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Short Communication

Capillary zone electrophoresis in polymer networks of polymerase chain reaction-amplified oligonucleotides: the case of congenital adrenal hyperplasia

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Abstract

The use of capillary zone electrophoresis (CZE) in polymer networks for the analysis of an 8 bp (base pair) deletion in congenital adrenal hyperplasia was investigated. Separations were performed in Tris–borate–EDTA buffer (pH 8.3) containing 6% liquid linear polyacrylamide as a sieving dynamic matrix and 10 μM ethidium bromide for improving DNA fragment separation. Easy analysis and detection of the 127 and 135 bp amplified fragments was accomplished. The capillary column can be used for >50 analyses before degradation and loss of resolution. The results are comparable to those obtained by gel-slab zone electrophoresis in a 12%T, 4%C polyacrylamide matrix. The sensitivity, by simple UV absorption at 254 nm, is similar to that obtained in gel slabs by dye intercalation staining.

1. Introduction

Genetic disorders exist for each of the steps in steroid hormone synthesis. 21-Hydroxylase (21-OH) deficiency is recessively inherited and accounts for over 90% of the genetic disorders of steroidogenesis (congenital adrenal hyperplasia) [1,2]. The severe form occurs in about 1 in 10 000–14 000 individuals; approximately 70% of classical 21-OH deficiency patients show an inability to conserve dietary sodium (salt wasting) while the remainder have been classified as simple virilizers. The mild, non-classical form (late onset) is more common, affecting 1 in 1000–2000 individuals [3]. The diagnosis of 21-OH deficiency is suggested by genital ambiguity in females, by a salt-losing episode in either sex, or by rapid growth and virilization in males. Plasma 17OHP is markedly elevated (>2000 ng/dl after 24 h of age) and hyper-responsive to stimulation with ACTH.

The P450c21B functional gene and the P450c21A pseudogene are linked, within the class III region of the HLA complex, to the complement genes C4B and C4A, respectively. The P450c21 genes are 3.4 kb (b = bases) long

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and contain 10 exons; they are highly homologous and differ in only 88 bp (base pairs) [4]. This close similarity indicates that these two genes are evolving in tandem. The duplicated region, about 30 kb long, includes a compound unit of a 21-OH gene plus C4 gene. The P450c21A gene appears to lack transcriptional activity and DNA sequencing has identified several deleterious mutations including a deletion of 8 bp normally found in exon 3, a T insertion in exon 7 and a C to T substitution in exon 8 [5]. The frequencies of the B gene deletion (ranging from 5 to 35%) and the large gene conversion (about 8%) were described in population studies [6–8]. About 75% of chromosomes encoding CAH do not carry alterations in the P450c21B gene detectable by Southern blotting, hence these genes appear to carry either smaller rearrangements or point mutations.

We have described the distribution of deletions and gene conversions at the P450c21B locus in the Italian population. Forty-five families affected by 21-OH deficiency were studied by multiple restriction analysis and *in vitro* amplification. In affected individuals the B gene deletion, the large gene conversion and the 8 bp deletion in exon 3 were found in 13.3, 7.7 and 2.2% of chromosomes, respectively [9]. Normally, the analysis of the 8 bp deletion is carried out by means of a PCR protocol which allows the selective amplification of the active gene using a B-specific forward primer. The amplified chains [a normal (135 bp) and a disease-linked (127 bp) fragment] are then usually separated by polyacrylamide gel-slab electrophoresis and detected by dye intercalation with ethidium bromide.

We propose here an alternative procedure, based on capillary zone electrophoresis (CZE) in polymer networks. It has recently been demonstrated that DNA electrophoresis in viscous polymer solutions allows high-resolution separations over a vast range of analyte molecular sizes and permits the repetitive use of the same column for multiple sample injections (well over 50 runs) [10,11]. Several applications have already appeared. Thus, Guttman *et al.* [12] proposed the separation of DNA restriction fragments by CZE in liquid linear polyacrylamide using field strength gradients. Del Principe *et al.* [13] demonstrated the analysis of a PCR-amplified product of the DSX 164 locus in the dystrophine gene (by CZE in 0.5% hydroxypropylmethylcellulose as sieving polymer). In the same sieving system, Schwartz and Ulfelder [14] have shown the possibility of laser induced-fluorescence detection of PCR fragments by using thiazole orange as intercalating dye. Kuypers *et al.* [15], have even demonstrated the feasibility of separation of point mutations in the p53 gene located in the short arm of chromosome 17 DNA by CZE in viscous solutions of linear polyacrylamide. We have also shown an excellent performance of this CZE technique for screening the delta F508 deletion in cystic fibrosis [16] and for separation of GATT microsatellites for linkage studies with cystic fibrosis [17].

2. Experimental

2.1. Reagents

Acrylamide, N,N′-methylenebisacrylamide, Tris(hydroxymethylaminomethane), ammonium peroxodisulphate and N,N,N′,N′-tetramethylethylendiamine (TEMED) were obtained from Bio-Rad Labs. (Richmond, CA, USA). 3-(Trimethoxysilyl) propylmethacrylate (Bind Silane), ethylendiaminetetraacetic acid (EDTA), boric acid and acetic acid were purchased from Aldrich Chemie (Steinheim, Germany). Bio Marker Low DNA molecular mass marker was obtained from Bio Ventures (Murfreesboro, TN, USA). Centricon 30 membranes were supplied by Amicon (W.R. Grace, Baltimore, MD, USA). Ethidium bromide was purchased from Sigma (St. Louis, MO, USA). Fused-silica capillaries (100 μm I.D., 370 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Capillary electrophoresis

CZE analyses were performed with a Waters Quanta 4000E capillary ion analyser from Millipore (Milford, MA, USA). A 37 cm × 100 μm

I.D. capillary, coated by the Hjertén protocol [18], filled with 6% acrylamide linear gel (in the absence of cross-linker) was used. After 12 h of polymerization, the capillaries were conditioned with separating buffer [TBE: 100 m*M* Tris–100 m*M* boric acid–2 m*M* EDTA (pH 8.3) with 10 μM ethidium bromide (ETBr) added for 1 h at 100 V/cm in order to remove charged catalysts. The samples and standards were loaded electrophoretically by applying 165 V/cm for 6–10 s depending on sample concentration. Separations were performed at 165 V/cm, a typical run lasting 30 min. Ultraviolet absorbance was monitored at 254 nm. The identification of the various DNA fragments by size was obtained by plotting log bp *vs.* migration time.

2.3. Sample preparation

The specificity of B gene amplification was confirmed in patients homozygotes for the P450c21B deletion. PCR was performed in a thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT, USA) in the presence of 50 pmol of each oligonucleotide, 1 μg of genomic DNA and 2.5 U of Taq polymerase in a final volume of 100 μl containing 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$ and 0.2 m*M* of each deoxynucleotide. Amplification was performed by 30 cycles (denaturation, 1 min at 94°C; annealing, 1 min at 59°C; extension, 1 min at 72°C). For CZE analysis the samples were desalted by ultrafiltering the sample solution through a Centricon 30 anisotropic membrane (note that salt removal is extremely important for DNA analysis when relying on intrinsic absorbance detection; otherwise, in electrokinetic injection, mostly salt and very little DNA will be loaded).

2.4. Polyacrylamide gel-slab electrophoresis

The amplified products were directly analysed by electrophoresis on 12% polyacrylamide gels in 0.5X TBE buffer (pH 8.3). The run was carried out at 15 V/cm for 3 h at room temperature in a Bio-Rad Mini Gel chamber. Detection by dye intercalation with ETBr.

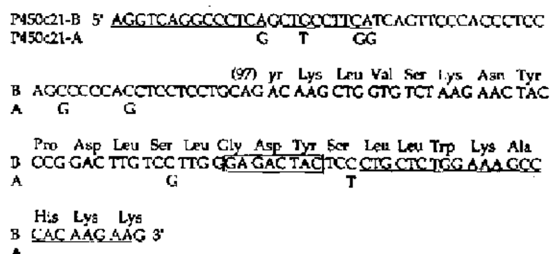

Fig. 1. P450c21B-amplified region encompassing the 8 bp deletion (boxed) in exon 3. Primer sequences are underlined. Only nucleotide differences in the pseudogene A sequence are indicated in the corresponding positions under the B gene.

3. Results and discussion

Fig. 1 shows the P450c21B-amplified region encompassing the 8-bp deletion (boxed) in exon 3. The primer sequences are underlined. In the corresponding pseudogene A sequence, only the nucleotide differences are indicated under the corresponding positions in the B gene.

Fig. 2 shows the polyacrylamide gel electrophoretic analysis of one patient heterozygous for the P450c21B gene 8 bp deletion. The father

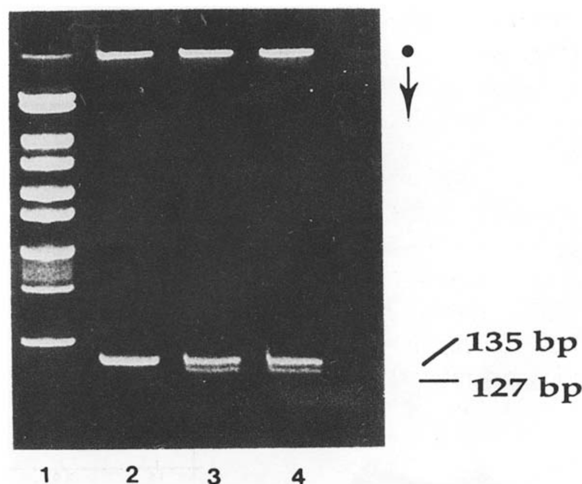

Fig. 2. 12% polyacrylamide gel electrophoretic analysis of the 8 bp deletion. The 135 and 127 bp amplified bands correspond to the absence or presence of the deletion, respectively. Lanes: 1 = molecular mass marker (Bio Marker Low); 2 = father; 3 = mother; 4 = affected child. Staining by dye intercalation with ETBr.

(lane 2) is a normal individual and shows only the 135 bp amplified fragment. The mother (track 3) is heterozygous for the condition and shows both the 135 and 127 bp chains. The son (track 4) inherited the mutation from his mother and thus exhibits the same band profile.

In Fig. 3, the results of the analysis of the same samples by CZE in polymer networks (6%T liquid linear polyacrylamide) are shown. In Fig. 3A, the profile of the father is shown, exhibiting only one chain of 135 bp. Fig. 3B gives the electropherogram of the mother, displaying both the 135 and 127 bp chains (heterozygous carrier). In Fig. 3C, the corresponding profile of the child (heterozygous carrier) is shown. In all instances, peaks 1–3 represent short oligonucleotides (15–20 bp) used as primers. Note that, in order to improve the resolution of the 127–135 bp couple, 10 μM ETBr was added to the background electrolyte in all runs, as suggested by, *e.g.* Nathakarnkitkool *et al.* [19].

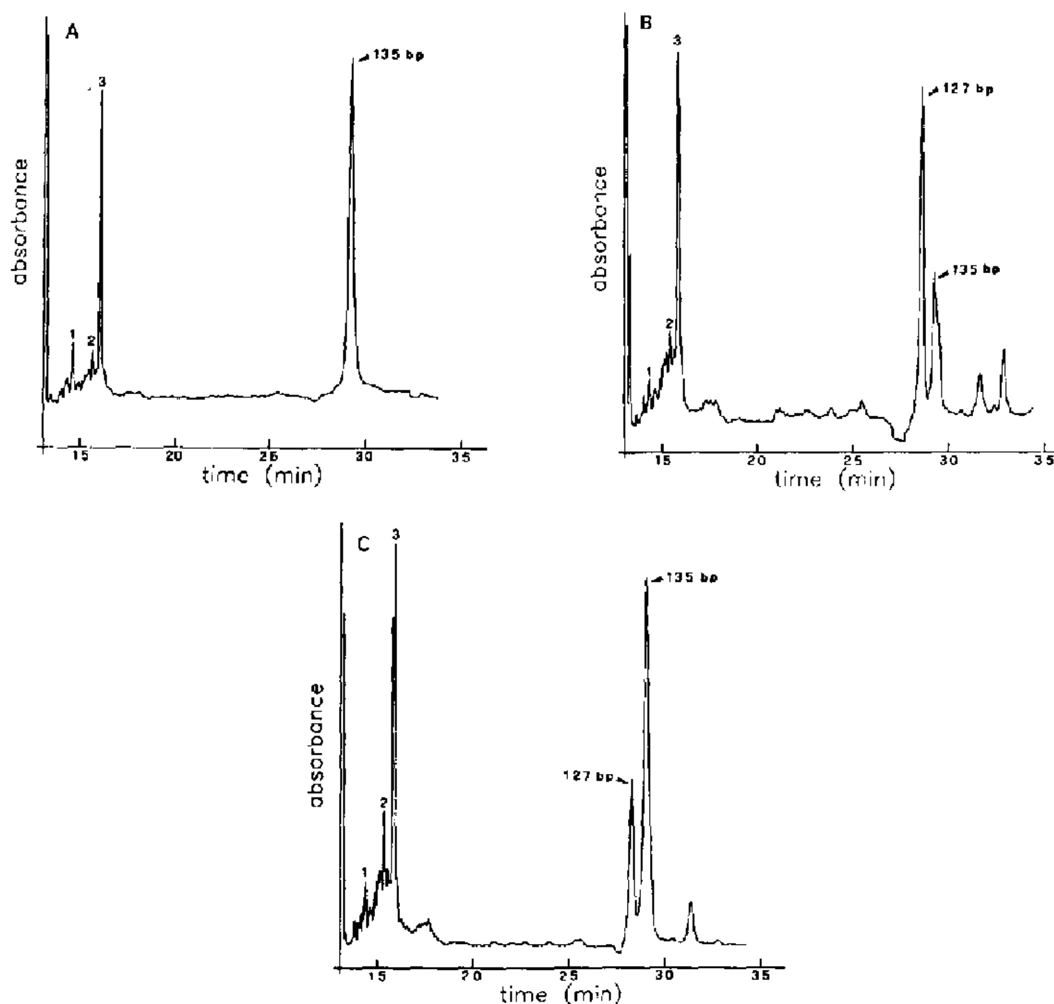

Fig. 3. CZE analysis of the 8 bp deletion. Conditions: 37 cm × 100 μm I.D. coated capillary, filled with 6% liquid linear polyacrylamide in TBE buffer, containing 10 μM ETBr. The samples were loaded electrophoretically by applying 165 V/cm for 6–10 s depending on sample concentration. Separations were performed at 165 V/cm, a typical run lasting for 40 min. Ultraviolet sample absorbance was monitored at 254 nm. (A) Father; (B) mother; (C) affected child. Peaks 1–3 – oligonucleotides used as primers (15–20 bp).

It is thus seen that CZE can offer resolution and diagnostic values comparable to those routinely obtained in zone electrophoresis in polyacrylamide gels slabs, the official technique routinely adopted in clinical chemistry laboratories. This will certainly not guarantee automatic switching over of clinicians from one technique to the other. There are still a number of advantages and disadvantages of both techniques to be accounted for before any final commitment. On the positive side of gel-slab electrophoresis is the multi-sample load ability of each slab, typically of the order of at least 10–15 samples. The horizontal sample alignment is also another unique advantage of zone electrophoresis in gel slabs, greatly facilitating sample comparison. The positive aspects of CZE are (a) automatic sample loading from a carousel, just as in HPLC; (b) on-line sample detection by intrinsic UV sample absorbance, thus eliminating the need for staining; (c) automatic storage of the electropherograms on a magnetic support; and (d) minute sample volume requirements (typically only a few microlitres in the sample vial, but only a few nanolitres of sample zone in the capillary!). The drawback of CZE at the moment is the possibility of analysing only one sample at a time, as only one channel is available. However, the situation might change rapidly, since for, *e.g.* DNA sequencing, a CZE unit comprising a battery of 20 capillaries (with real-time data acquisition in each channel) has been already described [20–22]. Clearly, when such units become commercially available, the challenge of CZE over existing techniques will be serious.

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