Protection of megabase DNA from shearing

Roger T. Kovacic¹, Luca Comai¹ and Arnold J. Bendich^{2,*}

Departments of ¹Botany and ²Botany and Genetics, University of Washington, Seattle, WA 98195, USA

Received July 28, 1995; Revised and Accepted August 29, 1995

Whereas intact chromosomal DNA molecules can be obtained using in-gel procedures (1), pipetting the DNA in solution shears these long molecules. We present a method with which condensed DNA can be manipulated in liquid medium without severely shearing it.

Two major forms of condensed DNA have been described. The ψ form of DNA (2,3) is induced by polyvinyl pyrrolidone or polyethylene glycol at high ionic strength. The circular dichroism (CD) spectrum of the ψ form is different from that of B-form DNA and the high concentrations of polymer and salt necessary for condensation should preclude ligation and restriction activities, rendering the ψ form undesirable for subsequent manipulation. The second form is cation-condensed DNA (4,5). The cations spermidine, spermine and $\text{Co}(\text{NH}_3)_6^{3+}$ (hexamine cobalt) at low concentration condense DNA into this form which has the CD spectrum of B-form DNA. Spermine has been used to protect lambda DNA (49 kb) from shearing (6). Cation-condensed DNA has the disadvantage that moderate concentrations of other cations such as Na^+ and Mg^{2+} will disrupt the complex, as do intercalating dyes such as ethidium bromide (7). We chose to use $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ because it offers fewer problems with aggregation of the DNA and sought conditions for both shear protection and enzyme compatibility.

Agarose gel slices (70 μl) containing DNA from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (1) and pea seedling nuclei (8) were equilibrated (six 1-ml changes) with 0.5 $\times$ TBE (Table 1) and then with other buffers including $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (three changes). Each slice was melted for 2 min at 65°C. Shearing was done by pipetting the liquid up and down either once or ten times through a standard 'yellow' pipette tip (0.51 mm orifice from Oxford) or a wide-bore tip (1.5 mm orifice from Phenix) attached to a 100 μl Pipetman pipetter. After reheating for 1 min, the liquid was transferred to a mold and hardened slices were (optionally) equilibrated to 0.5 $\times$ TBE before pulsed-field gel electrophoresis (PFGE). Ligation was in L (DNA ligation) Buffer (Table 1) + 0.1 mM ATP, and 0.5 mM DTT with 25 $\mu\text{g}/\text{ml}$ of lambda DNA and 1U T4 ligase (Boehringer) in 20 μl for 30 min at room temperature. Lambda DNA (0.5 μg) was digested at 37°C for 1 h with 1 U *EcoRI* in 21 μl of either *EcoRI* Buffer or 1, 10 and 100 U in LS (low-salt) *EcoRI* Buffer (Table 1). PFGE (BioRad CHEF-DR II in 0.5 $\times$ TBE) employed pulse times of 70 s for 15 h and 120 s for 12 h, or 3600 s for 96 h.

Pipetting once with a standard (narrow-bore) tip causes severe damage to chromosomal DNA of *S.cerevisiae* (Fig. 1A, lane 4), although not as much as pipetting ten times (lane 2). Ten pipettings with a wide-bore tip degrades the DNA almost as much as a single pipetting with a standard tip (lanes 5 and 4). With single pipettings and a wide-bore tip the chromosomal DNAs (200–2200 kb) were

largely preserved, but the larger DNAs showed some degradation (lanes 6 and 7). With the standard tip the degradation was more severe (lane 3) even when care was taken to pipette slowly. Use of a wide-bore tip avoids much of the shear degradation.

Table 1. Optimum $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ concentration for protection of DNA from shear damage

Buffer (composition)	$\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (mM)
0.5 $\times$ TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0)	0.06
TE (10 mM Tris, pH 8.0, 1 mM EDTA)	0.4
L (25 mM Tris-acetate pH 7.5, 5 mM MgCl_2)	0.4
<i>EcoRI</i> (50 mM Tris pH 8.0, 10 mM MgCl_2 , 100 mM NaCl)	6.0
LS <i>EcoRI</i> (25 mM Tris pH 8.0, 5 mM MgCl_2 , 20 mM NaCl)	0.6

We determined the protective concentration of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ to the nearest order of magnitude in each of five buffers (Table 1). Figure 1B shows the results for 0.5 $\times$ TBE and TE buffers. The lowest protective concentration is 0.1 mM for 0.5 $\times$ TBE (lane 5) and 1.0 mM in TE (lane 9). Separate experiments (not shown) progressively narrowed the protective concentrations. Table 1 summarizes the results which indicate that $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ protects DNA at salt concentrations compatible (see below) with restriction and ligation reactions. The 0.5 $\times$ TBE Buffer appears anomalous because hexamine cobalt chloride protects at very low levels in a buffer of moderate ionic strength. Perhaps the absence of strongly ionic cations and/or the size (charge density) of the cation is the reason that 0.5 $\times$ TBE Buffer is not more disruptive of the $\text{Co}(\text{NH}_3)_6$ -DNA complex. We infer that protection results from condensation of the DNA molecules. Widom and Baldwin (4) observed an increase in light scattering (a measure of the particle size for condensed DNA) at millimolar salt and micromolar $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ concentrations. At our higher salt concentrations the shear protective effect of $\text{Co}(\text{NH}_3)_6^{3+}$ may not require the cation to be bound as tightly as for the light scattering relationship.

There are two puzzling effects that we have observed. One is the apparent increased sensitivity of the DNA to shear at subprotective concentrations of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$: the DNA becomes sheared to smaller sizes than does unprotected DNA subjected to the same treatment. This effect occurs in all five buffers tested and is exemplified in Figure 1B (compare lanes 7 and 8 with 2). At concentrations below that causing cation-condensation of DNA, spermine is thought to decrease the flexibility of DNA molecules (9,10). Such a stiffening effect on DNA by a low level of

* To whom correspondence should be addressed

Figure 1. (A) Effect of bore size and manner of pipetting on shearing. DNA from YNN295 chromosomes was sheared in 0.5x TBE. Lanes 1 and 8, unsheared; lane 2, sheared 10 times, standard tip; lane 3, slow pipetting once, standard tip; lane 4, sheared once, standard tip; lane 5, sheared 10 times, wide-bore tip; lane 6, slow pipetting once, wide-bore tip; lane 7, sheared once, wide-bore tip. (B) Effect of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ concentration on shearing in 0.5x TBE and TE. YNN295 chromosomes were pipetted ten times using a standard tip. Samples 1–6, 0.5x TBE; samples 7–10, TE. The concentrations of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ in lanes 3–10 are given. Lane 1, control DNA, no shear; lane 2, control DNA, sheared; lane 3, 0.001 mM; lane 4, 0.01 mM; lane 5, 0.1 mM; lane 6, 1 mM; lane 7, 0.01 mM; lane 8, 0.1 mM; lane 9, 1 mM; lane 10, 10 mM. (C) Effect of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ on shearing of pea DNA in 0.5x TBE. Shearing was done by pipetting ten times using a standard tip. The concentrations of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ in lanes 5–10 are given. Lane 1, YNN295 marker DNA; lane 2, *S.pombe* marker DNA; lane 3, unsheared pea DNA; lane 4, sheared pea DNA control; lane 5, 0.001 mM; lane 6, 0.01 mM; lane 7, 0.1 mM; lane 8, 1.0 mM; lane 9, 10 mM; lane 10, 100 mM. DNA length in megabase pairs is shown for A, B and C.

$\text{Co}(\text{NH}_3)_6\text{Cl}_3$ could account for the increased susceptibility to shear that we observe. As for the second effect, although $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ protects large DNAs from shearing, this protective effect is not uniform with superoptimal concentrations of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$. At higher levels of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ gel bands representing chromosomal DNAs become more faint with the diminution greater for the larger chromosomes. A comparison of lanes 5 with 6, and 9 with 10 in Figure 1B shows the effect which is even more pronounced at higher levels of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (data not shown) where it appears that the largest DNA has been sheared to a smear of smaller size fragments. The rate at which DNA condensation occurs increases dramatically with the $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ concentration (4,11). Perhaps rapidly condensed DNA is not as compact as when more slowly condensed and this could explain the superoptimal effect we observe.

In order to establish the minimum protective concentration of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ on even larger DNAs we used nuclear DNA from pea seedlings. Lane 7 of Figure 1C shows that 0.1 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ is the lowest protective concentration. This is the same minimum concentration that protected *S.cerevisiae* DNA in 0.5x TBE indicating that the protective concentration is not dependent on the initial size of the DNA. Protection of very large DNAs by the hexamine cobalt cation will likely be minimal since fewer of the DNA molecules are protected for the large *S.cerevisiae* chromosomes than the small ones (compare the large and the small sized bands for unsheared and sheared/protected lanes in Figure 1A and B). Nevertheless, some DNA molecules as large as 4–5 megabases are protected (Fig. 1C, lanes 7–10).

Intermolecular DNA ligation can be stimulated by 1 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (12). Using *EcoRI* restriction fragments of lambda DNA we found that this stimulation was greater with 1.0 than 0.8 mM; 0.6, 0.4 and 0.2 mM did not affect the ligation reaction (data not shown). Since 1 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ in L buffer was nearly as effective in shear protection as the optimum concentration of 0.4 mM (Table 1), 1 mM might be best for ligating long DNA molecules.

We also tested the effects of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ on *EcoRI* restriction enzyme. No inhibition was observed at 0, 1, 2, 4 or 6 mM in *EcoRI* Buffer; inhibition was observed at 8 and 10 mM. The minimum protective concentration was 6 mM, offering little margin for variation of the $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ concentration. In an attempt to widen the gap between the protective and the inhibitory concentrations we adopted LS *EcoRI* Buffer. This change resulted in cleavage at

degenerate sites (star activity). When we tested $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ for inhibition, we observed that the star activity had disappeared. This result is in agreement with the findings of Pingoud (13) that spermidine suppresses cleavage at star sites. At 0.4 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ the enzyme was uninhibited; at 0.6 mM there was partial inhibition. The partial inhibition at 0.6 mM could be overcome by the addition of 10- or 100-fold amounts of enzyme without the return of star activity. Since the optimal degree of protection was somewhat greater in LS *EcoRI* than *EcoRI* Buffer, we favor LS *EcoRI* Buffer with 0.6 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ for doing *EcoRI* digests.

Protection of long DNA molecules from yeast and pea indicates that $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ is generally useful for preserving large DNAs in solution. In each of the five buffers tested protection works well, although the protective concentration varies due to their ionic strengths. Protection occurs at low enough concentrations that DNA-dependent enzymes such as ligase and *EcoRI* function normally. Therefore $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ would aid in preparing very large inserts during the partial digestion and ligation steps required for cloning DNA into yeast artificial chromosomes.

ACKNOWLEDGEMENTS

This work was supported by grants 92-37300-7677 from the USDA and R55HG00539 from the NIH.

REFERENCES

- Birren, B and Lai, E. (1993) *Pulsed Field Gel Electrophoresis: A Practical Guide*. Academic Press, San Diego, CA.
- Lerman, L. S. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1886–1890.
- Maniatis, T., Venable, J. H. Jr. and Lerman, L. S. (1974) *J. Mol. Biol.* **84**, 37–64.
- Widom, J. and Baldwin, R. L. (1980) *J. Mol. Biol.* **144**, 431–453.
- Widom, J. and Baldwin, R. L. (1983) *Biopolymers* **22**, 1595–1620.
- Kaiser, D., Tabor, H. and Tabor, C. W. (1963) *J. Mol. Biol.* **6**, 141–147.
- Widom, J. and Baldwin, R. L. (1983) *Biopolymers* **22**, 1621–1632.
- Luthe, D. S. and Quatrano, R. S. (1980) *Plant Physiol.* **65**, 305–308.
- Marquet, R., Houssier, C. and Fredericq, E. (1985) *Biochim. Biophys. Acta* **825**, 365–374.
- Marquet, R., Wyart, A. and Houssier, C. (1987) *Biochim. Biophys. Acta* **909**, 165–172.
- Bloomfield, V.A., He, S., Li, A-Z. and Arscott, P.G. (1992) In Harding, S.E., Sattell, D.B. and Bloomfield, V.A. (eds), *Laser Light Scattering in Biochemistry*. The Royal Society of Chemistry, Cambridge, pp. 320–333.
- Rusche, J.R. and Howard-Flanders, P. (1985) *Nucleic Acids Res.* **13**, 1997–2008.
- Pingoud, A. (1985) *Eur. J. Biochem.* **147**, 105–109.