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Identification of DNA molecules by pre-column hybridization using capillary electrophoresis

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ABSTRACT

In this paper, we demonstrate the feasibility of using gel-filled capillary electrophoresis to perform Southern blotting with on-line detection and to study parameters that affect the hybridization of DNA molecules in solution. A fluorescence-tagged oligonucleotide was used as the probe for hybridization in solution with complementary DNA molecules prior to electrophoresis. The reaction mixture was subject to capillary electrophoresis in a polyacrylamide gel-filled capillary, and the hybridized species was identified on-line by UV absorption or laser-induced fluorescence detection. The effects of probe concentration and annealing temperature were studied.

INTRODUCTION

Capillary electrophoresis is of great interest for the analysis of oligonucleotides, DNA restriction fragments, DNA sequence analysis, peptide mapping, pharmaceuticals, and carbohydrates. Because of the small inner diameter of the capillary (usually 50–100 μm) and the efficient heat dissipation capability of the fused-silica capillary, very high electric fields (100–500 V/cm) can be used, and consequently, a very high efficiency and fast separation can be achieved [1,2].

This laboratory has developed polyacrylamide gel-filled capillary for the analysis of nucleic acids [3,4]. The high resolving power and speed of capillary format has drawn great interest in the study of capillary electrophoresis for fast and large scale DNA sequence analysis [5–7]. Recently, this laboratory has also demonstrated high efficiency separation of DNA fragments of restriction digests using little or no cross-linking polyacrylamide gel-filled (*i.e.* physical gel) capillaries [8]. Our most recent results further indicate that fast separation of restriction fragments can be easily achieved in less than 5 min with such columns. Separation of restriction fragments can also be obtained with dilute hydrophilic polymer networks [9,10].

Conventionally, Southern blotting is performed in order to identify the nucleotide sequence of a DNA molecule [11]. The process involves separation of DNA

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species with gel electrophoresis, transfer and immobilization onto a membrane support, and hybridization with a radioactively labelled probe. An autoradiogram is developed, and the molecules carrying complementary sequence to the probe are identified. Although a powerful tool, this method is a time-consuming and laborious process.

The goal of this study was to investigate the possibility of using capillary electrophoresis to perform on-line Southern blotting and to study parameters that affect DNA hybridization in solution. Several potential advantages can be expected from such an approach: (1) high speed identification of DNA molecules (1–5 min), (2) use of fluorescent tags rather than hazardous radioactive materials, (3) simultaneous identification of several species using various fluorescence-tagged probes, (4) low detection limits. For example, subattomolar detection is achievable using a laser-induced fluorescence detection system [5–7]. Thus, capillary electrophoresis has potential in the development of an automated ultra-sensitive tool for the detection of genetic mutations and infectious diseases.

EXPERIMENTAL

Apparatus

The capillary electrophoretic apparatus with UV or laser induced fluorescence detection, and the preparation of gel-filled capillaries for the separation of DNA molecules have been described previously [3,8]. A 30-kV, 400-μA direct current high-voltage power supply (Model PS/MJ30P0400-11; Glassman, Whitehouse Station, NJ, USA) was used to generate the potential across the capillary. For the UV detection of DNA molecules at 260 nm, a Spectra 100 (Spectra-Physics, San Jose, CA, USA) was used, while for the laser-induced fluorescence detection, an argon ion laser (Model 532AT; Ominichrom, Chino, CA, USA) was employed with the power level regulated at 2–4 mW. The laser light was filtered through a narrow-band pass filter (Model D1-488; Corion, Holliston, MA, USA), directed by a beam steerer (Model M670, Newport), and focused into the capillary with a 25 mm focal length lens (Model KBX043, Newport). The emitted fluorescent light was collected with a 40 × objective lens (Model M-Set, Newport) and passed through an interference filter (Model S10-520-R, Corion) and a colored glass filter (Model OG520, Schott Glass Technologies, Duryea, PA, USA). A photomultiplier tube (Model R928, Hamamatzu, San Jose, CA, USA) operated at 700 V, and a photon counter (Model 7070, Oriel) were used to detect the fluorescence signal. The data were stored in a PC computer through an analog-to-digital converter (Model 760, Nelson Analytical, Cupertino, CA, USA).

Gel-filled capillaries

Fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA) with inner diameter of 75 μm, outer diameter of 375 μm, effective length of 20–40 cm, and total length of 40–60 cm was first treated with methacryloxypropyltrimethoxysilane (Petrarch Systems, Bristol, PA, USA) and then filled with a carefully degassed solution of 9% acrylamide solution in 25 m*M* Tris–borate buffer, pH 8.0, 25 m*M* EDTA added with ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED).

Oligonucleotides and chemicals

Oligonucleotides pC1 (17-mer: GTAAAACGACGGCCAGT, identical to −20 universal M13 sequence primer) and pC2 (34-mer: TCGAATTCACTGGCCGTCGTTTTACAACGTCGTG) were synthesized on a DNA synthesizer (Cyclone, Milligen/Biosearch, Burlington, MA, USA) and purified by agarose gel electrophoresis. Joe-labelled M13 sequencing primer was either purchased from Applied Biosystems (Foster City, CA, USA) or synthesized on the Cyclone. The 5′ end of the oligonucleotide to be fluorescently labelled was modified with N-TFA-C6 Aminomodifier (Clontech, Palo Alto, CA, USA) and attached with activated fluorescent dye, Joe (Applied Biosystems). Unincorporated dye was separated from larger DNA molecules by size-exclusion chromatography with G-25. The Joe-labelled molecules were further purified by reversed-phase high-performance liquid chromatography (HPLC) with a C_8 column. Ultra-pure Tris base, urea, acrylamide, and EDTA were purchased from Schwartz/Mann Biotech (Cleveland, OH, USA). TEMED and ammonium persulfate were purchased from Bio-Rad (Richmond, CA, USA). DNA size standards were purchased from Pharmacia LKB (Piscataway, NJ, USA).

RESULTS

Effect of salt on electrokinetic injection

Although salt is often used to enhance hybridization of DNA molecules, it was known that in the presence of a significant concentration of salt in the sample preparation, the amount of DNA sample introduced by electrokinetic injection was greatly reduced [12]. Therefore, prior to hybridization, we examined the effect of solvent conditions on injection. Restriction digest ϕX 174 *Hae* III was separated on a 9%T, 0%C column, as previously described [8][a]. A serial dilution of the sample was made with distilled water. Since the commercial restriction digest contained 10 m*M* Tris–HCl buffer, the more dilute the DNA concentration, the less the amount of salt in the sample preparation. Interestingly, as seen in Table I, a much higher signal was observed when the DNA concentration in the sample vial was actually much lower. This result is due to a higher electric field drop created in the low conductivity sample solution, with more sample being introduced into the capillary for the given injection condition. As further shown in Table I, the relative peak heights of three different fragments injected under the same conditions from a solution containing different buffer concentrations were compared. Nearly a 500-fold difference in the sample introduction could be observed due to the difference in buffer concentration. Therefore, in order to avoid any misinterpretation caused by the differences during injection, all the DNA preparations used in this study were diluted with distilled water or with TBE (Tris–borate–EDTA) buffer of constant concentration, and no salt was added to the hybridization mixtures.

Identification of hybridized species

Unless otherwise specified, annealing of DNA molecules was achieved by following conventional molecular biology protocols: heating at 65°C for 10 min fol-

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

TABLE I

EFFECT OF BUFFER CONCENTRATION ON ELECTROKINETIC INJECTION

Restriction digest, ϕX 174 *Hae* III, was separated on a 9%T, 0%C, non-denaturing gel as described in [8]. The peak heights corresponding to 234, 271, and 603 base-pairs (bp) fragments were compared. More DNA was injected when the sample was diluted with distilled water. A nearly 500-fold increase in peak height was observed when the sample was diluted 1000-fold. Injection was done electrokinetically at 15 kV for 5 s.

DNA concentration (μg/ml)	Tris–HCl concentration (m*M*)	Relative peak height		
		Fragment 234 bp	Fragment 271 bp	Fragment 603 bp
1000	10	1	1	1
500	5	5	6	5
100	1	33	27	24
20	0.2	139	139	119
5	0.05	436	442	364
1	0.01	346	282	282

lowed by slow cooling to room temperature in 30 min [13]. Equal amounts of Joe-labelled M13 universal sequencing primer and a complementary 34-mer, pC2, were mixed and annealed, as described above. As shown in Fig. 1, the species that was successfully hybridized was identified by UV absorption (Fig. 1C) and laser induced fluorescence detection (Fig. 1E). It is interesting to note that the molecules smaller than pC1 (Fig. 1D), (arising possibly from synthesis failure), were apparently also hybridized onto pC2, as seen by the extra peaks in Figure 1E. pC2, which served as the target molecule and was untagged, was not observed with laser induced fluorescence detection.

Dissociation of the hybridized species by urea and heat

Urea is often used as a denaturing reagent in the gels for DNA sequence analysis [13]. To demonstrate further that the third peak observed in Fig. 1C and E was indeed the hybridized species of Joe-labelled primer and pC2, we injected the same hybridization mixture as in Fig. 1C into a 7 *M* urea gel-filled capillary. As shown in Fig. 2, no hybridized species could be observed. The overall migration rate was slower in the urea gel than in the column without the denaturant due in part to the increased viscosity when urea was included.

To further demonstrate the identity of the third peak in Fig. 1C and E, the hybridization was driven to completion at low temperature, as described below. As shown in Fig. 3C, a single hybridized species was observed in the electropherogram. The same mixture was heated to 65°C for 5 min before injection and, as shown in Fig. 3D, two peaks corresponding to the single stranded species reappeared along with the residual hybridized species.

Effect of probe concentration and temperature on hybridization

With on-line detection, several parameters that affect DNA hybridization in solution can be conveniently studied using capillary electrophoresis. Various ratios of

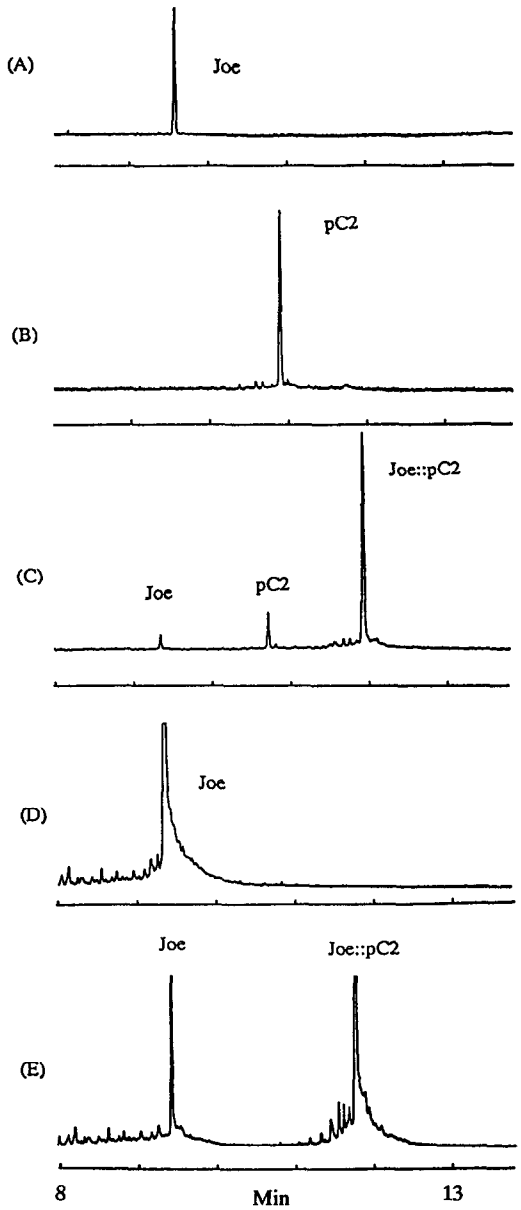

Fig. 1. Identification of DNA molecule by hybridization with a fluorescence-tagged oligonucleotide probe using capillary gel electrophoresis. (A) Joe-labelled 17-mer alone (5 μg/ml in 10 m*M* TBE). (B) pC2 alone (5 μg/ml in 10 m*M* TBE). (C) Equal amounts of Joe-labelled primer (10 μg/ml in 10 m*M* TBE) and pC2 (10 μg/ml in 10 m*M* TBE) were annealed prior to electrophoresis and were analyzed by UV absorption. (D) The same sample as (A) was analyzed by the laser system. (E) The same sample as (C) was analyzed by the laser system. The hybridized species was clearly identified either by UV (C) or by the laser system (E). The capillary was filled with 9%T, 0%C polyacrylamide gel with no urea. Total length of the capillary was 45 cm with an effective length of 25 cm. Injection was done electrokinetically at 13.5 kV for 5 s, and the electric field for electrophoresis was 300 V/cm.

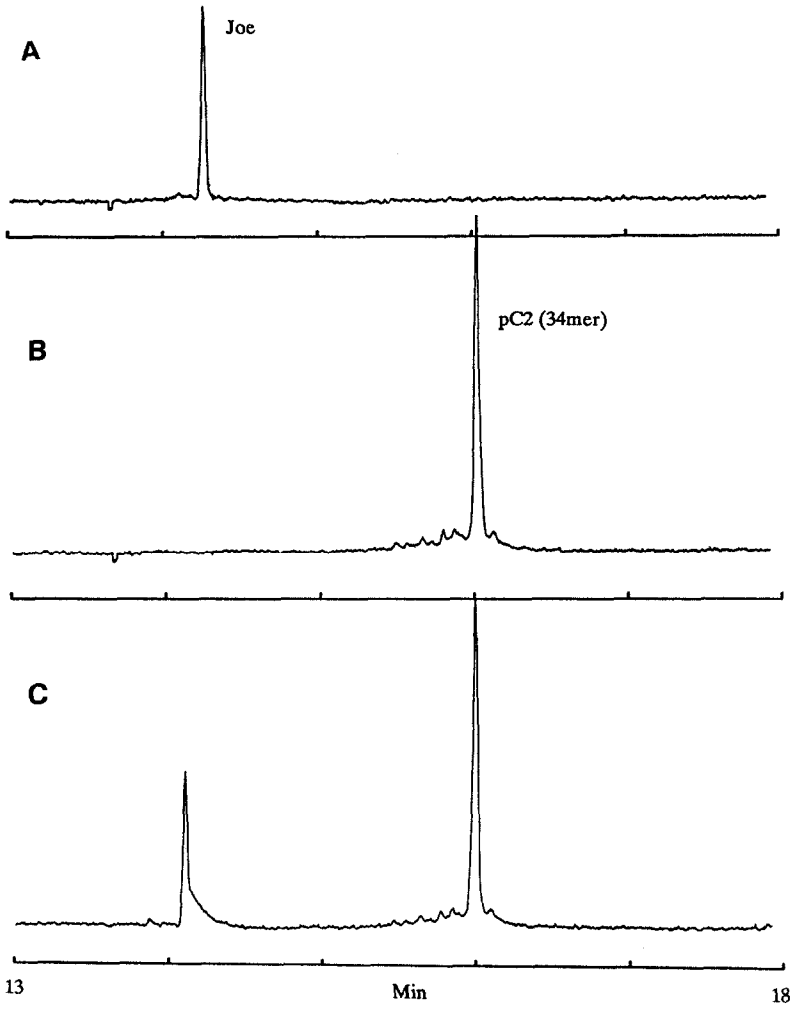

Fig. 2. Dissociation of the hybridized species in the urea gel. (A) Joe-labelled primer alone. (B) pC2 alone. (C) The same hybridization mixture as in Fig. 1 was analyzed in a 9%T, 0%C, 7*M* urea gel filled capillary with an effective length of 30 cm. Injection was done at 13.5 kV for 5 s, and the electric field for electrophoresis was 300 V/cm.

pC1 and complementary oligonucleotide, pC2, were annealed. As shown in Fig. 4, as expected, the amount of hybridized species increased as more probe was present. It is possible to drive the reaction to near completion with about a 10-fold molar excess of probe molecule.

In most molecular biology studies, heating followed by slow cooling is usually performed for the annealing of DNA molecules. However, as shown in Fig. 5, a much higher efficiency of hybridization is observed when the DNA mixture was incubated in dry ice without even heating. Indeed, a complete hybridization was often achieved with incubation in dry ice for *ca.* 10–15 min (data not shown).

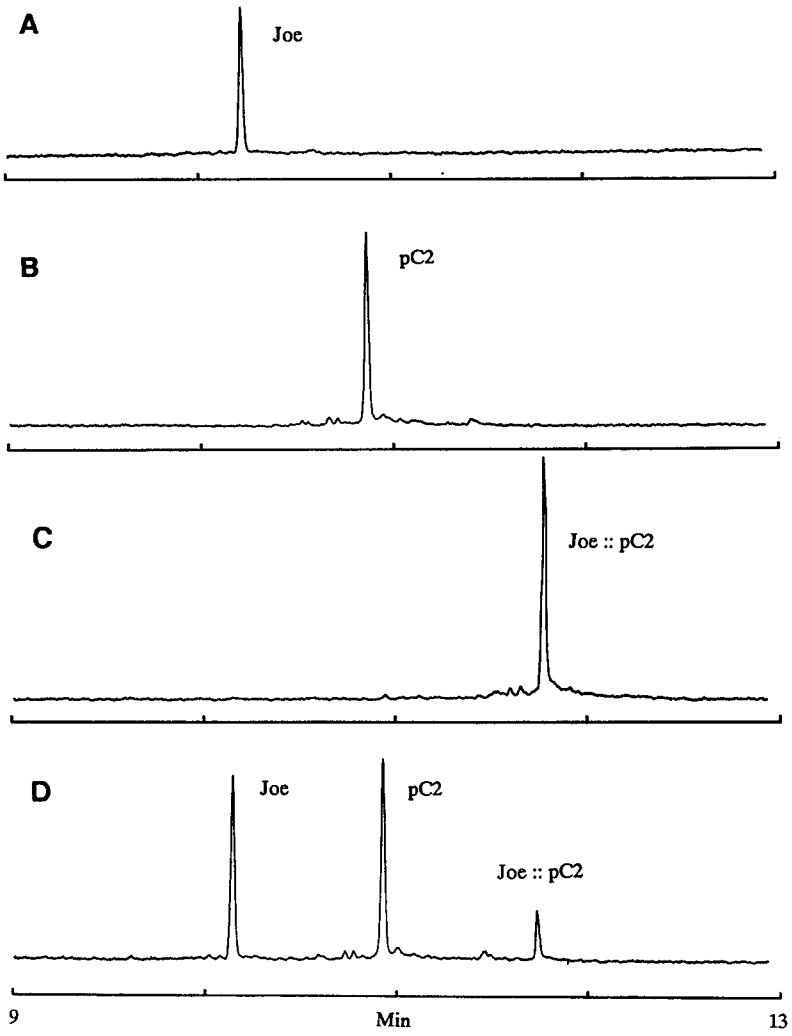

Fig. 3. Dissociation of the hybridized species by heat. (A) Joe-labelled primer alone. (B) pC2 alone. (C) Mixture of equal amounts of Joe-labelled primer and pC2 driven to a complete hybridization by incubating in dry ice. (D) After heating at 65°C for 5 min, the same mixture as in (C) was analyzed in the capillary. Two peaks corresponding to Joe-labelled primer and pC2 reappeared. The capillary was filled with 9%T, 0%C, non-denaturing gel. Total length of the capillary was 45 cm with an effective length of 25 cm. Injection was done at 13.5 kV for 5 s and the electric field for electrophoresis was 300 V/cm.

DISCUSSION

Since its development polymerase chain reaction (PCR) analysis has become one of the most important methodologies for molecular biology studies and also a powerful tool for the identification of genetic mutations and infectious agents [14]. However, very often, hybridization with a specific probe other than the primers used

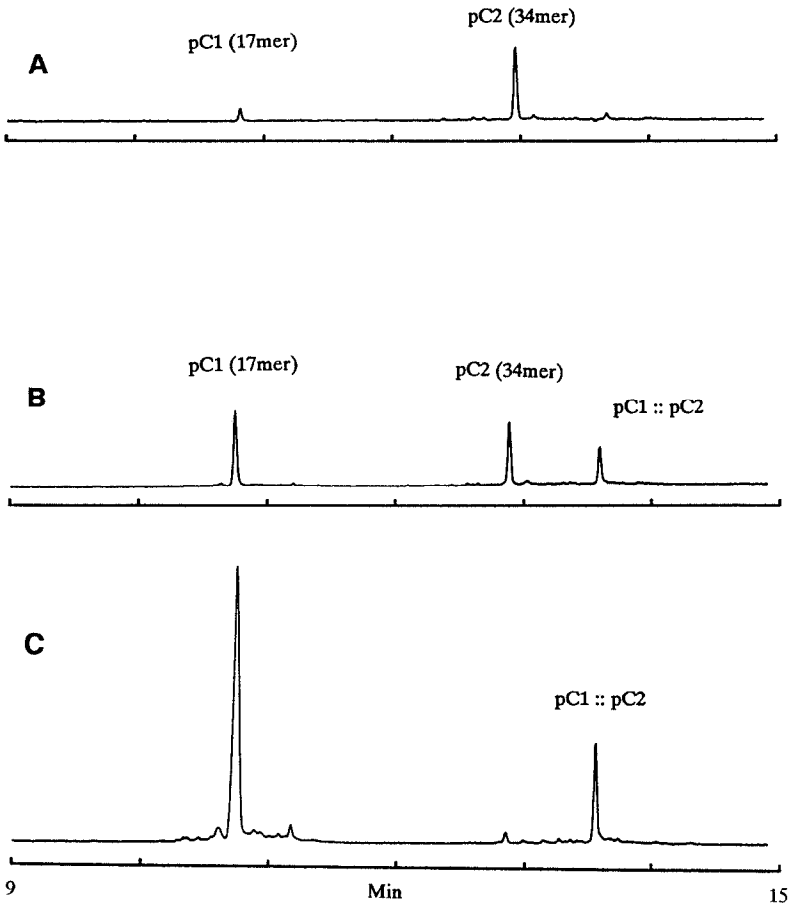

Fig. 4. Effect of probe concentration on hybridization. Various dilutions of pC1 and pC2 were prepared, and hybridization was performed with different molar ratios of pC1 and pC2. (A) Molar ratio of pC1 to pC2 is 2 to 5. (B) Molar ratio of pC1 to pC2 is 2 to 1. (C) Molar ratio of pC1 to pC2 is 10 to 1. The molar ratios of 1 to 5, 1 to 1, 4 to 1, and 20 to 1 have also been examined but not shown. A nearly complete hybridization was observed with a 10-fold molar excess of probe, as indicated by the disappearance of the peak corresponding to the target molecule, pC2. The electrophoretic conditions were the same as described in Fig. 3.

for the PCR reaction has been performed in order to assure the accuracy of the PCR products. Recent results obtained in this laboratory has shown that an ultra fast high-resolution separation of DNA fragments of less than 2000 bp can be easily achieved in less than 5 min. This makes capillary electrophoresis a potentially powerful tool for the analysis of PCR products. Moreover, with the highly sensitive laser induced fluorescence detection system and the possibility of performing on-line Southern blotting with a specific fluorescence tagged oligonucleotide probe, capillary electrophoresis has the possibility of becoming a screening tool for genetic mutations and infectious diseases in conjunction with PCR analysis. In this paper, we have demonstrated such a possibility with small single-stranded DNA molecules. For dou-

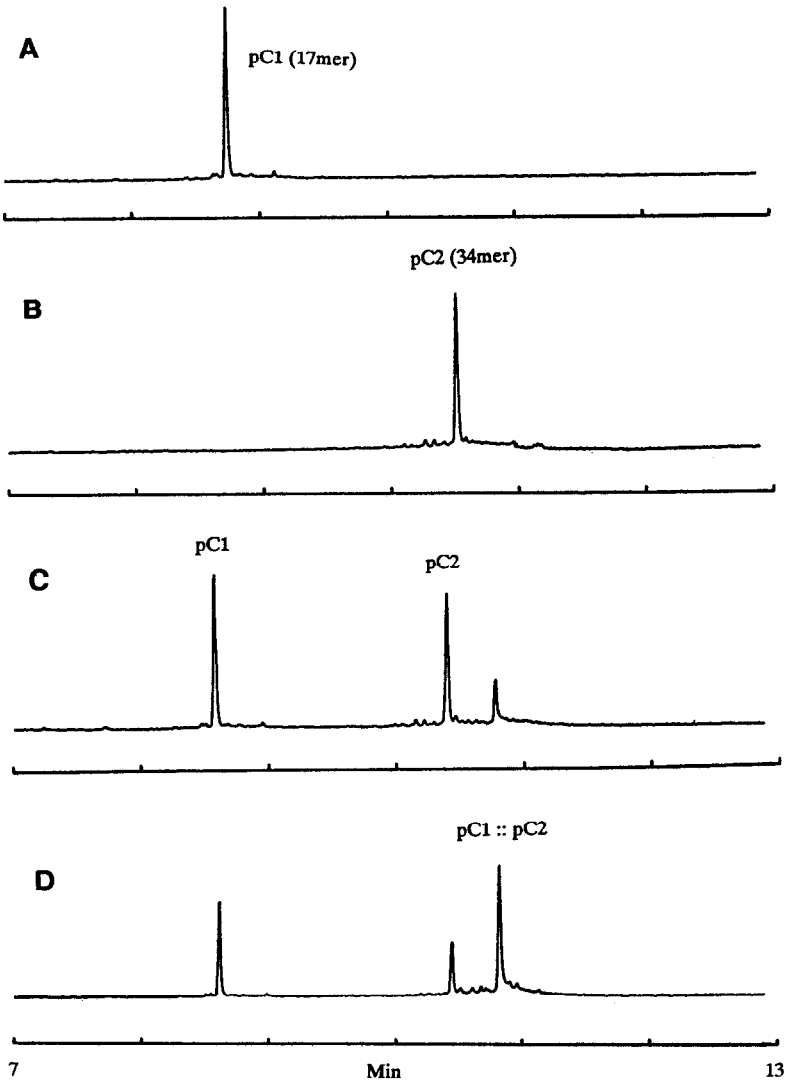

Fig. 5. Optimal incubation temperature for hybridization. (A) pC1 alone. (B) pC2. (C) Equal amounts of pC1 and pC2 were mixed and incubated in ice for 5 min before injection. (D) Equal amounts of pC1 and pC2 were mixed and incubated in dry ice for 5 min before injection. The extent of hybridization was greatly enhanced at low temperature and a nearly complete hybridization was achieved by incubating the mixture in dry ice for 10 min (data not shown). The electrophoretic conditions were the same as described in Fig. 3.

ble-stranded DNA molecules, however, we have observed rather inconsistent results. Further studies on the behavior of ds DNA and the possible effects of high electric field on "D-Loop" structure are needed. Nevertheless, potential applications can still be derived with similar approaches reported in this paper due to the fact that single-stranded products can be generated by asymmetric PCR reaction with appropriate

ratios of two primers and that single-stranded molecules are more accessible to hybridization with oligonucleotide probes.

It is well known that double-stranded DNA molecules migrate faster than single-stranded molecule of the same molecular weight. However, the hybridized species observed in this study apparently migrated more slowly than the single-stranded oligonucleotides. This may be due to the fact that the hybridized molecule still carried some single-stranded overhang on both ends.

It was quite surprising to note that a direct incubation at low temperature was actually the best annealing condition, although heating followed by slow cooling is recommended by most molecular biology protocols. However, in most molecular biology studies, hybridization has been done with membrane-bound DNA molecules, and any degree of annealing that sustains the washing procedure would generate identical positive responses on a subsequent autoradiogram. It is speculated that the high electric field (100–300 V/cm) used in capillary electrophoresis may disrupt molecules that are not perfectly annealed. Therefore, the higher annealing efficiency observed for low temperature may simply indicate the extent of perfectly annealed molecules. On the other hand, since the annealing in the study was performed without salt, it is also possible that low temperature provides a more favorable condition for DNA annealing in low ionic strength solutions. Interestingly, a similar result was reported by Casanova *et al.* [15] who found that −70°C was the best annealing condition for sequence analysis of double-stranded PCR products.

DNA molecules separated on a capillary can also be collected and immobilized on a membrane. Further confirmation with specific probes, similar to conventional Southern Blotting, could then be performed. Further studies are needed for identification of double stranded DNA molecules or even genomic DNAs using Southern Blotting with capillary gel electrophoresis.

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