ELSEVIER

Journal of Chromatography B, 714 (1998) 21–27

JOURNAL OF CHROMATOGRAPHY B

High-resolution capillary electrophoretic separation of supercoiled plasmid DNAs and their conformers in dilute hydroxypropylmethyl cellulose solutions containing no intercalating agent

David T. Mao[a,*], Joshua D. Levin[b], Liying Yu[a], Roy M.A. Lautamo[a]

[a] *J&W Scientific, 91 Blue Ravine Road, Folsom, CA 95630, USA*
[b] *Life Technologies Inc., Frederick, MD 21704, USA*

Abstract

The three conformers of plasmid pBR322, linear, supercoiled and nicked circular forms, were separated by capillary electrophoresis (CE) in 0.1% hydroxypropylmethyl cellulose (HPMC) solution in the absence of intercalating agents and the migration order was confirmed by co-migration of enzymatically prepared corresponding DNAs. The previously observed broad peaks of supercoiled DNAs in CE are results of unresolved peaks of topoisomers which differ only in the degrees of twisting. We have demonstrated the separation of an artificial topoisomer ladder made from pBR322 and topoisomerase I. The population of topoisomers of a supercoiled DNA is dependent on sample matrices and separation conditions.

Keywords: Topoisomers; DNA

1. Introduction

Many advances in modern molecular biology and biotechnology are essentially built on the recombinant technology of plasmid DNAs. Plasmids are circular bacterial DNAs which exist naturally in negatively twisted (supercoiled) forms. The supercoiling of plasmids has been associated with many critical biological processes, such as transcription, replication and recombination [1]. Plasmid DNA,which replicates independent of the chromosome, is ideal for the insertion and replication of desired DNA sequences. More recently, new approaches in gene therapy focus on the use of plasmid DNA for in vivo delivery, such as naked DNA vaccines and lipid-mediated gene transfers [2,3]. These developments have led to the increased interest in the purification and characterization of plasmid DNAs as therapeutic agents [4].

Capillary electrophoresis (CE) in polymer solutions has been used for the separation of supercoiled plasmid DNA and its major impurities, namely linear and nicked circular species [5–8]. Recently, Hammond et al. [9] separated the three plasmid conformers (linear, supercoiled and nicked circular forms) in dilute hydroxyethyl cellulose (HEC) solutions with concentrations below the entanglement point of HEC. The results are better resolved peaks and faster analysis time. The migration order of the nicked circular form and supercoiled form was reversed when compared to other reports. The authors also

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00069-3

noticed that the peak of a supercoiled DNA was significantly broader than that of a linear DNA of the same size. They suggested a different migration dynamics as the explanation for the unusual migration behavior of supercoiled DNAs based on the videomicroscopy evidence of DNA molecules migrating through a polymer solution. In all reported studies, intercalating agents such as ethidium bromide were invariably included in the separating solutions. The insertion of the planar ethidium bromide in supercoiled DNA was found to relax and distort the molecule, thus making the separation of topoisomers, which only different in the degrees of twisting, an unrealizable task. This difficulty was pointed out by Heller [10] in his effort to analyze plasmid DNAs by CE.

In this report, we use CE and dilute hydroxypropylmethyl cellulose (HPMC) solution (0.1% HPMC) in the absence of any intercalating agent to separate the supercoiled form from linearized and nicked forms found in a typical pBR322 plasmid sample. The migration order of these three forms, linear, supercoiled, followed by nicked circular forms, were confirmed by the co-migration of enzymatically prepared corresponding forms. Earlier studies of plasmid DNA samples using the electron microscopy [11] and two-dimensional slab-gel electrophoresis [13] suggested that a supercoiled DNA might exist in multiple topological forms. We believe that the previously observed broad peak width of supercoiled DNA is a result of unresolved peaks of topoisomers. Since the topoisomers of supercoiled DNA had not been separated previously by CE, an artificial topoisomer ladder of pBR322 was generated using a known nicking-closing enzyme, topoisomerase I. The separation of thus prepared topoisomers was carried out in a 0.1% HPMC solution containing no intercalating agent. Furthermore, under optimized conditions, topoisomers of individual supercoiled DNA in a commercial available supercoiled DNA standards were revealed. The observation that the larger supercoiled DNAs contain more topoisomers is consistent with the broader peak width previously reported [9]. The population of the topoisomers observed on CE are dependent on sample matrix and CE conditions, therefore it may not represent the actual supercoiled DNA at its natural state.

2. Experimental

2.1. Materials

pBR322 Plasmid DNA (1.3 mg/ml) was purchased from Sigma (St. Louis, MO, USA). All other biochemicals including supercoiled DNA ladder, *DNase* I, *Ava* I and topoisomerase I were obtained from Life Technologies (Gaithersburg, MD, USA). The polymer HPMC of a viscosity of 4000 cP at 2% was purchased from Sigma. Tris(hydroxylmethyl)aminoethane (Tris) and boric acid are products of Fluka (Milwaukee, WI, USA). Ethylenediaminetetraacetic acid (EDTA, disodium salt) was purchased from Aldrich (Milwaukee, WI, USA). The deionized water was generated from a Nanopure Ultrapure Water System by Barnstead.

2.2. DNA standards

Linear pBR322 was prepared by the digestion of DNA with *Ava* I following the manufacturer's suggested procedure. The final DNA concentration was 0.13 μg/μl.

The nicked circular pBR322 was prepared by incubating pBR322 plasmid and diluted *DNase* I (~50 pg/μl) at 37°C in the manufacturer's reaction buffer. The reaction was stopped after 20 min by adding EDTA. The final DNA concentration was 0.21 μg/μl.

The artificial topoisomer ladder of pBR322 was generated by reacting the pBR322 plasmid with topoisomerase I in the manufacturer's suggested reaction buffer (50 m*M* Tris–HCl, pH 7.5, 59 m*M* KCl, 10 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.5 m*M* DTT, 30 μg/ml BSA). The final DNA concentration was 0.26 μg/μl.

All the above DNA samples were dialyzed against deionized water at 4°C to remove excess salts using the following procedure. A drop of DNA sample (15 μl) was applied to the top of a membrane (Millipore VSWP 025 00) and floated on deionized water with the shiny side up. After about 20 min, the sample was piped to a vial for CE analyses.

2.3. Separation buffer

The separation buffers were prepared by adding appropriate amount of HPMC in 1×TBE (89 m*M*

Tris, 89 m*M* boric acid and 2 m*M* EDTA). The mixture was stirred initially at 70°C for 2 h then at room temperature overnight. The solution, thus prepared, looks clear and homogeneous but can not be used right away because it generates tailing peaks and poor resolution. This poor performance was related to polymer relaxation. The polymer requires some time to relax to reach true homogeneity. For the best result, the buffer is allowed to stay at room temperature for at least 24 h without disturbing such as agitation and transferring. This polymer solution is very stable. It can be used for at least six months if stored in a refrigerator.

2.4. Capillary electrophoresis

All experiments were performed on an instrument constructed in-laboratory. This instrument consists of a high-voltage power supply (Bertan, Model 230) and an UV absorbance detector (Model UVIS 200) from Linear (Reno, NV, USA). Detection wavelength was set to 260 nm. Sample was introduced by an electrokinetic injection at 7.5 kV for 5 s. A fan was installed to cool the capillary by forced air. Room temperature is around 21–23°C in our laboratory. A personal computer was used for data collection and calculation. The data acquisition board (Chrom-1 AT) and software were obtained from Galactic Industries Corporation (Salem, NH, USA). The capillary, μSil-FC (p/n 194-8111), 50 μm I.D, was obtained from J&W Scientific (Folsom, CA, USA).

3. Results and discussion

3.1. Linear, supercoiled and nicked circular plasmid DNAs

Plasmids are widely used for the manipulation of desired DNA sequences in recombinant DNA technology. Plasmid DNA is frequently purified from crude cell extracts for further molecular studies. Plasmids exist predominately in the supercoiled form and the major impurities due to degradation are linearized and nicked circular forms. These three forms have been separated by CE in a 0.1% HPMC solution in the absence of intercalating agent.

It is essential to remove excess salts in the sample in order to achieving the stacking effect for better resolution. The plasmid samples were dialyzed on a float membrane before injection. Fig. 1 shows an electropherogram of a typical sample of pBR322 (4.3 kb) (Fig. 1B). This sample consists of a major component as a broader peak and several minor components. The major component was confirmed to be the supercoiled pBR322 by comparing it electrophoretically with the supercoiled DNA ladder which consists of 11 supercoiled DNAs with size ranging from 2 to 16 kb (Fig. 1A). Two of the minor components were identified as linear and nicked circular forms of the pBR322 by comparing their

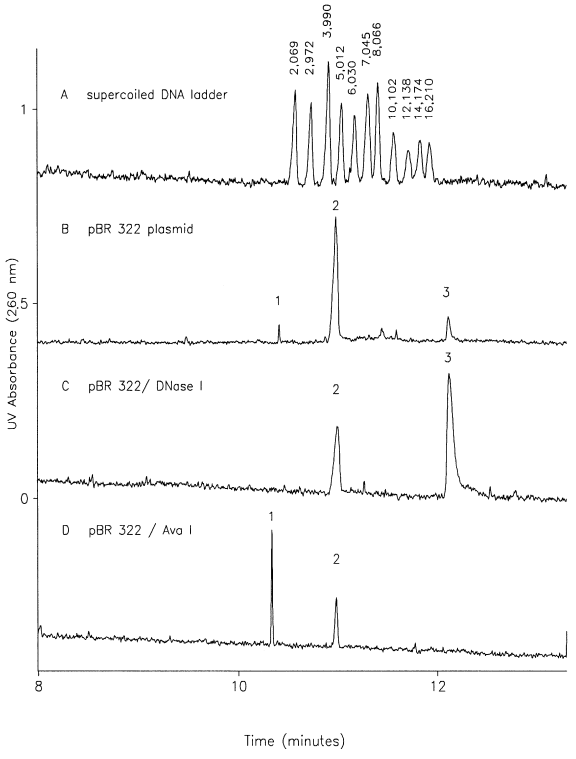

Fig. 1. Separation of linear, supercoiled and nicked circular pBR322 DNAs. (A) Supercoiled DNA ladder, (B) pBR322 plasmid DNA, (C) pBR322 plasmid DNA treated with diluted DNase I, (D) pBR322 plasmid DNA digested with *Ava* I. Peak 1=linear form, peak 2=supercoiled form, peak 3=nicked circular form. Conditions: μSil-FC capillary, 50 cm (20 cm to the detector)×50 μm I.D., 0.1% HPMC in TBE, −6.0 kV, room temperature. Electrokinetic injection at 7.5 kV for 5 s. All samples were dialyzed over a float membrane for 20 min at 4°C.

migration times with that of enzymatically prepared DNA standards. The linear form of pBR322 was prepared by digestion of pBR322 with a restriction enzyme, *Ava* I, which cleaved a single site on this plasmid. A diluted *DNase* I (~50 pg/μl) will nick or break one chain of the double-stranded DNA and the supercoiled DNA will unwind completely to the relaxed circular form. Both reactions were stopped before their completion, therefore the unreacted plasmid can be used as a reference. The linearized pBR322 (Fig. 1D), prepared as above, co-migrated with the peak 1 and the nicked and relaxed circular pBR322 (Fig. 1C) co-migrated with the peak 3. Therefore, the migration order of these three forms is linear, supercoiled and nicked circular forms. There is no general agreement on the migration order of these three forms in CE found in literature. This disagreement is partially due to the uses of different intercalating agents and their concentrations in different studies. However, in most cases, the relaxed circular form is the slowest-migrating form of a plasmid DNA because its ''floppy'' molecular shape impedes movement through the polymer solution. A similar slow migration of supercoiled DNA was observed in slab agrose electrophoresis.

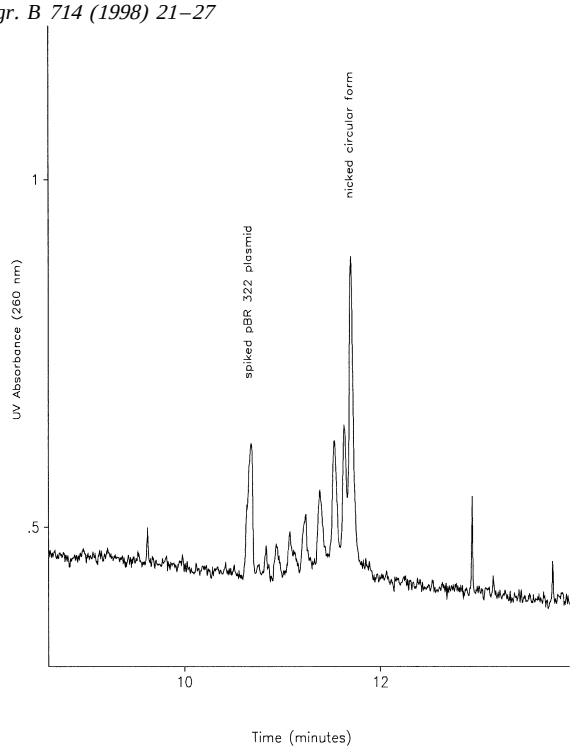

Fig. 2. Separation of topoisomers of pBR322. Topoisomers generated by using topoisomerase I were co-injected with the original pBR322 plasmid. Separation conditions as in Fig. 1.

3.2. Topoisomers

Electron micrographs of plasmids extracted from *E. coli* clearly showed that multiple topological forms, other than linear and nicked circular forms, co-existed in that sample [11]. Those topological forms were of similar twisted circular form but the degrees of the twisting were different. Since the separation of topoisomers of supercoiled DNA by CE has not been reported in the literature, an artificial topoisomer ladder of pBR322 was generated using a known nicking-closing enzyme, topoisomerase I, in order to demonstrating the feasibility. This enzyme is able to make a nick in one of the polynucleotides of a double-stranded DNA, relax the molecule by a few turns and then close the nick, thus creating a distribution of toposiomers. In a 0.1% HPMC solution without intercalating agent, these topoisomers were conveniently separated by CE with the last peak being the relaxed circular form (Fig. 2). All topoisomers migrated between the parent pBR322 and its nicked circular form. A similar topoisomer pattern was also obtained for a ϕx174 RF plasmid DNA (data not included).

3.3. Parameters affecting CE separation of topoisomers

The supercoiling of DNA is dependent on temperature, counter-ion concentrations and the binding of intercalating agents [14]. The characteristic broad peaks of plasmid DNAs observed by others could be a result of unresolved multiple peaks of the topoisomers. Therefore, under optimized conditions, they could be separated. A commercial supercoiled DNA ladder, which consists of 11 plasmids with sizes ranging from 2 to 16 kb, was chosen for this study.

3.3.1. Salt concentration in DNA samples

In a typical experiment, the supercoiled DNA ladder was diluted three-fold in deionized water

before injection. As shown in Fig. 3A, each supercoiled DNA started to show a multiple peak pattern. The larger DNAs contain more topological forms. Some of which are barely resolved. Conversely, if the DNA sample was dialyzed to remove most salts before the CE separation, sharper peaks resulted (Fig. 3B). The number of topoisomers was apparently reduced or not separable under these conditions. Similar sharper peaks of this supercoiled DNA ladder were also observed in the presence of ethidium bromide in the running buffer (Fig. 3C). It is well-established that the insertion of planar ethidium bromide changes the conformation of supercoiled DNAs [14]. Therefore, it is not unreasonably to believe that some sort of conformation changes also occur when the supercoiled DNA is exposed to a low salt environment.

3.3.2. HPMC concentration

A solution of 0.1% HPMC in TBE buffer is suitable for separation of most plasmid DNAs in the supercoiled DNA ladder. As the concentration was slightly increased to 0.15%, the larger DNAs were no longer separable (see Fig. 4). This effect of polymer concentration was also observed on the polyethylene glycol (PEG)-based polymers (data not included).

3.3.3. Applied voltage

One of several parameters which improves resolution in CE is applied voltage. According to an equation derived by Jorgenson and Lukacs [12], the resolution is proportional to the square root of applied voltage. Therefore, increases in applied voltage will increase resolution until the Joule heat-

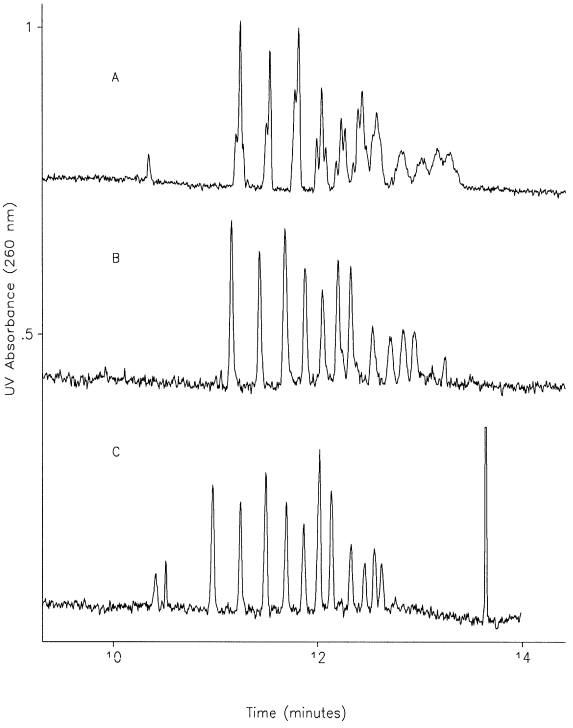

Fig. 3. Effect of salt concentration in the sample matrix on the population of topoisomers. (A) Supercoiled DNA ladder was diluted three-fold in d.i. water, (B) same sample was dialyzed for 30 min at 4°C to remove excess salts. Separation conditions as in Fig. 1. (C) Same sample separated in 0.1% HPMC containing 10 μ*M* ethidium bromide.

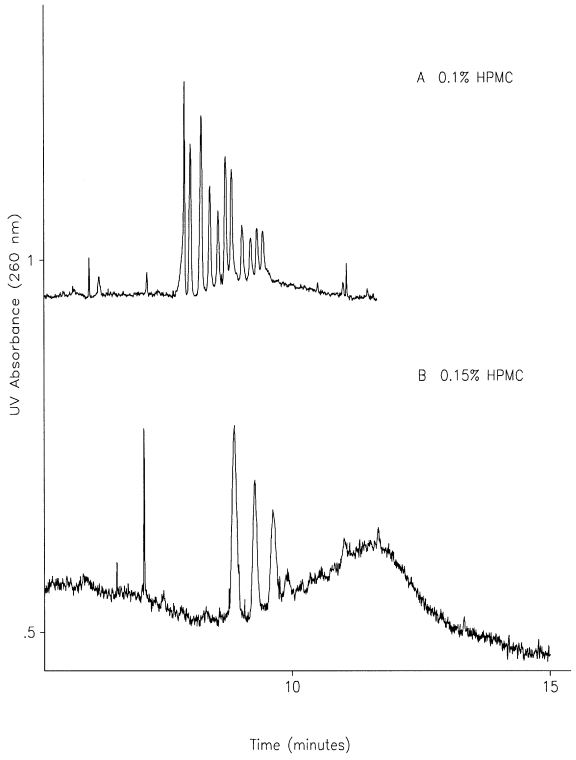

Fig. 4. Effect of HPMC concentration. (A) Supercoiled DNA ladder in 0.1% HPMC–TBE solution, (B) in 0.15% HPMC–TBE solution. Separation conditions: μSil-FC capillary, 60 cm (20 cm to the detector)×75 μm I.D., 9 kV. Other conditions as in Fig. 1.

ing becomes a problem. In a 60 cm fluorocarbon-coated capillary filled with 0.1% HPMC in TBE buffer, CE separations of a supercoiled DNA ladder were carried out at various applied voltages ranging from 6 to 12 kV. In general, the peak efficiency increases as the applied voltage increases as expected. For example, the separation efficiency of the 2.1 kb plasmid (the first peak from left) increases from 400 000 plates at 6 kV to 690 000 plates at 10 kV. Maximum resolution was achieved at about 10 kV (~160 V/cm) (Fig. 5). At that applied voltage, several of the larger plasmids (>7 kb) were beginning to show multiple peak patterns. Although the heat may alter the conformation of supercoiled DNA as described by Pulleyblank et al. [15], the current generated during CE separations was never over 10 μA. With air cooling, this heat will not be a contributing factor influencing the conformational changes of supercoiled DNAs.

4. Conclusions

CE using dilute polymer solutions has been an effective technique for the separation of various conformers of supercoiled DNAs. Linear and nicked circular forms were separated from its supercoiled parent. An artificially generated topoisomer ladder of pBR322 was separated for the first time by CE. The CE separation of supercoiled DNA could be influenced by sample matrices and separation conditions. In the future, CE could be a useful tool for the study of conformational changes of supercoiled DNAs exposed in different environments.

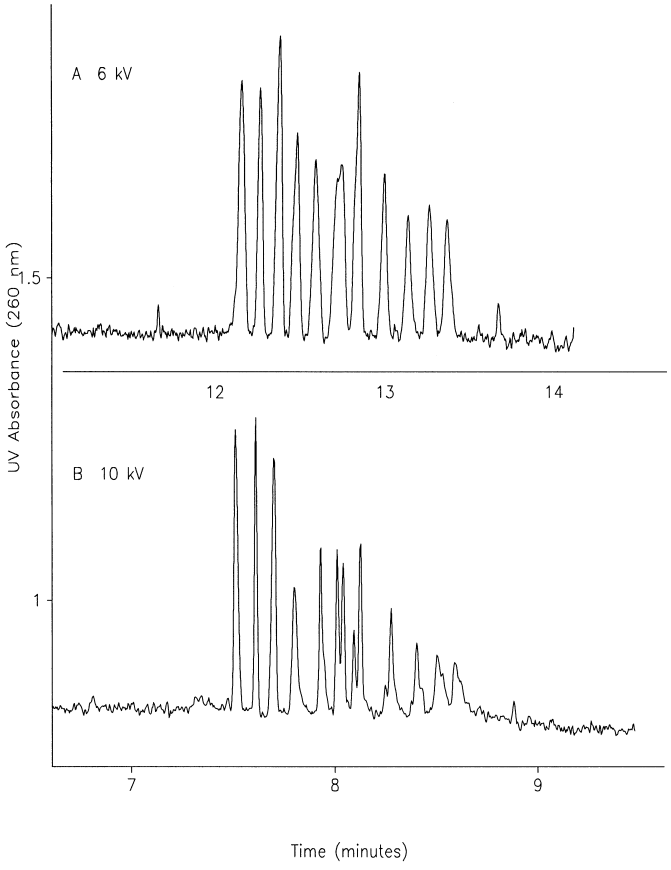

Fig. 5. Effect of applied voltage on separation of supercoiled DNA ladder. Supercoiled DNA ladder was dialyzed and the final concentration was 0.1 μg/μl. Separation conditions: μSil-FC capillary, 60 cm (20 cm to the detector)×50 μm I.D., 0.1% HPMC in TBE, room temperature.

Acknowledgements

We wish to thank Dr. Christoph Heller of the Max-Planck Institute for Molecular Genetics in Berlin-Dahlem, Germany for his helpful discussion and comments.

References

[1] N.R. Cozzarelli, Science 207 (1980) 953.
[2] J.M. Leiden, New Engl. J. Med. 333 (1995) 871–873.
[3] J.A. Wolff, R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, P.L. Felgner, Science 247 (1990) 1565–1568.
[4] M. Marquet, N.A. Horn, J.A. Meek, BioPharm May (1997) 42–50.
[5] P. Bocek, A. Chrambach, Electrophoresis 12 (1991) 620–623.
[6] Z. Nackerdien, S. Morris, S. Choquette, B. Ramos, D. Atha, J. Chromatogr. B 683 (1996) 91–96.
[7] K. Hebenbrock, K. Schugerl, R. Freitag, Electrophoresis 14 (1993) 753–758.
[8] B.C. Courtney, K.C. Williams, Q.A. Bing, J.J. Schlager, Anal. Biochem. 228 (1995) 281–286.
[9] R.W. Hammond, H. Oana, J.J. Schwinefus, J. Bonadio, R.J. Levy, M.D. Morris, Anal. Chem. 69 (1997) 1192–1196.
[10] C. Heller (Editor), in Analysis of Nucleic Acids by Capillary Electrophoresis, Vieweg, 1997, p. 303.
[11] W.R. Bauer, F.H.C. Crick, J.H. White, Scientific Am. 243 (1980) 118–133.
[12] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[13] R. Bowater, F. Aboul-Ela, D.M.J. Lilley, Methods Enzymol. 212 (1992) 105–121.
[14] J.C. Wang, Trends Biochem. Sci. 5 (1980) 219–221.
[15] D.E. Pulleyblank, M. Shure, D. Tang, J. Vinograd, H. Vosberg, Proc. Nat. Acad. Sci. USA 72 (1975) 4280–4284.