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Separation of 1–23-kb complementary DNA strands by urea–agarose gel electrophoresis

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ABSTRACT

Double-stranded (ds), as well as denatured, single-stranded (ss) DNA samples can be analyzed on urea–agarose gels. Here we report that after denaturation by heat in the presence of 8 M urea, the two strands of the same ds DNA fragment of ~1–20-kb size migrate differently in 1 M urea containing agarose gels. The two strands are readily distinguished on Southern blots by ss-specific probes. The different migration of the two strands could be attributed to their different, base composition-dependent conformation impinging on the electrophoretic mobility of the ss molecules. This phenomenon can be exploited for the efficient preparation of strand-specific probes and for the separation of the complementary DNA strands for subsequent analysis, offering a new tool for various cell biological research areas.

INTRODUCTION

Gel electrophoresis is a powerful, yet, convenient tool routinely used to separate nucleic acids on the basis of differences in their size, as well as local and global conformational characteristics. For molecules smaller than the pore size of the gel, the electrophoretic mobility is adequately described by the Ogston sieving mechanism (1). Longer molecules that exceed the volume of a single pore snake through the gel matrices in an end-on fashion (2–4), while the DNA molecules tend to stay oriented parallel to the electric field (5). The model for this latter electrophoretic process, called reptation, is based on the concept of a tube through which the nucleic acid chain passes via random stretching and shrinking [(6–8) and references therein]. In 1% agarose gels, the double-stranded (ds) DNA molecules appear to follow the Ogston approximation below ~4-kb size, while they are expected to behave as predicted by the reptation model at larger sizes (6,7). In addition to size and conformational characteristics, the handedness of supercoiling influences electrophoretic mobility (9), a phenomenon lacking molecular explanation. The relatively unstable single-stranded (ss) nucleic acid molecules appear to form coiled structures with size parameters that are sensitive not only to base composition, but also sequence in a size range of up to 2–300-bp length; this phenomenon is utilized in single-strand conformation analysis performed usually in polyacrylamide gels (10–12). Orientation of the gel matrix itself in the electric field has also been recognized as a factor influencing electrophoretic mobility (5). The average pore size is typically 200–500 nm for agarose, and it exceeds that of acrylamide gels that ranges from 5 to 100 nm, depending on the conditions and methods of assessment used (13). Polyacrylamide appears to be chemically inert, while the hydroxyl groups of agarose may participate in transient H-bonding during migration.

The electrophoretic separation of urea/heat-denatured and non-denatured (considered ss and ds, respectively) nucleic acids in the same 1 M urea-containing agarose gels was first described by Materna *et al.* (14): they observed a difference in the migration of ds vs. ss molecules of the same size and band duplication after denaturation in the case of one of the PCR fragments analyzed, without commenting on the strandedness dependence of electrophoretic migration documented and characterized in detail herein. The general belief still considers urea as a denaturant unsuitable for use in agarose gels (15). Here we demonstrate that this separation system can be very useful in applications requiring the separation of the complementary DNA strands in an unexpectedly broad size-range, opening new areas of application.

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MATERIALS AND METHODS

Agarose-embedded yeast genomic DNA

The *Saccharomyces cerevisiae* WDHY 199 (MATa, leu2-3,112 trp1-289 ura3-52 his7-2 lys1-1) cells were grown and the preparation of agarose-plugs containing the yeast spheroplasts was carried out as previously described (16). For restriction enzyme digestion, the plugs were preincubated in the appropriate 1× restriction enzyme buffers three times for 1 h each, then incubated with 150 U/ml SmaI or Nb.Bpu10I (Fermentas Life Science, Maryland, USA) in 200 µl of the same buffer at 37°C, for 1.5 h. For S1 nuclease treatment, 1× S1 buffer was used for washing of the plugs before digestion by 500 U/ml of the enzyme (Promega Life Science, Madison, USA) at 37°C, for 1.5 h. The plugs were finally equilibrated with TE buffer before electrophoresis.

PCR amplification of *S. cerevisae* rDNA segments

PCR was performed using 1.25 U of the Long PCR Enzyme mix (Fermentas) in 50 µl of 1× buffer supplemented with 1.5 mM $MgCl_2$, containing, 20 pmol of each primer (Integrated DNA Technologies, Coralville, IA, USA), the dNTPs (Promega) at 0.25 mM concentration and 300 ng *S. cerevisae* genomic DNA prepared as described earlier (16). Each forward primer (see Tables 1–2 of Supplementary data) was used in pair with the reverse p1R primer resulting in variable length of amplicons, overlapping at their 3′-ends defined by the common reverse primer.

Sample preparation for urea–agarose gel electrophoresis

Before loading the DNA samples on the gels, either 5 µl DNA (0.1–1 µg) solution was added to 25 µl urea–LB [0.5 mg/ml bromephenol blue (Sigma), 8 M urea (Sigma), 1% (v/v) NP-40 (Calbiochem), 1mM Tris pH 8] or when DNA was embedded into agarose plugs, the blocks were soaked into freshly prepared 8 M urea solution/TE at room temperature for 45 min. These samples were either loaded without denaturation, or were heat-denatured at 80°C for 5 min and then loaded on the same gel.

Standard and urea/heat-denaturing agarose gel electrophoresis

For standard, non-denaturing gel electrophoresis, 1.2% agarose gels (SeaKem) were prepared in 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8); the electrophoresis buffer was also 1× TAE. For urea/heat-denaturing gel electrophoresis, 1.2% agarose gels containing 1 M urea were prepared; the electrophoresis buffer was 1× TAE supplemented with 1 M urea. Electrophoresis was carried out in the cold room, at 4°C and 55 V, for 12 h. After gel electrophoresis agarose gels were stained with 0.5 µg/ml ethidium bromide (Ebr; Promega) for 30 min, but the urea–agarose gels were washed in 1× TAE to remove urea, then soaked in 100 mM NaCl solution and stained with 0.5 µg/ml EBr for 30 min. In reassociation experiments, agarose blocks containing ss and ds fragments were excised, heat treated at 95°C for 5 min, then allowed to renature at 45°C for 30 min before loading them on standard agarose gels. Molecular mass markers were lambda HindIII fragments and the 1 kb ladder of Fermentas.

Southern blot with rDNA-specific probes

The standard and urea/heat-denaturing agarose gels were transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) using a BIO-RAD vacuum blotter. The membranes were dried for 30 min at 80°C and UV cross-linked (1.2×10^5 µJ/cm^2). The blotted, denatured DNA was prehybridized for 3 hours at 55°C in 30 ml prehybridization solution (1 m/v% BSA, 0.5 M Na_2HPO_4, 7 m/v% SDS, 1 mM EDTA, 10 µg/ml salmon sperm DNA), and was hybridized for 15 hours with single-strand-specific probes for the rDNA gene cluster. The ds PCR product of 1405 bp length, synthesized using the p1F and the p1R primer pair (Supplementary Data), was used as template DNA for subsequent probe preparation. Labeling with ^{32}P was performed either by random primer labeling (ds probe; using a RediPrime Kit, Amersham) or 'linear amplification' (using a single primer) to prepare strand-specific probes: The p1F primer alone was applied for sense-specific, and the p1R primer for antisense strand-specific probe preparation. In these reactions 2.5 U Taq polymerase (Fermentas Life Science, Maryland, USA) was used, in 50 µl of 1× reaction buffer (10 mM Tris–HCl, 50 mM KCl, 0.08% N P-40, pH 8.8) supplemented with 3 mM $MgCl_2$, containing 50 ng template DNA, 20 pmol of primer and the nucleoside-triphosphates. dATP, dTTP and dGTP were used at 0.25 mM, dCTP at 5 µM concentration (all from Promega Life Science, Madison, USA), and for each labeling reaction 5 µl [$\alpha^{32}P$]-dCTP (6000 Ci/mmol, 10 mCi/ml; Institute of Isotopes LTD, Hungary) was added. In the first reaction cycle, denaturation was at 94°C for 3 min, annealing at 60°C for 1.5 min, polymerization at 72°C for 1.5 min; this was followed by 45 cycles when denaturation was at 94°C for 1.5 min, annealing at 60°C for 50 s, polymerization at 72°C for 1.5 min. The probes were purified on Sephadex G-25 (Amersham). After hybridization, the membranes were washed three times at 60°C with a washing solution (40 mM Na_2HPO_4, 1 m/v% SDS, 1 mM EDTA). The signal was detected by Phospho-screen (Kodak) and visualized by a BIO-RAD Phospho-Imager.

Photographic equipment and settings

Gel photos were taken by FinePix S602Zoom digital camera (Fujifilm) and prepared for publication using Paint Shop Pro 9.0.

RESULTS AND DISCUSSION

The solitary bands of ds PCR fragments are separated into two distinct bands upon urea/heat denaturation and agarose gel electrophoresis, in the presence of 1 M urea, in a size range of ~1000–10 000 nucleotides (Figure 1). The ss DNA fragments in both bands run much faster than the ds DNA they are derived from. ('ss' stands here for a single polynucleotide chain that has been separated from its complementary strand upon heat denaturation, regardless

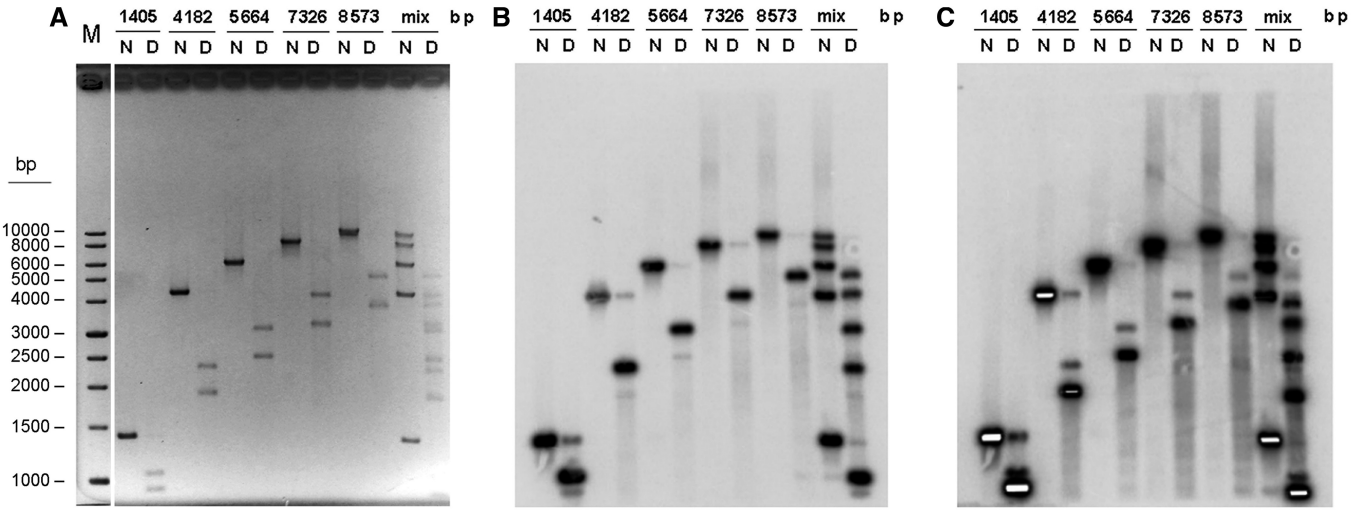

Figure 1. Urea–agarose gel electrophoretic analysis of ds and urea/heat-denatured ss fragments of varying length. The ds DNA fragments were prepared by PCR, using *S. cerevisiae* rDNA as template and a set of primers (see 'Materials and Methods' section) designed to yield PCR products of 1405, 4182, 5664, 7326 and 8573-bp length (overlapping at the 3′-end defined by the reverse primer). (**A**) EBr-stained urea–agarose gel. Lanes labeled 'N' and 'D' contain undenatured and denatured PCR products of increasing length, respectively. The numbers indicate the size of the PCR products analyzed in that lane; 'mix': mixture of the PCR products. M: undenatured 1 kb marker. (**B, C**) Southern blot of the gel in panel A, using a sense-strand-specific (B) or an antisense-strand-specific (C) probe (prepared as described in 'Materials and Methods' section).

of the likely presence of stable or transient ds regions in it, while 'ds' is used to designate the non-heat-denatured molecular species which may become partially ss while running in the urea–agarose gel.) Southern hybridization using ss-specific probes reveals that the two bands correspond to the complementary strands of the ds fragments. The presence of traces of isotope-labeled molecules recognizing the other strand allowed visualization of both strands. The minor band corresponding to the ds molecules in denatured samples (see Figure 1B and C) is apparently due to reassociation of the separated complementary strands. In line with this, Figure 2 demonstrates that after separation, the sense strand (co-linear with the coding strand of the 18S, 5.8S and 25S rDNA genes) and the antisense strand of the excised bands readily pair with each other, regenerating the original ds fragments (see Figure 2B, lane 5). The two strands maintain their differential migration properties even after re-running them, separately, in agarose devoid of urea (Figure 2B, lanes 3–4), suggesting that urea is necessary to separate the complementary strands upon heat denaturation, and its continued presence in the gel system is not required for their differential electrophoretic migration. A partial association between ss fragments of identical polarity is also visible in Figure 2B, lane 4 (especially after prolonged incubation; data not shown); these sense–sense and antisense–antisense complexes run similarly to the ds fragments composed of complementary strands. The presence of numerous hairpins, pseudoknots and entanglements in the folded single strands (simulated using the Kinefold program: http://kinefold.curie.fr/cgi-bin/form.pl) and the structures derived in molecular dynamics simulations of ss oligonucleotides (12) are in line with the possibility that such complexes may arise when two identical molecules interact. As Figure 3 shows, strandedness-dependent

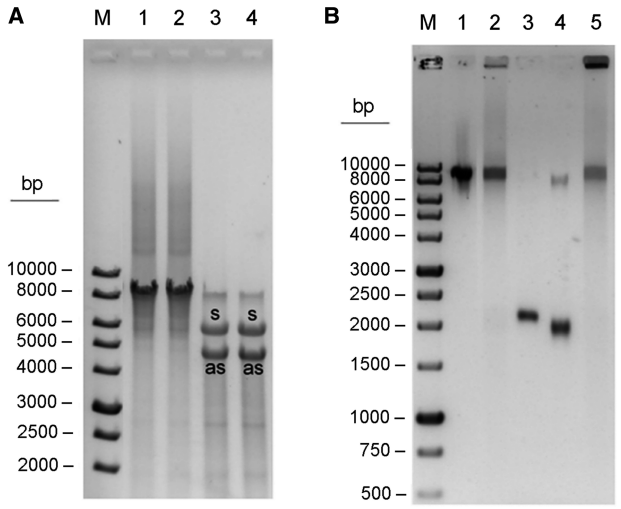

Figure 2. Reassociation of the complementary strands. (**A**) Urea–agarose gel electrophoretic analysis of the 8573 bp PCR product. Lanes 1–2 (duplicate sample): undenatured PCR products; lanes 3, 4 (duplicate sample): sense (s) and antisense (as) strand, run after heat denaturation. M: 1 kb ds marker. The gel was washed in 1× TAE, renatured in 100 mM NaCl solution and stained with 0.5 μg/ml EBr. The bands, already devoid of urea, containing the ds PCR product and the two complementary strands were excised and re-run on a standard agarose gel, as shown in panel B. (**B**) Non-denaturing gel electrophoretic analysis. Lane 1, and lanes 3–4: 8573 bp ds PCR product and the separated complementary strands, respectively, cut out from the first gel (from lane 1 and 3, respectively) and re-run directly without heat denaturation. Lane 2: the excised block of the ds fragments of panel A lane 2 was heat denatured then allowed to renature (as described in 'Materials and Methods' section), and loaded on the gel. Lane 5: the samples of the excised agarose blocks containing the complementary strands (bands labeled 's' and 'as' in lane 4 of panel A) were united, heat denatured, then allowed to renature (as described in 'Materials and Methods' section), to allow reassociation of the two strands before analysis. M: 1 kb ladder, analyzed without heat denaturation (both panels).

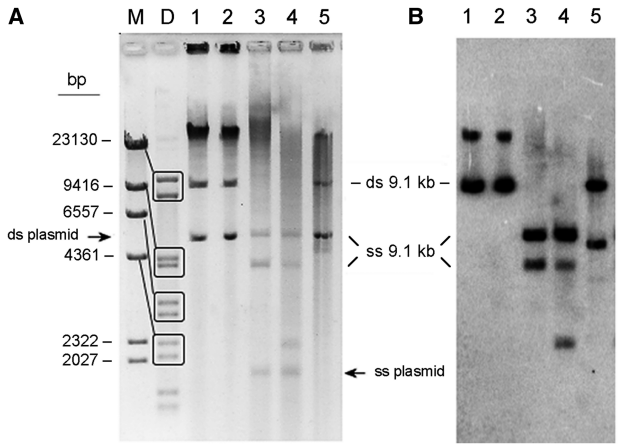

Figure 3. Urea–agarose gel electrophoretic analysis of λ HindIII fragments and agarose-embedded *S. cerevisiae* genomic DNA. (**A**) EBr-stained urea–agarose gel. Lanes 1 and 3: SmaI-digested *S. cerevisiae* genomic DNA, undenatured and denatured, respectively. Lanes 2 and 4: SmaI- and Nb.Bpu10I-digested *S. cerevisiae* genomic DNA, undenatured and denatured, respectively. Lane 5: SmaI- and Nb.Bpu10I-digested *S. cerevisiae* genomic DNA, run without denaturation but after S1 nuclease digestion. Restriction enzyme digestions were partial in the agarose blocks. M and D: λ HindIII fragments without and after heat denaturation, respectively. The numbers on the left side of the panel indicate the size of the λ Hind III ds fragments in bp. The different size ds fragments match the different ss fragment pairs as indicated by the connecting lines in the figure, verified by rerunning in denaturing conditions of individual ds λ fragments isolated from a standard agarose gel. The *arrows* point at the 2 μ plasmid also present in the yeast nuclei. (**B**) Southern blot of panel A, hybridized with a ds probe hybridizing equally with the sense and antisense rDNA strands (prepared as described in 'Materials and Methods' section). The bands corresponding to the ds and denatured, ss 9.1 kb genomic rDNA repeat are indicated. The identity of the two complementary strands was confirmed based on the decreased intensity of the band corresponding to the antisense strand after digestion of the agarose-embedded DNA with a nickase enzyme (Nb.Bpu10I) specific for this strand (lanes 3–4). The size of the new ds fragment formed upon S1 digestion of the Nb.Bpu10I-pretreated blocks (lane 5) is in line with the nickase activity expected.

electrophoretic separation is not restricted to particular DNA sequences. The complementary strands of the λ DNA HindIII fragments are also separated, particularly in the case of the 23 kb fragment. The identitiy of the two strands was determined using single-strand-specific probes (see Supplementary Data, Figure S1). In *S. cerevisiae* genomic DNA the rDNA cluster, i.e. the template used for the PCR reactions (Figures 1 and 2), contains repetitive 9.1 kb segments that can be excised with SmaI. This '*in vivo* amplified' DNA is also separated into differentially migrating complementary strands (Figure 3A and B, lanes 3–4). The identity of the two complementary strands was confirmed based on the decreased intensity of the band corresponding to the antisense strand after digestion of the agarose-embedded DNA with a nickase enzyme (Nb.Bpu10I) specific for this strand (Figure 3A and B, lanes 3–4). The size of the new ds fragment formed upon S1 digestion of the Nb.Bpu10I-pretreated blocks (Figure 3, lane 5) is in line with the (partial) nickase activity expected. (The nicking character of this enzyme could be demonstrated by hybridization using strand-specific probes, as shown in Supplementary Figure 3, and by translating the nicks out of their specific site using DNA polymerase I and nucleotide triphosphates; data not shown.) Comparison of the nucleotide composition of the opposing strands for all the DNA fragments analyzed above reveals that their separation depends both on the C/G and (A + T)/(C + G) ratios (Supplementary data). When the latter ratio is similar, the higher the C/G ratio, the more retarded the electrophoretic mobility of an ss fragment will be.

In contrast with the results obtained with agarose gels, the electrophoretic mobility of the two strands was indistinguishable in urea–polyacrylamide gels (data not shown); thus the effect described herein has no relevance for the two-dimensional strandedness-dependent electrophoresis approach (17), which is based on PAGE. The possible reasons for the difference between the two gel systems may be related to (i) agarose-specific chemical interactions with ss DNA, (ii) the different average pore size of the two gel types (see above) or (iii) architectural differences in the gel matrix. The retardation of the ds fragments relative to the ss molecules in TAE-buffered gels was larger at lower agarose concentration (data not shown), underlining the role of sieving mechanism in differentiating between the two strands. Interestingly, when run in TBE buffer instead of TAE (used throughout the experiments shown), the denatured DNA fragments generally migrate slower than the corresponding ds DNA in 1.0–1.6% agarose gels [data in accordance with (14); not shown].

Urea is expected to completely deproteinize chromatin samples in agarose blocks (18) and is also known to reduce T_m by approximately 3 K per molar urea added, independent from base composition (19). It seems that 8 M urea is not sufficient to permanently or completely melt duplex regions, as raised in ref. (20). This conclusion is in line with the presence of a minor band containing undenatured fragments in Figure 2A, lanes 3–4. It is also in agreement with the fact that the purified two strands readily reassociate (Figure 2B, lane 5), and also with our futile efforts to keep homopolymers of poly(dG)–poly(dC) denatured while running these samples in urea–agarose gels (data not shown). The fact that the differential mobility of the two strands is maintained in agarose gels devoid of urea (Figure 2B, lanes 3–4) argues against the possibility that masses of bound urea would significantly contribute to these effects. Different base composition on its own may result in different overall conformational characteristics. On the other hand, a dynamic interaction between the bases and the denaturant could occur and influence the conformation of the two strands differentially. Interaction of urea with T bases exposed upon denaturation has been invoked from thermodynamic considerations (10,19); H-bonding between urea and the other three bases is also possible (19). In the presence of urea, intrastrand pairing is not expected to be hampered more than reassociation of the opposing strands that certainly takes place even in the presence of urea (see Figure 2B, lane 5), so differences in base composition may lead to different 3D structures and different mobility even in the presence of the denaturing agent. The mobility of ds DNA

in gel electrophoresis is primarily dependent on strand size, and to a much lesser extent, the particular nucleotide sequence (21). The mobility of single strands, however, is considerably influenced by small changes in sequence. The sequence sensitivity of the 3D structures formed is the basis of the single-strand conformation polymorphism (SSCP) technologies applicable in the $\leq$300 bp range (22). Based on the above considerations, and in view of the similar interpretation of SSCP phenomena in the short fragment ranges, sequence-dependent conformational differences between the opposite strands are proposed to explain their differential migration in urea–agarose gels.

Analogous observations using alkaline denaturation have also been reported without being characterized with the purpose of general applicability (23,24). The novel application of urea–agarose gel electrophoresis is primarily recommended as an easy way to prepare ss probes. In addition, it offers a simple procedure for the strand-specific analysis of CpG methylation, discontinuities (including nicks) present in the DNA strands (see Figure 3, lanes 4–5) and separation of differentially labeled two strands for subsequent analysis. Our method is not influenced by the presence of alkali-sensitive sites and it can be utilized in various cell biological research areas, including the distinct mechanisms of leading and lagging strand DNA synthesis, the puzzle of 'immortal strand' hypothesis (25), analysis of strand-specific repair processes, the mechanism of imprinting leading to the mating-type switch in yeast (that was attributed to a lagging-strand-specific nick generated by unknown mechanism) (26–29), the analysis of topoisomerase-mediated cleavages and the mechanism involved in the generation of nicks upon class switch recombination at the immunoglobulin heavy chain region (30).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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