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Analysis of polymerase chain reaction product by capillary electrophoresis and its application to the detection of single base substitution in genes

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Abstract

Capillary gel electrophoresis (CGE) was studied for the direct analysis of polymerase chain reaction (PCR) amplified samples. A low cross-linked polyacrylamide gel (3%T, 0.5%C) was used for CGE with treated and untreated silica capillaries. CGE showed high reproducibility and resolution in the separation of DNA fragments (*ca.* 100–1000 base pairs) produced by PCR. The CGE system was applied to the detection of an amplification refractory mutation system (ARMS) and PCR–restriction fragment length polymorphism (PCR–RFLP), which are detection methods of single base substitution in genes using PCR. With the CGE system, full automation of PCR product detection is feasible.

1. Introduction

DNA analysis is an analytical technique with great potential for the diagnosis of genetic and infectious diseases. The polymerase chain reaction (PCR) has been used to increase enzymatically the specific segment of a target gene, which produces 10^5 more target sequences [1]. Detection of the PCR product is generally accomplished by ethidium staining or hybridization with a radiolabelled DNA probe after separation by slab gel electrophoresis. The development of an automated PCR procedure that involves a detection system is desirable, but no detection system has yet proved suitable for automated PCR because of the problems of rapidity, quantification, reproducibility and the repeated use of slab gel separation. Analysis of double-stranded DNA fragments by capillary electrophoresis (CE) has used low- and zero-cross-linked polyacrylamide [2,3], agarose [4] and cellulose derivatives [5–8] as buffer additives for molecular sieving. CE with hydroxypropylmethylcellulose as the methylcellulose derivative has great potential, because CE conditioning can be performed by changing the sieving buffer. However, the PCR product must be submitted to ultrafiltration to remove PCR byproducts (dNTP, primers and enzyme) before analysis by CE, because PCR components co-migrate with the PCR product of interest in CE. On the other hand, capillary gel electrophoresis (CGE) with low- and zero-cross-linked polyacrylamide, which has a theoretical plate number as high as that of CE with a cellulose derivative, can be used for

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analysis of the PCR product directly without an ultrafiltration step.

We have studied the direct analysis of PCR products by CGE. A low-cross-linked polyacrylamide gel (3% T, 0.5% C)[a], which was developed by Heiger *et al.* [2], was used as a CGE system with treated and untreated silica capillaries. This system permitted standard DNA fragments [72–1315 base pairs (bp) of ΦX174 RF DNA-*HaeIII* digest], which correspond to specific segments amplified by conventional PCR, to be separated within 30 min. In this work, CGE was applied to the detection of an amplification refractory mutation system (ARMS) and PCR–restriction fragment length polymorphism (PCR–RFLP), which are detection methods of single base substitution in genes by PCR. The results show that analysis of PCR products by CGE is rapid, reproducible and quantitative and has promise for development into an automated PCR system capable of diagnosing genetic and infectious diseases.

2. Experimental

2.1. Apparatus

A Model 270A capillary electrophoresis system (ABI, Foster City, CA, USA) was used. The separations were monitored on-column at 254 nm. A P/ACE 2000 system with a laser-induced fluorescence (LIF) detector (Beckman, Fullerton, CA, USA) was used. Excitation was at 488 nm and a 530-nm band-pass filter was used for emission.

2.2. Materials

ΦX174 DNA-*HaeIII* digest (DNA MW Marker 4) was obtained from Nippon Gene (Osaka, Japan). γ-Methacryloxypropyltrimethoxysilane was purchased from Sigma (St. Louis, MO, USA) and acrylamide, Bis, N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium peroxodisulphate (APS) from Wako (Osaka, Japan). Silica capillary tubing [50 cm (effective length 30 cm) × 100 μm I.D. × 375 μm O.D.) was obtained from GL Sciences (Tokyo, Japan). A Gene Amp PCR reagent kit with Amplitaq DNA polymerase was supplied by Perkin-Elmer (Norwalk, CT, USA). Primers A (PKU1), B (PKU2), PKU15w and PKU16m for phenylalanine hydroxylase gene and dried blood spot of phenylketonuria with $Arg^{413} \rightarrow Pro^{413}$ mutation site of exon 12 were generously donated by Professor K. Narisawa (Tohoku University, Medical School, Miyagi, Japan), Primer 1, primer 2 and dive E gene were generously donated by Professor R. Ohki (Kyourin University, Tokyo, Japan). *HaeIII* was purchased from Toyobo (Osaka, Japan). Thiazole orange was generously donated by Dr. T. Satow (Beckman Instruments, Tokyo, Japan). Other chemicals were of analytical-reagent grade.

2.3. CGE

Fused-silica capillary tubing [50 cm (effective length 30 cm) × 100 μm I.D.] was used for CGE. Acrylamide polymerization was accomplished in the capillary according to the methods of Paulus and Ohms [9] and Baba *et al.* [10]. The polymer was covalently attached to the walls of the fused-silica capillary by a bifunctional reagent, as follows. The capillary was rinsed with distilled water for 5 min, then a mixture of methanol and γ-methacryloxypropyltrimethoxysillane (50:50, v/v) was injected into the capillary and left there for 3 h. A 5-ml volume of a solution consisting of acrylamide and Bis dissolved in 100 m*M* Tris–250 m*M* borate buffer (pH 7.8) was carefully degassed by vacuum and introduced in the capillary after adding 2 μl of TEMED solution and 50 μl of 10% (w/v) APS. The polymerizing solution was quickly introduced into the treated capillary by a vacuum injection system equipped with an ABI Model 270A CE system for 10 min and left for at least 2 h. In a CGE experiment, the electrode of the injection side was attached to the negative side of a power supply. Sample injections were performed electrophoretically for typically 2 s at 5 kV. Electrophoresis was per-

[a] C = g N,N′-methylenebisacrylamide (Bis)/%T; T = (g acrylamide + g Bis)/100 ml solution).

formed with 100 mM Tris–borate buffer (pH 8.3) at 10 kV and 30°C. The DNA fragments were detected at 260 nm.

2.4. PCR

Amplification of λ phage DNA as a control was performed according to the procedure of the Gen Amp PCR kit (Perkin-Elmer, Norwalk, CT, USA).

2.5. ARMS for PKU

This process was carried out according to the method of Narisawa and co-workers [11,12]. DNA was extracted from dried blood specimens on Guthrie cards that are generally used for neonatal screening. Three discs (3 mm diameter) containing about 10.8 μl of blood were punched from a specimen with a steel punch and placed in a 1.5-ml polypropylene tube. The discs were methanol-fixed with 30 μl of methanol for 5 min at room temperature, dried under vacuum and incubated in 90 μl of a solution containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin and 50 μg/ml proteinase K at 55°C for 1 h. The mixture was then heated at 99°C for 10 min and centrifuged at 15 000 g for 10 min. A 10-μl volume of the supernatant (extracted DNA) was subjected to PCR with primers A and B [13] to amplify 245 bp DNA fragments containing $Arg^{413} \rightarrow Pro^{413}$ at the mutation site of exon 12 in a hepatic phenylalanine hydroxylase (PAH) gene. The PCR reaction mixture consisted of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs (dATP, dCTP, dTTP and dGTP), 1 μM each of the amplification primers, 10% dimethyl sulphoxide, 10 μl of extracted DNA and 1.5 units of Taq DNA polymerase in a total volume of 30 μl. Nucleotide sequences of the amplification primers were primer A and primer B. Primer A is complementary to the antisense DNA strand of intron 11, 58–77 nucleotides upstream from exon 12. Primer B is complementary to the sense DNA strand of intron 12, 33–52 nucleotides downstream of exon 12. A DNA thermal cycler (ATTO; Zymoreactor, Tokyo, Japan) was used to carry out 30 cycles of PCR according to the following programme: 1 min denaturation at 94°C, 1 min annealing at 56°C and 1 min extension at 74°C. This first amplified products were diluted 1:100 with water and amplified again with the primer-specific mutation site. A second PCR was separately amplified using primer A and PKU15w primer and primer A and PKU16m primer. This second PCR condition was the same as for the first PCR except for 20 cycles. The PCR product obtained was analysed by CGE with UV detection.

In the measurement of the PCR product with a high-sensitivity LIF detector, the first PCR is omitted. A 10-μl volume of the extracted DNA (supernatant) was subjected to PCRs of ARMS with primer A and PKU15w primer and primer A and PKU16m primer. The PCR condition was similar to that described above. The product obtained was analysed by CGE with LIF detection and the intercalating dye. Electrophoresis was performed with 100 mM Tris–borate buffer (pH 8.3) containing 0.1 μg/ml thiazole orange, 2 mM EDTA and CGE with 3% T, 0.5% C [capillary 37 cm (30 cm to detector) × 100 μm I.D.] at 10 kV and 30°C.

2.6. PCR–RFLP

This experiment was carried out with dive E 42 gene. DNAs, which were extracted from *E. coli* containing dive E gene, were generously donated by Professor R. Ohki. The PCR was performed according to the method and Ohki and co-workers [14,15]. Wild and mutant DNA of dive E were subjected to PCR with primer 1 and Primer 2. Primer 1 is complementary to the antisense DNA strand of dive E 42 gene (19–48 nucleotides). Primer 2 is complementary to the sense DNA strand (448–477 nucleotides). The PCR reaction mixture consisted of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 1 μM each of amplification primers 1 and 2, 5 ng of extracted DNA and 1.5 units of Taq DNA polymerase in a total volume of 50 μl. PCR was carried out for 30 cycles according to the following programme:

1 min at 94°C, 2 min at 50°C and 5 min at 72°C. Two PCR products, which were generated from wild and mutant genes, were separately digested with *HaeIII*. A 21-μl volume of PCR product was incubated in 26.7 μl of solution containing 10 m*M* Tris–HCl (pH 7.5), 7 m*M* $MgCl_2$, 60 m*M* NaCl, 7 m*M* 2-mercaptoethanol, 0.1 mg/ml BSA and 52 U *HaeIII* for 3 h at 37°C. After incubation, 2.9 μl of 200 m*M* EDTA solution were added to inactivate the *HaeIII* enzyme. A sample was then analysed by CGE with UV detection.

3. Results and discussion

DNA restriction fragments and PCR product can be separated by CE in the presence of methylcellulose derivatives as buffer additives. Schwartz and co-workers [6,7] applied CE with a sieving buffer containing hydroxypropylmethylcellulose (HPMC) and ethidium bromide for the detection of a PCR-amplified restriction fragment length polymorphism of ERBB2 oncogene and PCR products of the human immunodeficiency virus 1. The PCR products were separated by this CE system in less than 30 min, and the capillary tube could be re-used by changing the sieving buffer. However, the PCR product could not be directly analysed with this CE system, because the PCR reaction mixture components (primer, dNTP) co-migrated with the PCR product of interest. Therefore, the system required an ultrafiltration step with the Centricon system before CE analysis. Although this method is very useful, the purification step makes the routine assay tedious. Therefore, we now describe the use of CGE for direct analysis of PCR products and its application to the detection of an ARMS and PCR–RFLP, which are detection methods of single base substitution in genes using PCR.

3.1. Migration and separation by CGE

It is well known that the resolving power of CGE is very high. The separation of ds DNA by CGE has been reported by Heiger *et al.* [2]. A gel-filled capillary was prepared as follows: first the inner surface of the capillary (50 cm × 100 μm I.D.) was treated with methacryloxypropyltrimethoxysilane as described by Paulus and Ohms [9], then a polyacrylamide gel-filled column was prepared by the method of Baba *et al.* [10] with minor modifications.

The effect of gel concentration (3%–9% T at 0 and 0.5% C) on the separation of the ds DNA fragment (ΦX174 RF DNA–*HaeIII* digest) was studied. Conditions of 3% T, 0.5% C and 8% T, 0% C polyacrylamide provide good resolution of ds DNA restriction fragments. Typical electropherograms obtained by ΦX174 RF DNA–*HincII* digest on 8% T, 0% C polyacrylamide and ΦX174 RF DNA–*HaeIII* digest on 3% T, 0.5% C are shown in Figs. 1 and 2, respectively. As can be seen, the separation of the ds DNA fragment obtained at 8% T, 0% C polyacrylamide gel, which showed the separation of fragments differing by 5–10 bp in length, was better than that at 3% T, 0.5% C. However, the analysis time (migration time) was long (about 40 min) and the mean precision of the migration time of each fragment on the separation of ΦX174 RF DNA–*HaeIII* digest on 8% T, 0% C (data not shown) was 3.42%, within-run ($n = 8$). These results were inferior to those with 3% T, 0.5% C described below. The condition of 3% T, 0.5% C polyacrylamide for the separation of ds DNA was used in the following experiment. The efficiency of this CGE (3% T, 0.5% C) was evaluated by the separation of ΦX174 RF DNA–*HaeIII* digest. The results showed large theoretical plate numbers of $2.5 \cdot 10^6$ for the 118 bp and $1 \cdot 10^6$ for the 310 bp. The precision of the migration time obtained by this CGE for ds DNA fragments ranging from 72 to 1353 bp was examined. The within-run ($n = 10$), day-to-day ($n = 8$) and gel-to-gel ($n = 12$) results are given in Table 1. The mean relative standard deviations obtained within-run, day-to-day and gel-to-gel were 0.47, 2.77 and 0.83%, respectively.

The detection limit of PCR-amplified λ phage obtained with this CGE method was 1 pg of a sample of PCR. This value was the same as that obtained for ethidium bromide-stained slab gel (data not shown). The life of this capillary gel

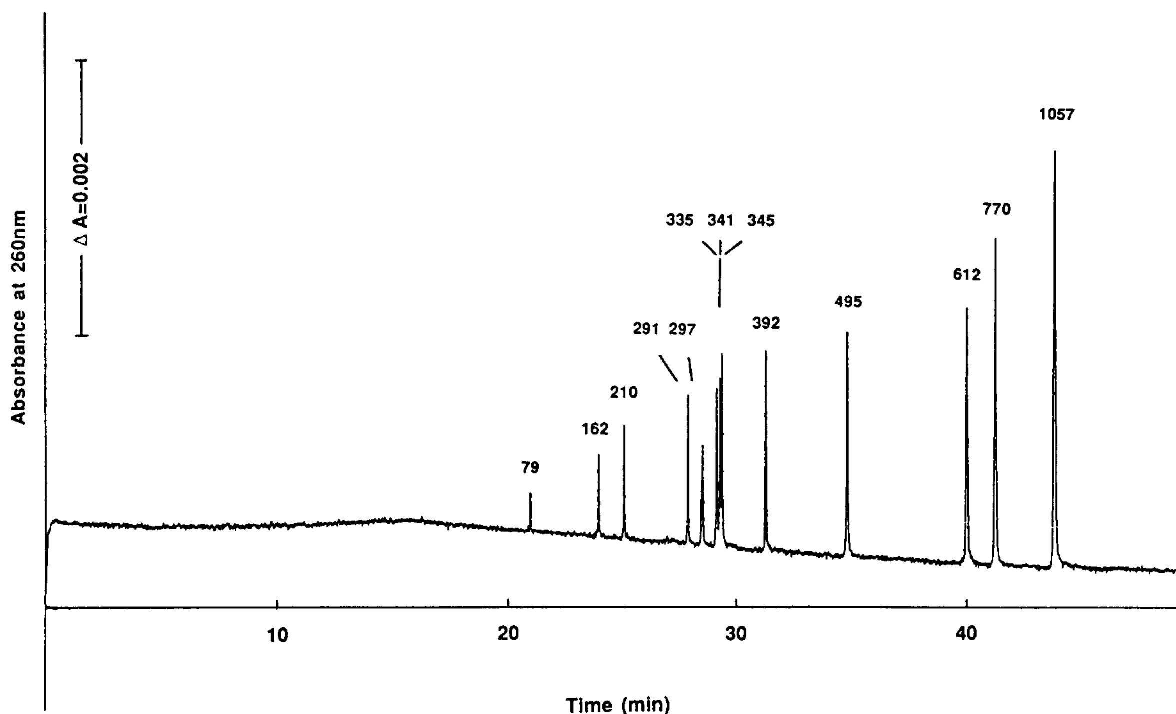

Fig. 1. Separation of *HincII* digest of ΦX174 RF DNA on a 8% T, 0% C polyacrylamide capillary.

was about 80 separations. In contrast, the life of a capillary gel without methacryloxypropyltrimethylsillane pretreatment is about 20 separations under the same conditions. Hence the use of a pretreated capillary gel appears to be a good method for the determination of PCR product.

3.2. Application of CGE analysis to the detection of single base substitutions in genes

Two methods using PCR have been developed to detect single base substitution in a gene. The ARMS is one method for the determination of point mutation in a gene [16]. This method depends on whether the PCR product is observed in slab gel electrophoresis after PCR with a primer-specific mutation site. The basis of this system is that oligonucleotides with a mismatched 3′-residue do not function. Narisawa and Matsubara have developed ARMS for the phenylketonuria mutation (PKU) ($Arg^{111} \rightarrow Ter$, Sp4, $Tyr^{204} \rightarrow Cys^{204}$, $Arg^{243} \rightarrow Gln^{243}$, $Arg^{413} \rightarrow Pro^{413}$, $Arg^{261} \rightarrow Gln^{261}$). We tried to apply CGE analysis to the detection of this ARMS technique for PKU. As a model, the PCR product obtained by ARMS of Arg^{413}(CGC)$\rightarrow$$Pro^{413}$(CCC) of exon 12 in the hepatic PAH gene was detected by CGE (3% T, 0.5% C). DNA was extracted from dried blood specimens on Guthrie cards according to the method of Matsubara *et al.* [11]. The method is described in detail under Experimental and a scheme for ARMS is shown in Fig. 3.

Extracted DNA was first subjected to PCR to amplify the 245 bp fragment in exon 12. This region contains the mutation site $Arg^{413} \rightarrow Pro^{413}$. The 245 bp fragment was amplified by 30 cycles of PCR, and part of the amplified products were then further subjected to a second PCR according to the method of Matsubara *et al.* [11]. The second PCR was carried out separately using primer A and PKU15w primer, and primer A and PKU16m primer. PKU15w primer corresponds to the normal sequence at Arg^{413}(CGC) and PKU16m primer corresponds to the point mutation se-

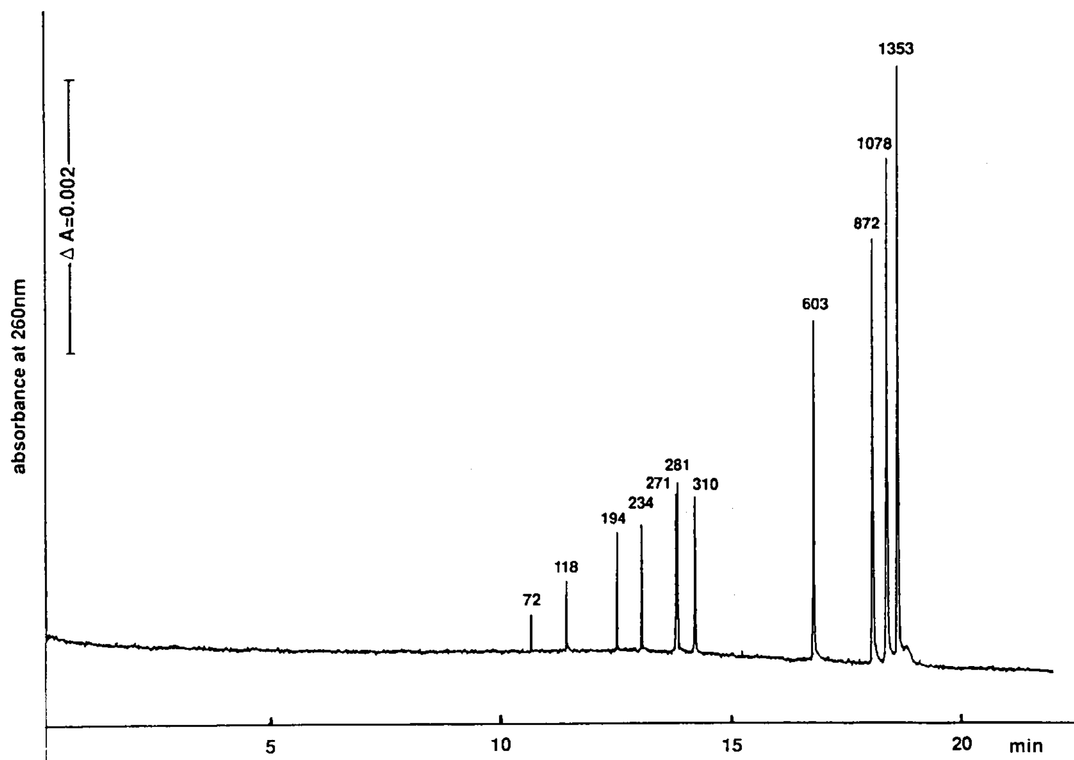

Fig. 2. Separation of *HaeIII* digest of *Φ*X174 RF DNA on a 3% T, 0.5% C polyacrylamide capillary.

quence at Pro^{413}(CCC). In a normal subject, the product (138 bp) was derived only from primer A and PKU15w, and no product was generated when the PKU16m primer was applied to normal DNA. On a patient's sample containing a point mutation at Pro^{413}, no product was generated by

Table 1
Precision of migration times for various DNA fragments on the separation of *Φ*X174 DNA-*HaeIII* digest

Base pairs	Within-assay (n = 10)		Day-to-day (n = 8)		Gel-to-gel (n = 12)	
	Mean (min)	R.S.D. (%)	Mean (min)	R.S.D. (%)	Mean (min)	R.S.D. (%)
72	10.53	0.46	10.99	2.49	10.97	0.74
118	11.29	0.47	11.78	2.50	11.75	0.70
194	12.38	0.46	12.90	2.61	12.88	0.73
234	12.92	0.45	13.46	2.66	13.43	0.70
271	13.67	0.44	14.23	2.71	14.19	0.74
281	13.71	0.44	14.28	2.72	14.24	0.77
310	14.08	0.44	14.66	2.75	14.62	0.76
603	16.61	0.47	17.30	2.93	17.24	0.92
872	17.85	0.48	18.61	3.02	18.54	1.03
1078	18.13	0.53	18.92	3.03	18.86	1.01
1353	18.37	0.52	19.17	3.02	19.11	0.99

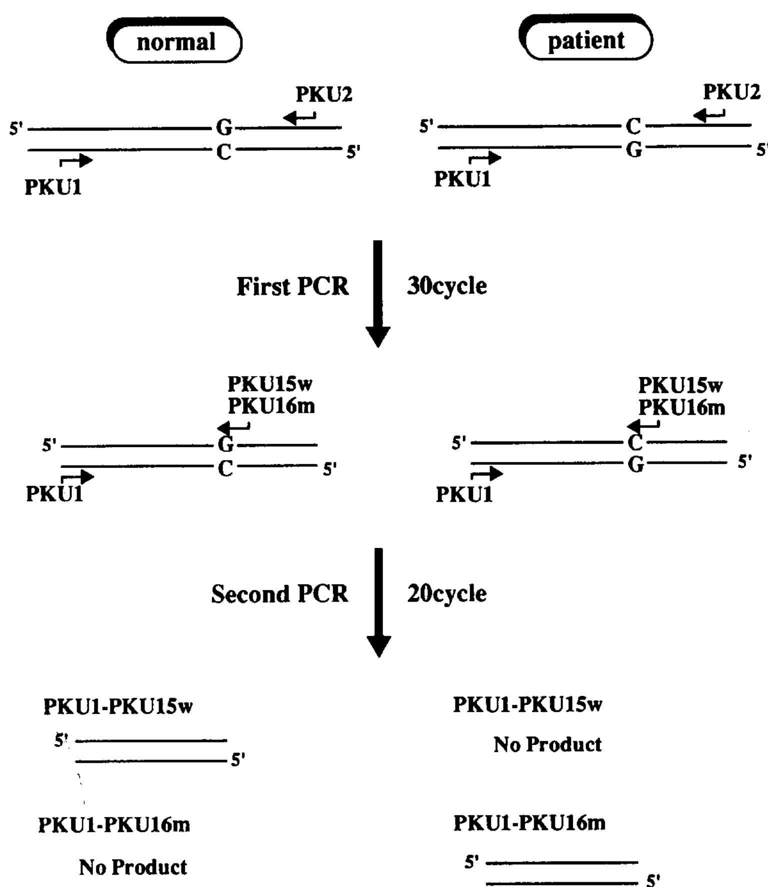

Fig. 3. Scheme of the method of ARMS.

primer A and PKU15w primer. However, product was observed when PKU15w primer was replaced with PKU16m primer. The PCR products thus obtained were analysed by CGE.

As shown in Fig. 4, the PCR product migrated to about 12.30 min. Comparison of these results should indicate whether or not the DNA sample has a point mutation at exon 12. In this work, the detection limit of DNA by CGE with UV detection was not adequate to detect a specific gene from a small amount of human DNA, even after 30 cycles of PCR. Therefore, the two PCR systems described above were required (the first PCR produced a 245-bp fragment which contained a mutation site, and this first PCR product was then used as a template for the second PCR). This system requires long reaction time, which might result in contamination of the DNA. This problem can be resolved by using the high-sensitivity CGE with LIF. Schwartz and Ulfelder [17] recently developed a highly sensitive method of separating PCR fragments by CE which used hydroxypropylmethylcellulose as sieving buffer with LIF. This method using thiazole orange as an intercalating dye is *ca.* 400 times as sensitive as UV detection. In this work, PCR products obtained directly from ARMS without the first PCR were analysed by CGE (3% T, 0.5% C) with LIF and the dye. The results are shown in Fig. 5. This CGE system with LIF and thiazole orange as an intercalating dye had higher sensitivity (λ phage 100 fg, PCR 25 cycles) and higher resolution (baseline resolution at 271 and 281 bp) than the CGE system

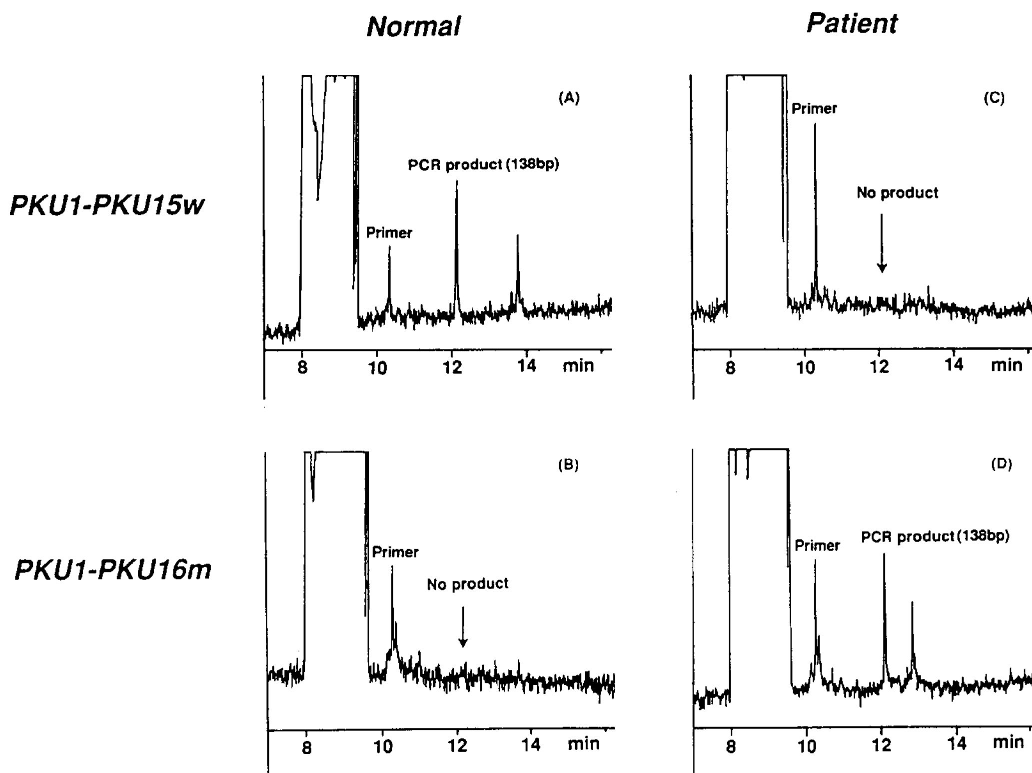

Fig. 4. Electropherograms of the products obtained by ARMS. (A) and (B) are the results for a normal subject with primer set primer A–PKU15w and primer A–PKU16m, respectively; (C) and (D) are the results for a PKU patient with primer set primer A–PKU15w and primer A–PKU16m, respectively.

with UV detection. The results will be described in detail elsewhere. This method has high potential for PCR product obtained from a human genome.

Another method to determine a single base mutation in a gene is PCR–RFLP using the restriction enzyme. PCR–RFLP has been used to diagnose many diseases. In this work, we tried to apply CE analysis to the detection of PCR–RFLP. The model used was the dive E 42 gene carrying wild- and mutant-type DNA. The mutant type of this DNA contains G→A point mutation at the 141 site. Wild and mutant DNA were subjected to PCR to amplify the 359 bp fragment in the dive E 42 gene. This region contains the mutation site of $G^{141} \rightarrow A^{141}$. Two PCR products generated from the wild and mutant gene are the ^{140}GGCC and ^{140}GACC sites in the 359 bp fragment. The restriction enzyme, *HaeIII*, cuts the GGCC site in the PCR product generated from a wild gene, whereas the PCR product of a mutant is not cut by this enzyme. Hence this *HaeIII* recognition site is lost in mutated DNA. The PCR reaction was carried out according to the method of Ohki and co-workers [14,15] described under Experimental. The amplification product was directly digested by the restriction enzyme, *HaeIII*, for 3 h at 37°C, and the sample was subsequently analysed by CGE in 3% T, 0.5% C. As predicted from the dive E sequence, *HaeIII* restriction enzyme cut the GGCC site in the PCR product of wild genes and produced two peaks (122 bp) at 11.35 min and (237 bp) at 12.98 min. In

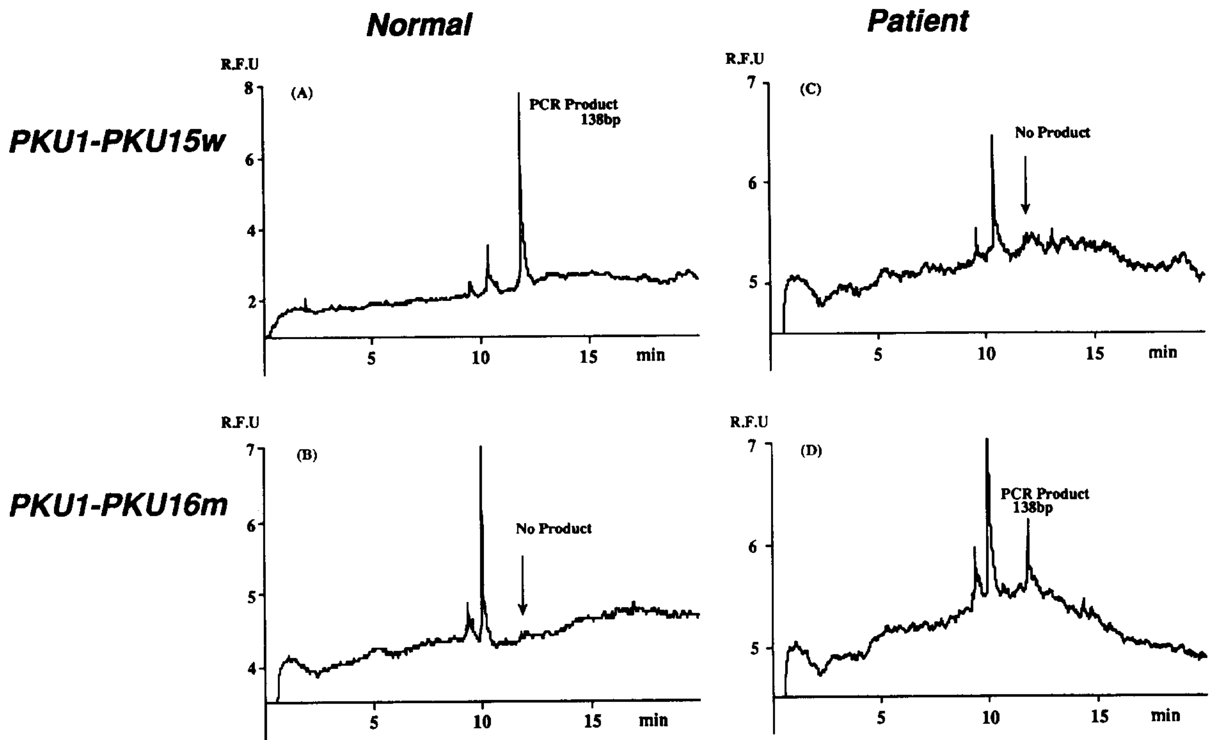

Fig. 5. CGE with LIF detection of *HaeIII* digest of ΦX174 DNA and the products obtained by ARMS. (A) and (B) are the results for a normal subject with primer set primer A–PKU15w and primer A–PKU16m, respectively; (C) and (D) are the results for a PKU patient with primer set primer A–PKU15w and primer A–PKU16m, respectively.

contrast, the PCR product of a mutant gene, digested by *HaeIII*, showed only one peak (359 bp) at 14.54 min. The results are shown in Fig. 6.

The results described above show that CE analysis of PCR–RFLP is a useful method to determine the point mutation in a gene.

4. Conclusions

We developed a method for the analysis of the PCR product by CGE. The separation of the DNA fragment obtained by CGE showed a high theoretical plate number, short analysis time and high precision. This method provides an efficacious means for the determination of PCR product. CGE was applied to the determination of single base mutation of a gene amplified by PCR. It was found that CGE is a useful technique for detection by the ARMS and PCR–RFLP. With this method, the PCR procedure for the diagnosis of genetic and infectious disease could be automated in the near future.

5. Acknowledgements

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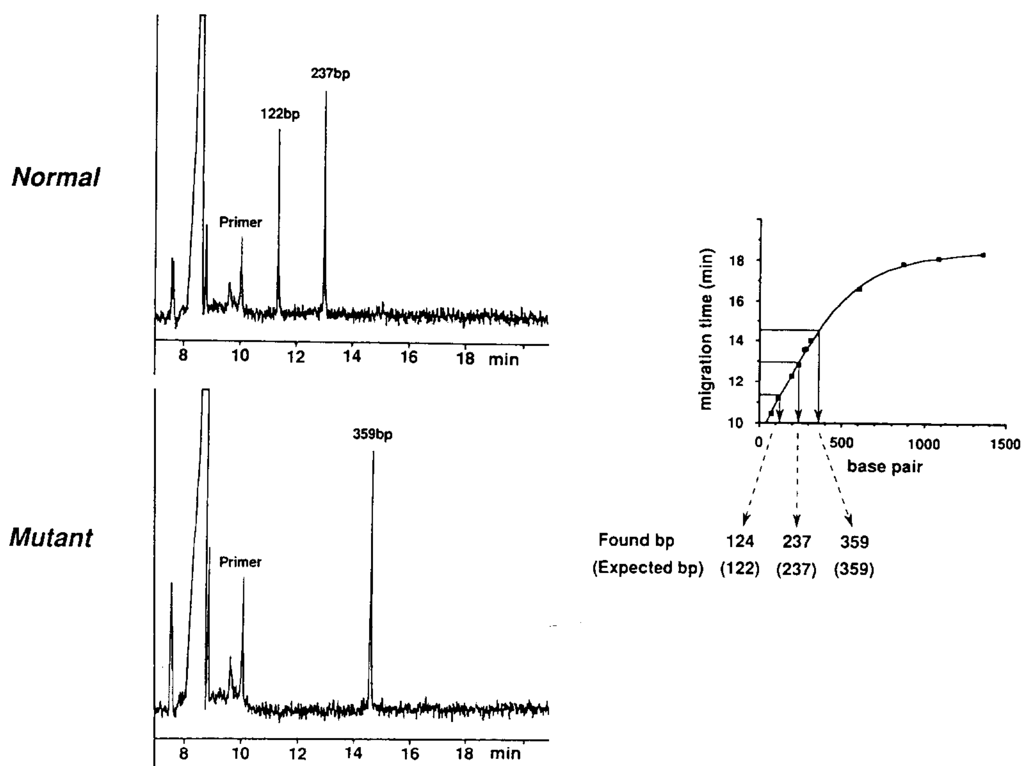

Fig. 6. Electropherograms of an *HaeIII* restriction digest of PCR products obtained from wild and mutant dive E gene.

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