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CAG triplet analysis in families with androgen insensitivity syndrome by capillary electrophoresis in polymer networks

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Abstract

The potential use of capillary zone electrophoresis in polymer networks (linear polymers above the entanglement threshold, added to the background electrolyte for sieving purposes) for analysis of DNA fragments amplified by a polymerase chain reaction, is shown. In typical runs, the capillary is filled with Tris–borate–EDTA buffer, at pH 8.3, containing 6% linear polyacrylamide as a dynamic sieving matrix. Such formulations allow replenishing the capillary with fresh sieving solution when resolution decays after prolonged use (typically >30 injections per capillary are obtained). The DNA fragments are detected by their intrinsic absorbance at 254 nm. This system has been applied to the analysis of CAG triplet polymorphism in families carrying the androgen insensitivity syndrome. While easy separation is obtained for fragments 139 base pairs (bp) and 160 bp (in families carrying a difference of 7 CAG repeats) even more difficult cases (such as those of families exhibiting fragments of 136 and 139 bp, thus differing by only one CAG repeat) are resolved with precision and diagnostic value.

1. Introduction

The androgen receptor (AR) is a DNA-binding, transcription regulating protein. Mutations in the AR block the normal pathway of androgen action (testosterone and 5α-dihydrotestosterone) and result in a number of phenotypic abnormalities of male sexual development. A spectrum of different phenotypes is known: the complete androgen insensitivity syndrome (CAIS, also called Morris disease), the partial androgen insensitivity syndrome (PAIS), the infertile male syndrome, and the undervirilized fertile male, each of which is transmitted as an X-linked trait [1]. The androgen receptor abnormalities have been characterized in cultured genital skin fibroblasts from patients with androgen resistance. These defects span from normal binding, qualitative abnormalities to complete absence of androgen binding. Cloning of cDNA encoding the androgen receptor [2,3] has made possible to elucidate the molecular defects causing androgen resistance. In most patients mutations are single nucleotide substitutions within the coding region of the androgen re-

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ceptor, indicating a heterogeneous mechanism causing androgen resistance [4].

The AR consists of different domains: a carboxyl terminus responsible for the hormone binding; a central region containing the DNA-binding domain with two Zinc-finger motifs and an amino terminus domain specific for transcriptional activation. The latter is characterized by the presence of three homopolymeric repeats of amino acids. In particular the CAG trinucleotide repeat in exon A was found to be polymorphic and ranging from 17 to 28 Gln residues [4]. The highly polymorphic CAG repeat in exon A represents a useful marker for determination of affected, unaffected and carrier members within pedigrees showing a recessive X-linked transmission of AIS. Inheritance of polymorphic fragments represents a marker for following transmission of the disease within a family, allowing the two maternal X chromosomes to be distinguished [5]. For this purpose, a nested polymerase chain reaction (PCR) has been developed for analysis of CAG repeats. The amplified fragments are usually resolved by conventional polyacrylamide gel electrophoresis (PAGE) either on 12% PAGE or on 6% T[1], 8 *M* urea sequencing gels.

In the present report, we demonstrate the applicability of capillary zone electrophoresis (CZE) to the resolution and detection of PCR-amplified DNA fragments in the AIS. Separation is achieved in novel sieving matrices, consisting of a flexible polymer network, rather than rigid gel structures, as proposed long ago by De Gennes [6]. Dynamic sieving matrices [7] are rapidly coming of age, and have been now successfully applied to the screening of cystic fibrosis [8,9] and of congenital adrenal hyperplasia [10].

2. Materials and methods

2.1. Subjects studied

Three generation families of patients diagnosed as suffering from complete or partial AIS

[1] T = (g acrylamide + g N,N'-methylenebisacrylamide)/100 ml solution.

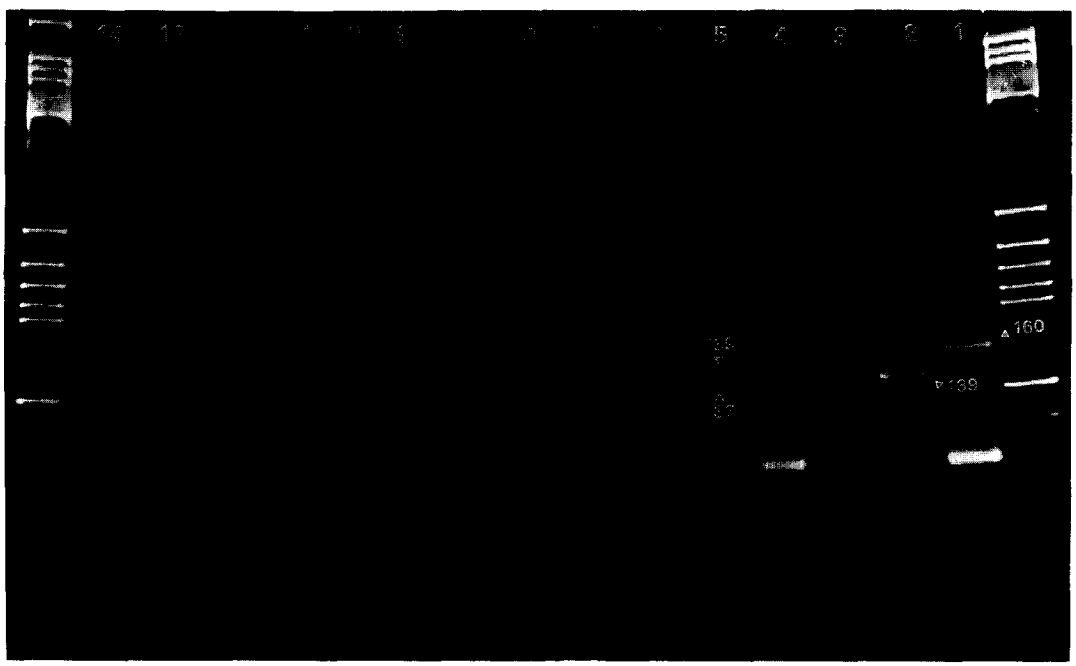

Fig. 1.

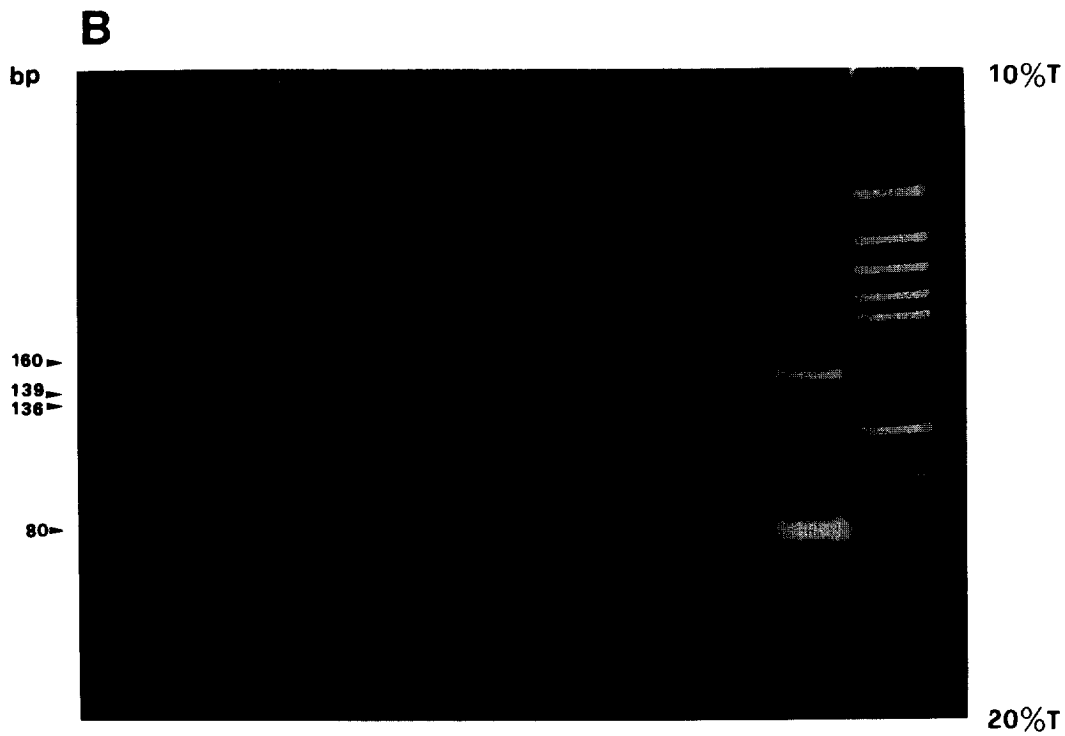

Fig. 1. Separation of nested PCR products in a 10–20% PAGE gradient gel in TBE buffer. The pBr 322/Hae III DNA was used as molecular mass standard. (A) Lanes: 1–3 = father, PAIS (affected child) and mother, respectively —the mother shows the presence of the two bands, the lower one being associated with the disease; 4–8 = father, mother, CAIS, CAIS (sister of the mother) and grandmother, respectively; 9–14: grandfather, grandmother, father, mother, CAIS and sister of the CAIS, respectively. In this last family the mother seems to be homozygous for the number of repeats and thus not informative. In order to obtain the exact size of the PCR fragments, electrophoresis of radiolabelled products was done in sequencing gradients, resulting in values of 136 bp for the smaller, 139 bp for the intermediate and 160 bp for the larger fragment (data not shown). (B) Close-up of a section of (A).

were studied. These patients are followed by the Department of Pediatrics, Endocrine Unit, S. Raffaele Hospital.

2.2. PCR amplification

Determination of CAG polymorphisms in the pedigrees of patients affected by AIS was carried out by two rounds of PCR. A first round of in vitro amplification was performed in the presence of primers A1 and A2, previously reported by La Spada et al. [11] for studying the CAG repeat expansion in spinobulbar muscular atrophy. A nested PCR was carried out by using primer 221 (5′-ACCTCCCGGCGCCAGTTTGCT-3′) and 360 (5′-AGAACCATCCTCACCCTGCT-3′) designed according to the sequence reported by Lubahn et al. [12]. The first round and nested PCR contained 250 ng of genomic DNA and 1 μl of first round PCR, respectively, with either 25 pmol of each primer or 3 pmol of the 5′ end ^{32}P-labelled 221 primer and 22 pmol of unlabelled, 400 μM each dNTP, 10 mM Tris · HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 5 units Taq polymerase (Taq = *Thermophilus aquaticus*) in a 100-μl final volume. Before addition of the enzyme, reactions were hot started for 3 min followed by 30 cycles (denaturation: 94°C for 1 min; annealing: 65°C for 1 min; extension: 72°C for 1 min) in a thermal cycler (Perkin-Elmer Cetus). The amplified fragments were analysed on 4% gels (3% agarose, 1% Nusieve), or on sequencing gels.

2.3. Capillary electrophoresis

CZE analyses were performed with the Waters Quanta 4000E capillary ion electrophoresis instrument from Millipore (Milford, MA, USA). We used 37 cm × 100 μm or 28 cm × 100 μm I.D. capillaries, coated by Hjertén's protocol [13], but with our novel monomer N-acryloylaminoethoxyethanol, offering extreme resistance to alkaline hydrolysis [14]. The capillary was then filled with a degassed solution of 6% acrylamide monomer (in the absence of cross-linker) in running buffer added with 1 μl of 40% persulphate and 1 μl of pure N,N,N',N'-tetramethylethylenediamine (TEMED) per ml of gelling solution. After 90 min of polymerization, the capillaries were conditioned with separation buffer (TBE: 89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.3), for 30 min at 25°C and 100 V/cm in order to remove charged catalysts. The samples (desalted and concentrated with Centricon 30 membranes from Amicon, Beverly, MA, USA) were loaded electrophoretically by applying 140 V/cm for 30 s, a typical run lasting 35 min. The ultraviolet absorbance was monitored at 254 nm.

2.4. Polyacrylamide gel slab electrophoresis

The amplified products were analyzed by electrophoresis on a 10–20% polyacrylamide gradient gel in TBE buffer, pH 8.6. It should be noted that a constant-concentration gel (12% T), as routinely adopted in clinical practice, is unable to resolve fragments differing by only one CAG triplet. Thus, in those cases, one had to resort to sequence gels. In contrast, a 10–20% T gradient, as adopted here, has been found to solve the problem. The run was at 180 V for 4 h followed by 300 V for 1 h. Staining was by dye intercalation with ethidium bromide.

3. Results

Fig. 1 shows the screening of three different families in a 10–20% polyacrylamide gradient gel. As shown in the close-up of Fig. 1B, while in some individuals (see lane 3) heterozygous for two fragments, 139 base pairs (bp) vs. 160 bp in length, thus differing by 7 CAG repeats, the

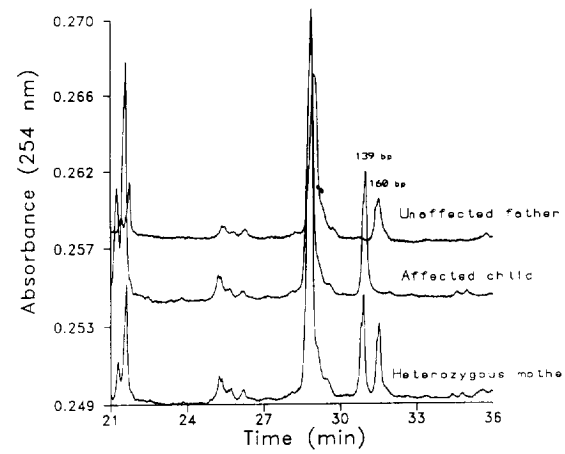

Fig. 2. Analysis of the samples in lanes 1–3 of Fig. 1 by CZE in polymer networks. Capillary length: 37 cm. Upper tracing: unaffected father, exhibiting the chain of 160 bp; intermediate tracing: affected child (chain of 139 bp); lower tracing: heterozygous mother, displaying both the 139 and 160 bp fragments (lane 3 in Fig. 1). In all cases the early peaks (ca. 21–23 min) correspond to the primers and the band located at ca. 29 min is a 80 bp constant amplification product.

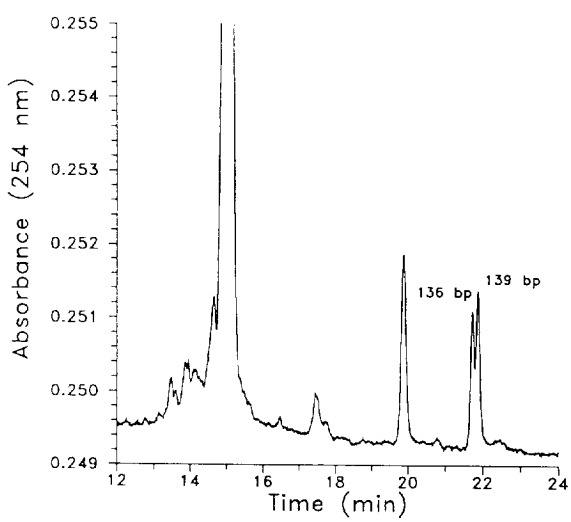

Fig. 3. CZE profile of the sample in lane 5 in Fig. 1 (heterozygous mother) displaying the two fragments sized 136 bp and 139 bp. Capillary length: 28 cm.

resolution of the two bands and diagnosis is quite easy, in other conditions, such as in lane 5, showing a carrier heterozygous for two bands differing by only one CAG triplet (136 vs. 139 bp), diagnosis is more difficult and could only be performed by using high-resolution, gradient gels and long running times.

As shown in Fig. 2, CZE could be a valid alternative for screening of PCR products from putative AIS patients. This figure represents the screening of the same family analyzed in lanes 1–3 in Fig. 1A. The upper tracing shows the pattern of the unaffected father, exhibiting only the 160 bp fragment. The intermediate tracing represents the affected child, carrying only the 139 bp DNA fragment. The lower tracing presents the electropherogram of the heterozygous carrier mother, with both 139 and 160 bp fragments. In all tracings, the early eluting peaks (ca. 21–23 min) represent primers, while the large peak at ca. 29 min represents an 80 bp constant fragment (primer dimers), visible in all sample tracks in Fig. 1A and B.

CZE in polymer networks can be utilized for resolution of more difficult cases, such as the sample in lane 5 of Fig. 1, where the two fragments are spaced only 3 bp apart. As shown in Fig. 3, also in this case the two 136 vs. 139 bp fragments can be separated, although not to baseline (note that, in this family, the 136 bp fragment represents the normal allele, whereas the 139 bp fragments represents the affected allele).

4. Discussion

CZE in polymer networks is rapidly emerging as a unique separation tool of extreme versatility. Dynamic sieving matrices are immune from the noxious problems of air-bubble formation (which would automatically open the electric circuit in such tiny channels) and from sample precipitation at the injection port. Due to the lack of a fixed-pore geometry, even large DNA fragments can open a pore in their wake, while they inevitably precipitate at the origin in cross-linked polyacrylamide gels. This allows repeated use of the same matrix (typically >30 runs). Even upon matrix fouling, the viscosity of a 6% polymer network still allows refilling of the capillary at the normal pressures utilized in CZE for, e.g., sample injection. As an extra bonus, the amount of sample required is truly minute (a few μl at the injection port, but only a few nl in the moving zone). Additionally, CZE does not require intercalating dyes (such as ethidium bromide, EtBr, mutagenic) for sample detection, as customary in slab-gel electrophoresis (although, occasionally, EtBr is added to the background electrolyte for modulating DNA velocities and thus increasing peak spacing). While at the moment CZE might not be so attractive for routine analyses, due to the availability of only a single channel, the novel generation of CZE equipment will provide batteries of channels (typically from 20 to 100), thus allowing for large sample handling abilities. This, coupled to the fully instrumental approach of CZE (with automatic storage of electropherograms on a magnetic support), might soon render this technique a challenge to conventional slab-gel electrophoresis.

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