 214 (1993) 172–179.
[4] M. Strege and A. Lagu, *Anal. Chem.*, 63 (1991) 1233–1236.
[5] N. Bianchi, C. Mischiati, G. Feriotto and R. Gambari, *Nucleic Acids Res.*, 21 (1993) 3595–3596.
[6] T. Satow, T. Akiyama, A. Machida, Y. Utagawa and H. Kobayashi, *J. Chromatogr. A*, 652 (1993) 23–30.
[7] V. Sheffield, D.R. Cox, L.S. Lerman and R.M. Myers, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 232–236.
[8] M Orita, Y. Suzuki, T. Sekiya and T. Hayashi, *Genomics*, 5 (1989) 874–879.
[9] R.M. Myers, Z. Larin and T. Maniatis, *Science*, 230 (1985) 1242–1246.
[10] R.G.H. Cotton, N.R. Rodrigues and D.R. Campbell, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 4397–4401.
[11] M.B. White, M. Carvalho, S. O'Brien, D. Derse and M. Dean, *Genomics*, 12 (1992) 301–306.
[12] R. Sorrentino, I. Cascino and R. Tosi, *Hum. Immunol.*, 33 (1992) 17–23.
[13] V. Chowdhury, R.J. Olds, D.A. Lane, J. Conard, I. Pabinger, K. Ryan, K.A. Bauer, M. Bhavani, U. Abildgaard, G. Finazzi, G. Castaman, P.M. Mannucci and S.L. Thein, *Br. J. Haematol.*, 84 (1993) 656–661.
[14] D.J. Wilkin, K.E. Koprivnikar and D.H. Cohen, *Genomics*, 15 (1993) 372–375.
[15] I. Dianzani, C. Camaschella, A. Ponzone and R.G.H. Cotton, *Trends Genet.*, 9 (1993) 403–405.
[16] K. Khrapko, J.S. Hanekamp, W.G. Thilly, A. Belenkii, F. Foret and B.L. Karger, *Nucleic Acids Res.*, 22 (1994) 364–369.
[17] T. Kasuga, C. Woods, S. Woodward and K.R. Mitchelson, *Curr. Genet.*, 24 (1993) 433–436.
[18] R. Singhal and J. Xian, *J. Chromatogr. A*, 652 (1993) 47–56.
[19] E. van den Akker, J.E.F. Braun, G. Pals, M.V.M. Lafleur and J. Retel, *Nucleic Acids Res.*, 20 (1992) 6745–6746.