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Capillary electrophoresis as a technique to analyze sequence-induced anomalously migrating DNA fragments

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ABSTRACT

Sequence-induced anomalous migration of double-stranded (ds) DNA in native gel electrophoresis is a well known phenomenon. The retardation of migration is more obvious in polyacrylamide compared with agarose gels, and is greatly affected by the concentration of the gel and the temperature. This anomalous migration results in a difference between calculated and actual sizes of the affected DNA fragments. A low viscosity polymer solution (DNA Fragment Analysis Reagent) under investigation for use in dsDNA analysis by capillary electrophoresis is shown to be useful for the visualization of anomalies in migration of dsDNA fragments. Comparable with traditional slab gel systems, the retardation effect, indicative of bent or curved DNA, is strongly dependent on polymer concentration and separation temperature. These dependencies have implications on the accurate sizing of dsDNA fragments with unknown sequences and secondary structures.

INTRODUCTION

Over the last several years the number of papers reporting the use of capillary electrophoresis for the analysis of double-stranded DNA (dsDNA) has rapidly increased. The aim is to improve the speed of analysis and simplify the necessary steps leading to a successful separation of nucleic acids. Promising results were shown for the analysis of restriction fragment length polymorphisms (RFLP; 1), polymerase chain reaction (PCR) products (2), variable number of tandem repeats (VNTR; 3), ultra sensitive plasmid mapping (4) and DNA/DNA hybridization (5,6).

Because of the linear charge density of nucleic acids (7), a sieving mechanism has to be employed in the electrophoretic separation of these molecules. Usually, cross-linked polyacrylamide gels (8), low or zero cross-linked polyacrylamide gels (9), cellulose derivatives (10,11), or agarose (12) are used.

We are investigating the use of a novel sieving polymer (DNA Fragment Analysis Reagent) for the analysis of dsDNA by capillary electrophoresis (13). This polymer provides both separation of nucleic acids and coating of the silica capillary in one step in a low viscosity formulation. This overcomes the very limited lifetime of gel-filled capillaries, avoids sample carryover from previous runs by replacing the capillary with fresh polymer after each analysis and allows the use of uncoated capillaries, saving time and resources.

During the characterization of this novel polymer, we discovered its potential for the separation of sequence-induced anomalous migration of dsDNA ('bent' or 'curved' DNA). Bent DNAs are nearly ubiquitous and have been correlated to a variety of biological processes (14). Traditionally, sequence-induced anomalies in migration are analyzed in non-denaturing cross-linked polyacrylamide gels, usually at low temperatures (reviewed in 15,16). This article shows that the polymer provides the high performance necessary to resolve even small differences in mobility and relates polymer concentration and temperature to the accurate sizing of dsDNA.

MATERIALS AND METHODS

Instrumentation

For all experiments, an ABI 270A-HT capillary electrophoresis instrument (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) was used. The reverse polarity mode was used, with the cathode on the injection side. Temperature over most of the separation length of the capillary was controlled in a thermostatted compartment between ambient and 60°C, with an accuracy of ±0.1°C. For electrophoretic runs below room temperature the instrument was transferred to a 4°C cold room. Separated DNA fragments were monitored on-line, using UV detection at 260 nm. Data were collected on a HP 3396 series II integrator (Hewlett-Packard Co., Fullerton, CA, USA).

DNA

The restriction fragments in the pBR 322 *Msp*I digest and the ΦX174 *Hae*III digest (New England Biolabs, Beverly, MA, USA) were diluted to a concentration of 50 ng/μl in deionized H_2O, or 1×TE, pH 8.0. The plasmids pON WT and pON subSPA (17) were purified by cesium chloride gradient (18). Plasmid DNAs were digested with the restriction enzyme *Bsp*1286I (USB, Cleveland, OH, USA). After completion of the digest, samples were desalted by dialysis (19) and diluted 1:4 with H_2O to a final concentration of 25 ng/μl. The 271, 281 and 310 bp fragments of ΦX174 were synthesized by PCR amplification. To ensure identical geometry between the PCR amplified and wild-type fragments, primers were chosen to generate fragments, which were about 20 bp longer in size on either side and included the *Hae*III site. After desalting the PCR reactions, the DNA fragments were digested with the restriction enzyme *Hae*III (Promega, Madison, WI, USA), to generate the fragments with the final length.

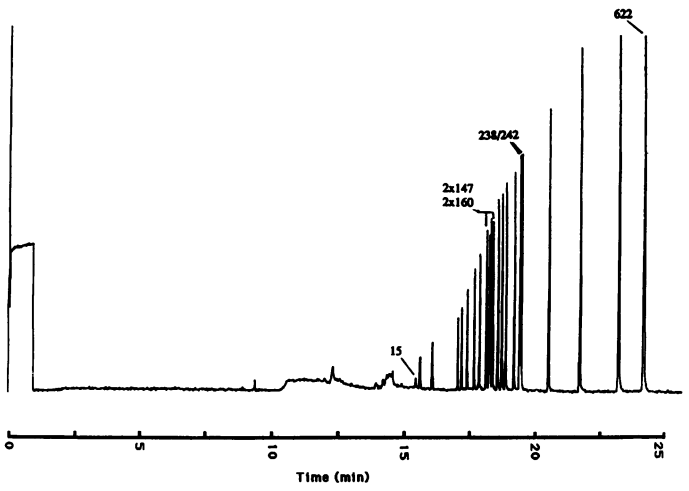

Figure 1. Capillary electrophoresis of a pBR 322 *MspI* digest at 30°C. The sample was injected for 5 s at 100 V/cm. DNA fragments were separated at a field of 214 V/cm at 30°C. The polymer concentration was 3.5%.

Capillary electrophoresis

Seventy-five μm uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were used. If not otherwise indicated, the total capillary length was 70 cm, with an effective length of 50 cm. The polymer solution (13) (available as DNA Fragment Analysis Reagent: Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) was filtered through a 0.2 μm filter disc (Gelman Sciences, Ann Arbor, MI, USA) and degassed for 2 minutes by vacuum. After installation, the capillary was conditioned by flushing, using the 20'' vacuum mode, for 30 s each with 0.3 N NaOH, H_2O, 1 N HCl, and finally for 60 s with H_2O. The capillary then was filled for 7 min (for temperatures above 25°C) or 15 min (for temperatures below 25°C) with the polymer, typically at a concentration between 3 and 4% (w/w). The conditioning procedure was repeated after each second injection. The DNA samples were typically electrokinetically injected for 5 s at a field of 140 V/cm. The applied field for electrophoresis is indicated in the respective figure legends.

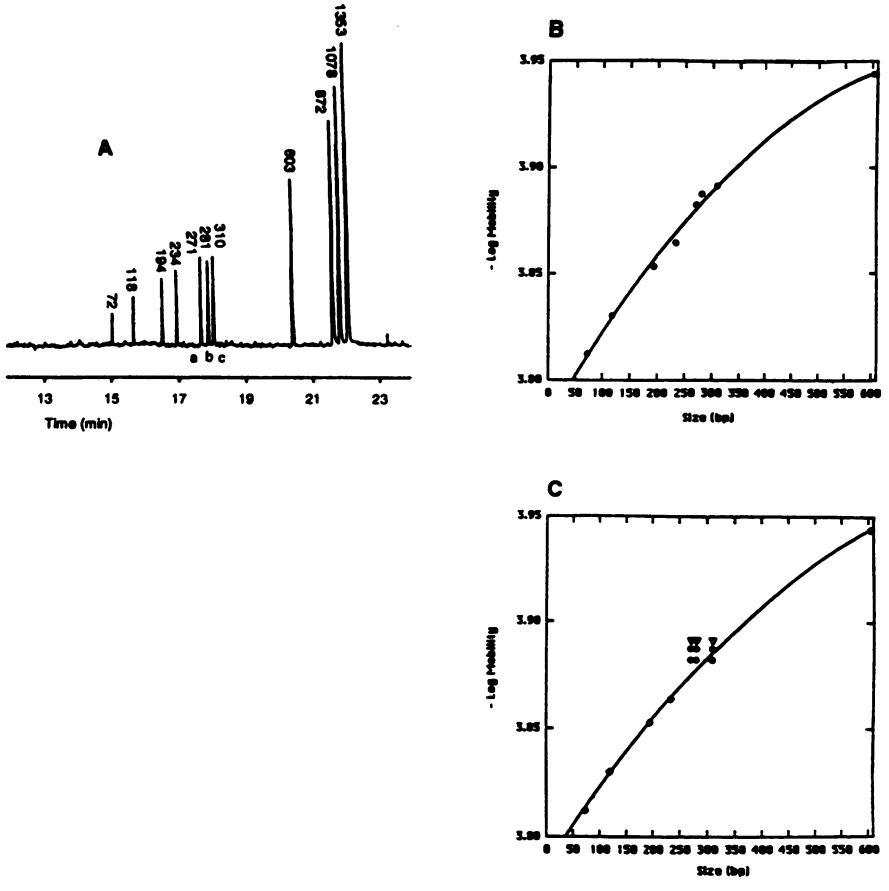

Figure 2. Capillary electrophoresis of a ΦX174 *Hae*III digest at 30°C. (**A**) Electropherogram after capillary electrophoresis of ΦX174 *Hae*III digest at 229 V/cm in a 3.3% polymer solution. Fragment sizes (bp) are indicated in order of increasing fragment lengths; fragments a, b and c are indicated. (**B**) Plot of the negative log of mobility (μ) over size for all fragments from 72 to 603 bp. (**C**) The negative logs of μ for fragments 72, 118, 194, 234 and and 603 bp were plotted over their respective size. Fitting curves in (B) and (C) were constructed by using a second-order regression. Each negative log of μ for fragments 'a' (o), 'b' (●) and 'c' (—) were plotted corresponding to fragment sizes of 271, 281 and 310 bp.

RESULTS

Two commonly used DNA size standards, both in capillary and conventional electrophoresis, are the *Msp*I digest of pBR 322 and the *Hae*III digest of the phage ΦX174. The electropherogram of the separation of the fragments of the *Msp*I digest of pBR 322 by capillary electrophoresis at 30°C is shown in Figure 1. It is noteworthy, that several fragments in this digest have, based on the published sequence (20), identical fragment lengths. Interestingly, both sets of these identical length fragments, 147 and 160 bp, are separated into two individual peaks each by using a 3.5% polymer solution with an effective capillary length of 50 cm, in less than 25 min. The anomalous migration of one of the 147 bp fragments was already observed in a conventional 6.9% T acrylamide gel (21).

Based on the additive effect of the absorbance at 260 nm of the nucleosides in a mixed sequence fragment of DNA, one would expect a monotonic increase in peak height with increasing fragment size assuming equimolar representation of each fragment in a complete restriction digest of plasmid DNA. The analysis of the *Hae*III digest of ΦX174 (22) at ambient temperature by capillary electrophoresis consistently shows that at least one of the peaks (Figure 2A, peak 'b') migrates to a position which appears to not follow this monotonic peak height increase.

A plot of the negative log of mobility against fragment size, assigning each mobility to a fragment in the order of increasing fragment size, should result in a concave, smooth curve (Figure 2B). The slightly concave curve results from the mobility of fragments <400 bp, separated by the Ogston regime (23), and fragments >400 bp, separated according to the reptation model (24) in this polymer system. The separation of the ΦX174 *Hae*III digest fragments by capillary electrophoresis does not follow these predictions.

A deviation in the mobility for some of the fragments from the expected curve is observed (Figure 2B). A plot of the negative log of mobility for the 72, 118, 194, 234 and 603 bp fragments over their respective size shows that all of them fall on the curve, indicating a normal mobility (Figure 2C). The mobility fo fragments a, b and c (Figure 2A) were plotted to positions corresponding to the sizes 271, 281 and 310 bp. The aim was to determine if any of the mobilities for the three fragments aligns with the curve, indicating a strictly size-dependent mobility. None of the mobilities for the three fragments a, b and c convincingly aligns with the curve connecting the fragments with the sizes of 72, 118, 194, 234 and 603 bp. This indicates that possible secondary structures within the DNA fragments, e.g. the presence of A tracts, may cause them to migrate anomalously in this polymer system.

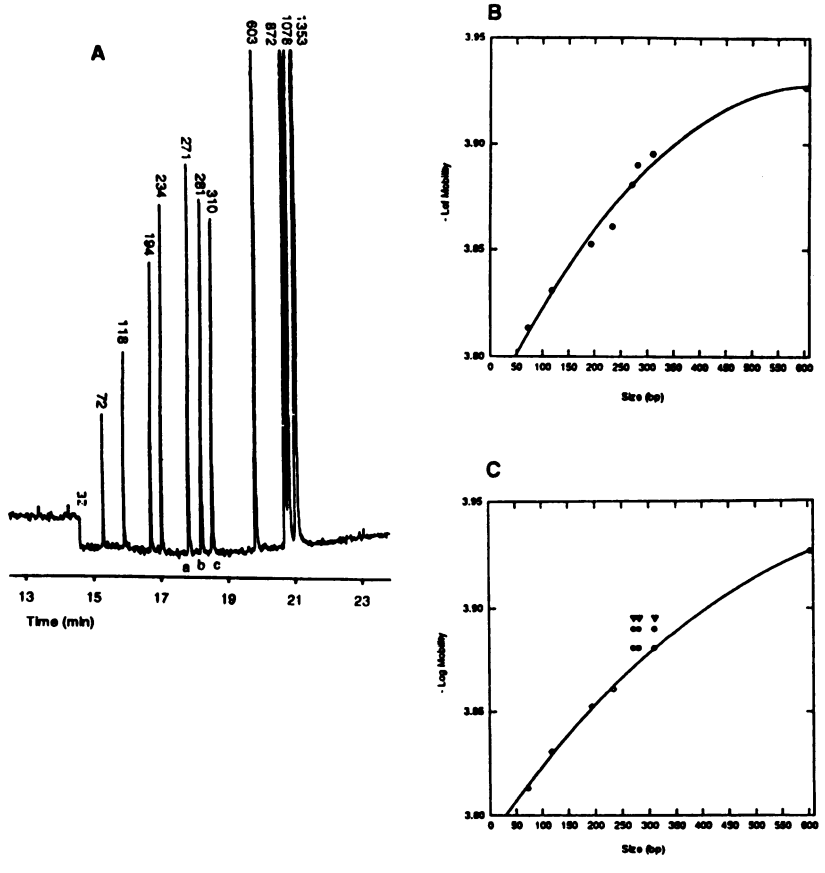

Figure 3. Capillary electrophoresis of a ΦX174 *Hae*III digest at 10°C. (**A**) Electropherogram after capillary electrophoresis of ΦX174 *Hae*III digest at 357 V/cm in a 3.3% polymer solution. See Figure 2 for additional information.

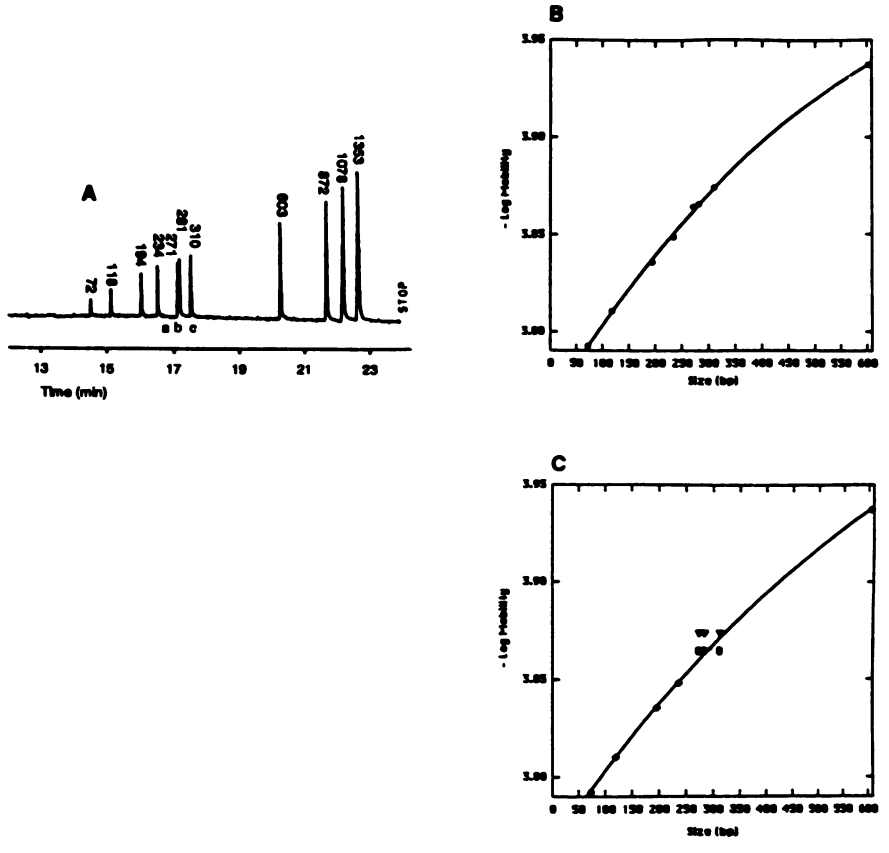

Figure 4. Capillary electrophoresis of a ΦX174 *Hae*III digest at 60°C. (**A**) Electropherogram after capillary electrophoresis of ΦX174 *Hae*III digest at 157 V/cm in a 3.3% polymer solution. See Figure 2 for additional information.

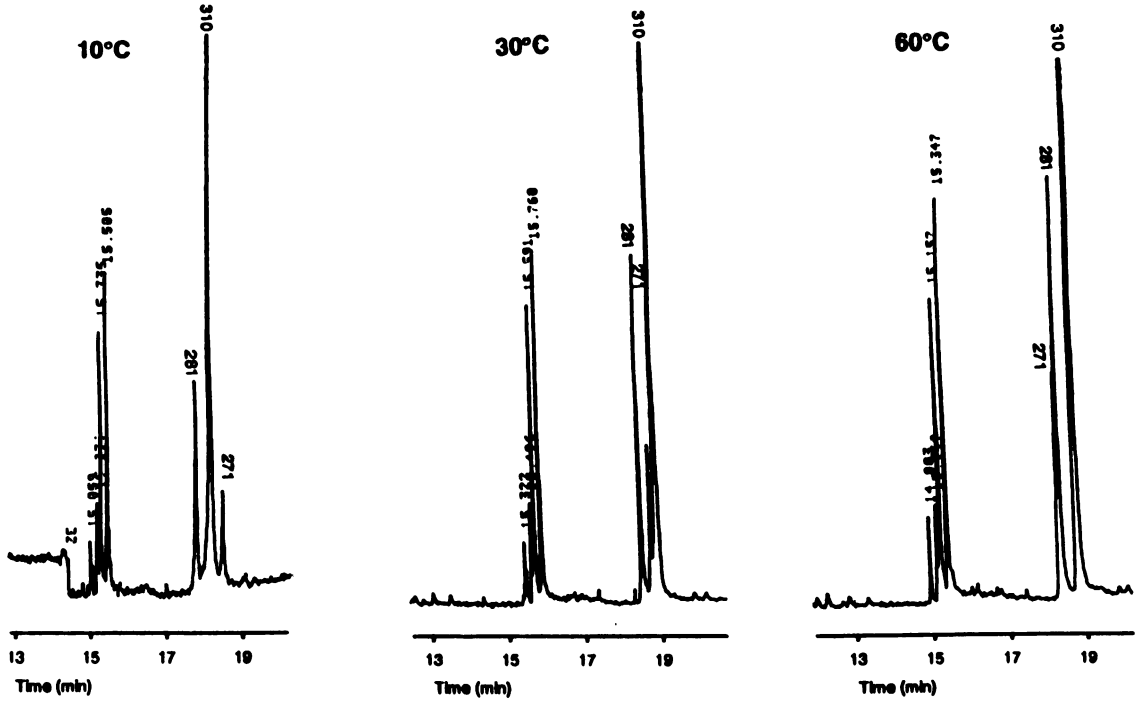

Figure 5. Capillary electrophoresis of PCR-generated fragments. The 271, 281 and 310 bp fragments were synthesized by PCR amplification. The desalted fragments were mixed in a ratio of 1:2:4 (271:281:310 bp) and separated at the indicated temperatures, using the same conditions as described in the legends to Figures 2–4.

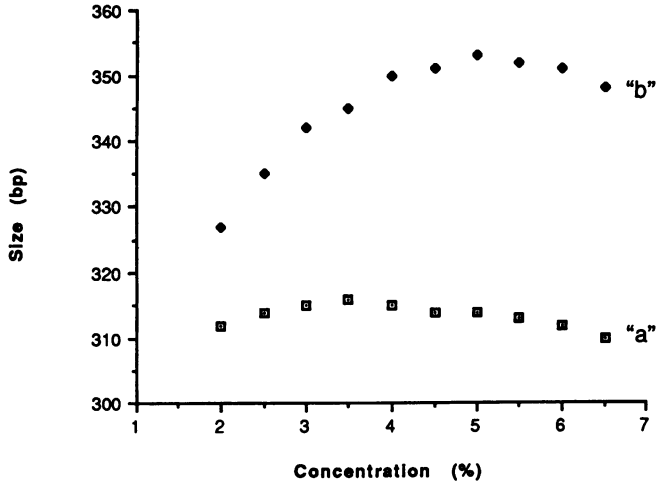

Figure 6. Relationship between polymer concentration and size calculation for fragments 'a' and 'b'. Conditions for capillary electrophoresis are as in Figure 2. The negative logs of mobility for fragments 72, 118, 194, 234 and 603 bp were plotted over their respective sizes. Second-order curve fitting was used to calculate the sizes for fragments 'a' and 'b'.

A possible wrong peak assignment for the 271, 281 or 310 bp fragments was recently discussed in a different polymer system used for capillary electrophoresis (25).

This finding prompted us to more closely examine the usefulness of this polymer system in combination with capillary electrophoresis for the visualization of anomalously migrating dsDNA.

It is known that the electrophoretic behavior of bent DNA is influenced by temperature (15). Therefore, the *Hae*III digest of ΦX174 was separated by capillary electrophoresis at various temperatures.

After the separation of the fragments by capillary electrophoresis at 10°C, the plot of the negative log of mobility for all the fragments from 72 to 603 bp shows an even more extensive deviation in the mobility for some of the fragments from the expected curve than at 30°C (Figure 3B). An attempt to align the mobility of fragments a, b and c with a position corresponding in size to 271, 281 and 310 bp, indicates that fragment 'a' migrates with a size of 310 bp, whereas the mobilities for fragments 'b' and 'c' correspond to even bigger fragment sizes (Figure 3C).

At 60°C, a temperature which releases the sequence-induced conformational strain on bent DNA, the electropherogram shows a monotonic increase of peak height with increasing fragment length (Figure 4A) and the negative log of mobility for all fragments between 72 and 603 bp corresponds to the DNA fragments in the order of increasing fragment length (Figure 4B). The assignment of the mobilities for fragments a, b and c to fragment sizes shows that the mobility for fragment 'c' now corresponds very well to the size of 310 bp. The mobilities for fragments 'a' and 'b' align well with sizes of 271 and 281 bp (Figure 4C).

No significant difference in peak width and peak order was seen when injecting the DNA fragments either out of H_2O or 1×TE and performing electrophoresis at 30°C or 60°C, indicating that denaturation of the DNA fragments at low ionic strength is not responsible for the anomalous migration of the fragments (data not shown). The injection out of a low/no salt buffer was preferred in order to increase the signal intensity (19).

While these results clearly demonstrate an abnormal migration pattern for fragments a, b and c, especially at below ambient temperatures, these data do not indicate the size identity of these fragments. In order to unambigously identify fragments a, b and c (see Figures 2–4) by their sizes, irrespective from their electrophoretic mobility, we synthesized the 271, 281 and 310 bp fragments by PCR amplification (see Materials and Methods). In order to discriminate the fragments from each another, they were mixed in a ratio of 1:2:4 (271:281:310 bp). No additional peaks were observed after spiking a *Hae*III digest of ΦX174 with a mixture of the PCR generated fragments, therefore assuring the identity of these fragments (data not shown).

The mixture of the three PCR generated fragments was then subjected to separation by capillary electrophoresis at 10°C, 30°C and 60°C (Figure 5). The peaks at about 15 min represent PCR reaction components, which are well separated from the 271, 281 and 310 bp fragments. The order of peaks now clearly establishes the identity of the fragments based on the peak height. At 10°C the 271 bp fragment trails the 281 and 310 bp fragments, whereas at 30°C the 271 bp fragment migrates in between the 281 and 310 bp fragments. Only at 60°C the three fragments migrate strictly according to their respective size.

Shifts in mobility of sequence-induced bent DNA in conventional electrophoresis show a strong correlation with the percentage of the polyacrylamide gel employed (26). Therefore, we examined the separation of the fragments of ΦX174 *Hae*III by capillary electrophoresis, using different concentrations of the polymer, varying between 2 and 6.5%, at 30°C. The sizes for fragments 'a' and 'b' (see Figure 2A) were calculated (Figure 6). Fragment 'a', at 30°C, migrates in a 3.5% polymer with an approximate size of 310 bp (see Figure 2C). For this fragment, at all polymer concentrations examined, a size of 313 bp (SD 1.8) was calculated. Fragment 'b', which was identified as a fragment having an anomalous migration behavior, exhibited a size which was strongly influenced by the polymer concentration. At 30°C, the calculated size (327 bp) was closest to its actual size (281 bp) at the lowest polymer concentration examined. With increasing polymer concentration the calculated fragment size even further deviated from the actual size. It is interesting to note that the maximum difference is reached at a concentration of 5%, thereafter the difference starts to decrease again (Figure 6).

However, more important than the polymer concentration is the influence of the separation temperature on the mobility of anomalously migrating dsDNA fragments and therefore the accurate size determination. We examined how separation temperatures between 10°C and 60°C influenced the sizing for the 'b' fragment of ΦX174 *Hae*III digest at a polymer concentration of 3%. Whereas the calculated size for the 'b' fragment at 60°C is very close to its actual size (297 versus 281 bp respectively), at 10°C the calculated size (382 bp) differs by more than 100 bp from its actual size (Figure 7).

Taken together, these data suggest that this polymer system performs comparably with polyacrylamide gels in reducing the mobility of sequence-induced bent DNA in a concentration- and temperature-dependent fashion.

To further demonstrate the usefulness of this polymer system for the visualization of bent DNA, we separated two plasmids from the pON series (17,27) by capillary electrophoresis. pON WT contains 17 bp of SV40 origin region 1, which is involved in binding the T antigen. Two direct repeat pentanucleotides are

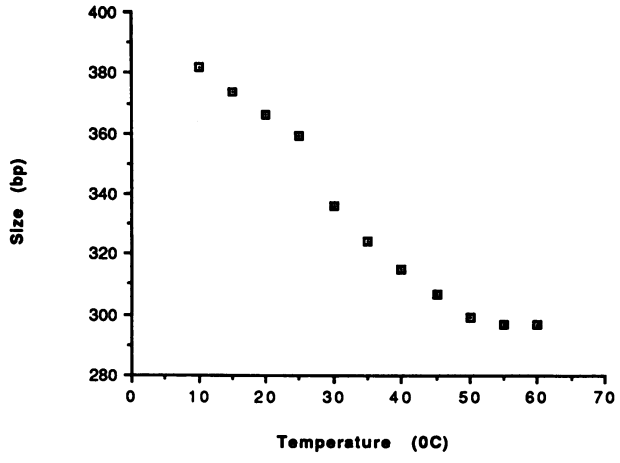

Figure 7. Temperature-dependent calculation of size for the 281 bp fragment. A ΦX174 *Hae*III digest was separated in a 3% polymer by capillary electrophoresis at a field of 229 V/cm. Separation temperature was increased in 5°C increments from 10°C to 60°C. The size for the 'b' fragment was calculated as described in the legend to Figure 6.

separated by an asymmetric spacer region, containing six consecutive As. The spacer region assumes a conformation which facilitates the binding of the T antigen to the pentanucleotides (17). We analyzed the plasmids pON WT and pON subSPA, which have conformational differences in a 220 bp fragment that can be discriminated by different mobilities on a 12% polyacrylamide gel at 5°C (17) by capillary electrophoresis.

Figure 8A shows the electropherograms of *Bsp*1286I restricted plasmids pON WT and pON subSPA together with the *Hae*III digest of ΦX174. The 220 bp fragments containing the wild type or mutated SV40 origin region I sequences are indicated. Figure 8B shows a portion of the electropherograms with the 220 bp fragment and an adjacent plasmid derived fragment after mixing a digest of both plasmids at a ratio of 2:1 (subSPA:WT) and performing separation by capillary electrophoresis at 10°C, 30°C and 60°C. While the plasmid-derived fragment from both plasmids comigrates at all temperatures, the 220 bp fragments, harboring the wild type or mutated SV40 origin region I sequences, are separated at 10°C and 30°C. This separation of both fragments is most evident at 10°C, but can be seen up to 50°C (data not shown). At 60°C both fragments have identical mobilities and comigrate.

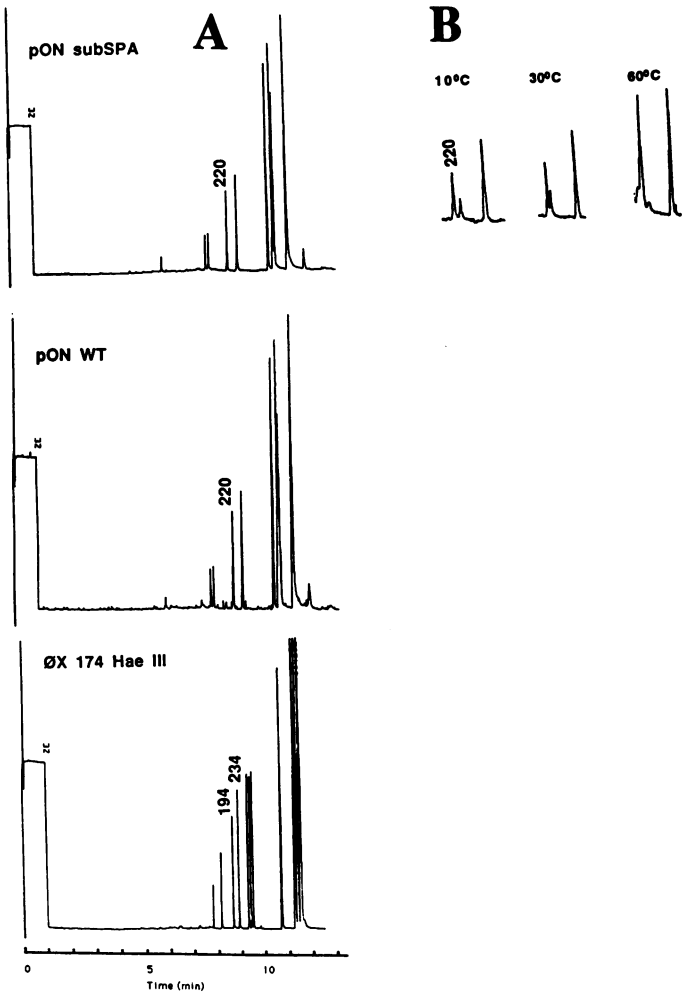

Figure 8. Capillary electrophoresis of pON plasmids. The total capillary length was 50 cm with an effective capillary length of 30 cm. The polymer concentration used was 3%. The applied field was 240 V/cm. (**A**) Separation temperature was 26.2°C. Electropherograms of the *Bsp*1286I digests of pON subSPA, pON WT and ΦX174 *Hae*III are shown. The 220 bp fragments containing the SV40 origin region I sequences are indicated. (**B**) The *Bsp*1286I digests of pON subSPA, pON WT were mixed in a ratio of 2:1 (subSPA:wt) and electrophoretically separated at the indicated temperatures. The 220 bp subSPA/WT fragments together with an adjacent reference plasmid fragment are shown.

DISCUSSION

We have shown the potential of capillary electrophoresis in combination with a novel sieving polymer for the visualization of sequence-induced anomalous migration of dsDNA. The polymer shows characteristics comparable with conventional polyacrylamide gel electrophoresis. Additionally, it provides several advantages over conventional electrophoresis for the analysis of bent/curved DNA: (i) rapid set up for analysis, only about 20 min are necessary before the first sample can be analyzed; (ii) analysis times are fast, usually well below 25 min (see Figures 1 and 2); (iii) retardation in migration can be visualized even at above ambient temperatures (see Figure 8B); (iv) the polymer permits discrimination of even minor changes in mobility. The 220 bp pON WT fragment migrates at 5°C in a conventional 12% polyacrylamide gel to a size, corresponding to 231 bp. This is an 11 bp difference to the 220 bp size for the pON subSPA fragment (17). This small difference in size can be well resolved by this sieving polymer (Figure 8B).

The demonstrated overall sensitivity of capillary electrophoresis for sequence-induced anomalous migration should make this system amenable for the analysis of minor changes in mobility as influenced by the location, the composition, or the number of A tracts that are responsible for the degree of a bend and therefore the resulting retardation (14,16,28,29).

Another important aspect of these experiments is the demonstration of the effect of the applied experimental conditions on the accuracy of determining the exact size of a given dsDNA fragment. The mobilities and therefore the accuracy of sizing of random sequence dsDNA fragments are predictable, allowing

the use of certain dsDNA fragments to generate a calibration curve (Figure 2C). This results in a very precise size determination of the 'a' fragment to 313 bp (RSD 0.97%) (Figure 6). As this paper and others report, the difference between actual and calculated sizes for certain dsDNA fragments can differ substantially (21,30). Differences of several hundred base pairs in polyacrylamide gels have been reported (30). It is evident that both the percentage of the sieving polymer and the applied temperature during the separation by capillary electrophoresis have an influence on the accuracy of sizing. It is clear that while the applied polymer concentration influences the mobility of anomalously migrating dsDNA fragments (Figure 6), the major effector on mobility of these fragments is the separation temperature (Figure 7). If an accurate estimation of size for a dsDNA fragment with unknown sequence and possible secondary structure has to be determined, the analysis of this DNA should be performed with at least two different temperatures, ambient and above 50°C. If the position of the unknown DNA is shifted relative to standard sizing DNA fragments at the different temperatures, then an anomalous migration can be assumed. This also implies that for an accurate size determination a DNA size marker standard has to be used, which has no anomalously migrating DNA fragment(s) present. Commercially available 'ladder' DNA, consisting of repetitive units of a defined DNA fragment, should prove useful.

Taken together, this paper demonstrates the potential of the relatively new technology of capillary electrophoresis for the visualization of sequence-induced anomalous migration of dsDNA and provides a method to increase sizing accuracy for certain dsDNA fragments.

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