Benchmarks

Dye-Induced Denaturation of DNA Dissolved in Water

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Restricted or linearized DNA fragments are routinely used in recombinant DNA technology to produce desired DNA constructs for a variety of biological studies. Purified DNA fragments are commonly dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or purified water (pH 5.5–6.0) before further manipulations. DNA fragments, in terms of their physical structures, are generally thought to be stable in either solution for prolonged storage at 4°C. Agarose gel electrophoresis is routinely performed by laboratory personnel to separate or characterize DNA molecules. To carry

out agarose gel electrophoresis, two dyes, xylene cyanol FF (XC) and bromophenol blue (BPB) (Sigma Chemical, St. Louis, MO, USA and Merck Ltd., Darmstadt, Germany), are predominantly used as the visible components of the gel-loading buffers (8). These dyes are chosen because of their small and well-documented molecular sizes, behaviors and structures. Also, they are anionic and relatively inert. They are, therefore, suitable for tracing the migration of DNA samples on electrophoretic gels.

Under identical electrophoretic conditions, the migration rates of DNA fragments are normally dependent on their molecular sizes (8). However, we have repeatedly observed during routine gel electrophoresis that regular DNA fragments that were dissolved by reverse osmosis, deionized or dissolved in double-distilled, purified water (pH 5.5–6.0), but not TE buffer, and mixed

with the common gel-loading buffer containing 30% glycerol, 0.25% each of XC and BPB (hereafter termed water dye), as described by Sambrook et al. (8), migrated much faster than expected on the basis of their molecular size. Figure 1 shows that a variety of water-dissolved linear duplex DNA fragments with molecular sizes ranging from 1.7 to 4.5 kb, isolated from a variety of organisms, exhibited additional bands that showed faster electrophoretic migration if the DNA fragments were mixed with water dye (the even-numbered lanes), whereas those fragments mixed with EDTA dye (water dye supplemented with 50 mM EDTA) migrated normally (the odd-numbered lanes) relative to molecular marker migration rate. Thus, the fast-migration property vanished if the gel-loading buffers contained EDTA or if DNA fragments were dissolved in TE buffer prior to mixing with water dye for gel electrophoresis

(data not shown). In contrast, this phenomenon has never been observed when covalently closed circular (plasmid) DNA was under the identical electrophoretic conditions (data not shown). Moreover, the fast-migrating DNA molecules could be purified and converted back to the original conformation with regular mobility (data not shown). Interestingly, all fast-migrating DNA bands on the gel exhibited some properties of single-stranded (ss)DNA fragments. They were more diffuse, bound less ethidium bromide (EtdBr; Sigma Chemical) and migrated approximately twice as fast as their slow-migrating counterparts (Figures 1 and 2A).

To identify active components in the gel-loading buffers to induce the fast-migrating DNA bands, we prepared different buffers as previously described (8) with or without XC or BPB, mixed the buffers with the DNA fragments and analyzed DNA mixture behavior by agarose gel electrophoresis. The results revealed that either of the two dyes was

required for the conversion of DNA fragments to the fast-migrating DNA molecules, whereas the major components of the gel-loading buffers, such as glycerol, Ficoll® and sucrose, did not play a role in this process (data not shown). Many other organic dyes and chemicals (and their final concentrations, wt/vol), such as acridine orange (0.25%; Sigma Chemical), EtdBr (0.5 µg/mL), Evans blue (0.25%; Sigma Chemical), dichlorofluorescein (0.25%; Merck Ltd.) and Coomassie® Brilliant Blue R250 (0.25%; Sigma Chemical), were substituted for XC and BPB in the gel-loading buffer and also tested for their ability to induce the production of fast-migrating DNA molecules on a gel, but none possessed such capability (data not shown).

We then analyzed the nature of these fast-migrating DNA bands by asymmetric polymerase chain reaction (PCR) (6) and Southern hybridization (10). Asymmetric PCR of a known gene, the mouse *Egr1* cDNA (11), yielded two ssDNA bands (Figure 2A, lanes 3 and 4) that had gel electrophoresis mobilities similar to those of fast-migrating bands produced from the water-dissolved PCR product of the *Egr1* gene and mixed with water dye (Figure 2A, lane 2). This finding indicates that the faster-migrating DNA bands are ssDNA molecules derived from denaturation of linear double-stranded (ds) DNA. To further demonstrate that this is the case, DNA samples identical to those on the gel in Figure 2A were transferred to nitrocellulose membranes for Southern hybridization. One of these gels was treated according to the Southern blotting procedure, whereas the other was transferred without prior denaturation-neutralization treatments (10). As shown in Figure 2B, all DNA bands on the gel, which had been treated with denaturing agent, hybridized specifically to the ³²P-labeled probe (lanes 5–8), while only the ssDNA bands on the gel without prior denaturation were able to hybridize to the probe (lanes 1–4). Taking together the results of Figure 2, A and B, we conclude that the fast-migrating DNA bands must be ssDNA molecules that have been produced by dissociation of the DNA duplex in an XC- or BPB-induced denaturation (electrophoretic

Figure 1. Identical DNA fragments exhibit differential mobilities during agarose gel electrophoresis. Restriction enzyme-digested DNA fragments isolated from puffer fish (*Tetraodon fluviatilis*) chromosome (C. Li, unpublished data) (4.6 kb, lanes 1 and 2), hepatitis B virus (a gift from P.-J. Chen, National Taiwan University Medical School) (3.2 kb, lanes 3 and 4), linearized pBluescript® plasmid (Stratagene, La Jolla, CA, USA) (2.9 kb, lanes 5 and 6), *Hind*III-digested bacteriophage λ (2.3 kb, lanes 7 and 8), human ILF cDNA (3) (1.7 kb, lanes 9 and 10) and mouse *Egr1* cDNA (1.6 kb, lanes 11 and 12) were mixed with EDTA dye (odd-numbered lanes) or water dye (even-numbered lanes) and analyzed on EtdBr-containing agarose gels. When performing agarose gel electrophoresis, samples were prepared by mixing 10 µL of each DNA solution (approximately 50 ng of DNA) with 2 µL of appropriate 6×-concentrated gel-loading buffer (water dye or EDTA dye) and immediately loading onto a 1% agarose gel containing 0.5 µg/mL EtdBr. Electrophoresis was then performed in 1× TAE or TBE buffer until BPB reached two thirds of the gel length (8). Molecular weight marker (MW) was *Pst*I-digested *E. coli* bacteriophage λ; molecular sizes in base pairs of several DNA fragments are indicated at left of figure.

Benchmarks

dye-induced denaturation [EDID]) process.

It is well-known that denaturation of the DNA duplex requires treatment with either strong alkaline solution, urea, formamide or heat, which illustrates the stability of dsDNA in an aqueous solution. On the other hand, EDID is a mild and effective process (Figures 1 and 2) that occurs at room

temperature. It is also interesting that the addition of a variety of neutral salts and compounds (all purchased from Sigma Chemical) to certain concentrations (such as 10 mM MgCl₂, 10 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM sodium citrate, pH 7.0, 30 mM sodium acetate, pH 4.8, 10 or 35 mM 2-[*N*-morpholino]ethanesulfonic acid [MES], pH 5.5–6.7 and 10 or 35 mM 3-[*N*-

morpholino]propanesulfonic acid [MOPS], pH 6.5–7.9) abolished the ability of XC or BPB to denature DNA molecules (data not shown). Consistent with these findings, DNA fragments dissolved in TE buffer were protected from denaturation induced by XC and BPB. Therefore, the interactions between the catalysts and substrates seem to be so delicate that introduction of charged molecules destroys the interactions and causes changes in the physical structures of the DNA duplex.

To further analyze EDID, we used circular dichroism (CD) to monitor XC- or BPB-induced structural changes of the DNA fragments. We chose CD instead of the commonly used spectrophotometry for quantitation of EDID because XC and BPB absorb light strongly around 260 nm, which is also the wavelength most useful for detecting double- to single-stranded conversion (hyperchromicity) (1). The result is consistent with the findings from gel electrophoresis, asymmetric PCR and Southern hybridization analyses that were observed in the CD spectra of EDID-denatured DNA and unstacked (coiled) ssDNA molecules (1,2) (data not shown), indicating that the linear DNA duplex was disrupted into random single-stranded coils by these two agents.

Experiments undertaken in our laboratory revealed that room-temperature EDID of regular DNA fragments to fast-migrating DNA bands could be accomplished with all DNA fragments tested so far, the largest being a 23.1-kb *Hind*III-digested bacteriophage λ fragment (data not shown). This result suggests that EDID is a universal process that is independent of the size, origin and G/C composition, or nucleotide sequence of DNA. The results further compel a reevaluation of the use of water to dissolve DNA pellets and how to improve gel-loading buffers for gel electrophoresis. For example, many procedures for DNA manipulation (especially for handling genomic DNA in the human genome project and its related research) and commercial DNA purification kits recommend using water as the solvent to dissolve purified DNA fragments and analyze them by gel electrophoresis with a commonly used gel-loading buffer such as water dye. As demonstrated, these conditions

Benchmarks

Figure 2. Asymmetric PCR and Southern hybridization analyses of fast-migrating DNA. (A) Gel electrophoresis of the PCR products obtained by amplification of mouse *Egr1* cDNA with appropriate ratios of the specific primers (sense: 294–313, 5′-TCCGCTGCAGATCTCTGACC-3′ and antisense: 1284–1263, 5′-GGACTCGACAGGGCAAGCATAT-3′) (11). PCR was performed with an Omnigene® Thermal Cycler (Hybaid, Teddington, Middlesex, UK) programmed for 25 cycles at 95°C for 1 min, 55°C for 2 min and 72°C for 2 min, followed by an extension at 72°C for 10 min. 10 µL of each PCR product were mixed with 2 µL 6×-concentrated EDTA dye for electrophoresis. Order on gel: lane 1, PCR performed with equal molar primers (50 pmol each); lane 3, 50:1 pmol ratio of sense to antisense primers, respectively; and lane 4, 1:50 ratio of sense to antisense primers. On lane 2, an equal amount of amplified DNA from lane 1 was purified, dissolved in 10 µL water and mixed with 2 µL 6×-concentrated water dye. “DS” indicates position of dsDNA fragments on all lanes; “SS” represents ssDNA bands on lanes 2, 3 and 4. Molecular weight marker (in bp) is indicated at left of figure. (B) Two identical gels as in Panel A were blotted to nitrocellulose membranes for Southern hybridization. Labeling of 1.3-kb *Bgl*II-restricted mouse *Egr1* cDNA was performed with the Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN, USA) and [α - 32 P]dCTP (Amersham International plc, Little Chalfont, Bucks, England, UK). Approximately 10^6 counts per minute (cpm) of labeled DNA probe per mL of hybridization solution were used. Gel on left (lanes 1–4) was soaked in 20× standard saline citrate (SSC) immediately before blotting, whereas gel on right (lanes 5–8) was treated with denaturation and neutralization solutions (10) before transfer to membrane.

should give rise to EDID, resulting in additional fast-migrating bands. Thus, it is surprising to us that the dramatic alteration in DNA structure induced by the two dyes in the gel-loading buffer has escaped detection. To maintain and insure the original conformation of the DNA fragments during gel electrophoresis, the analyzed DNA should be dissolved in TE buffer, or the DNA solutions should only be analyzed with gel-loading buffer containing EDTA, because it is one of the additives that inhibit EDID.

BPB is a triphenylmethane compound, and other triphenylmethane derivatives are carcinogenic (7,9). Although BPB binds some cellular proteins (5,13), it is usually regarded and handled as nontoxic (4,12). Since this report demonstrated that XC and BPB possess DNA denaturation capability, these two compounds might need to be reassessed for their toxicity toward humans. We may also need to reconsider the safety procedures for laboratory personnel handling these compounds on a daily basis. Yet, because of the ability of XC and BPB to denature DNA fragments in water, EDID may be useful as an alternative to

boiling when denaturing radioactive probes in Southern hybridization (10). This alternative method, which would reduce the isotopic aerosol and other contaminants in the environment caused by boiling, is being tested now.

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