Journal of Chromatography, 296 (1984) 379–385
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 346

INCREASE OF CLONING EFFICIENCIES BY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-PURIFIED VECTORS AND LINKERS

RALPH DORNBURG*
Max-Planck-Institut für Biochemie, 8033 Martinsried (F.R.G.)
PETER FÖLDI
LKB Instruments GmbH, 8032 Gräfelfing (F.R.G.)
and
PETER HANS HOFSCHNEIDER
Max-Planck-Institut für Biochemie, 8033 Martinsried (F.G.R.)

SUMMARY

High-performance liquid chromatography (HPLC) was applied to the purification of vector and linker DNAs used in molecular cloning experiments. The data clearly demonstrate that HPLC (Blue Column from LKB/TSK G 4000 SW) based on steric exclusion is a very rapid and convenient method for the size seperation of DNA fragments that are virtually free of any contamination. Application of vector and linker DNAs, purified by this method, enabled us to clone nanogram amounts of avian myeloblastosis virus RNA, which was used for the evaluation of a recently described cloning procedure.

INTRODUCTION

Molecular cloning of a DNA complementary to a specific mRNA (cDNA), has become an important tool in modern biology. Indeed, the development of cloning techniques[1–3] paved the way for the understanding of eukaryotic gene organization, arrangement and expression[4–9]. However, very often the RNA of interest is available only in nanogram amounts and/or cannot be separated from large amounts of contaminant RNA species with the usual techniques. Further, it was very difficult and time consuming to clone such small amounts of mRNA, and full-length cDNA clones in particular were extremly difficult to obtain. This difficulty was overcome by the introduction of a very elegant and highly efficient cloning procedure by Okayama and Berg[10], who used a bacterial plasmid as vector-primer for cDNA synthesis and a special linker for the required vector circularization. Both vector-primer and linker have to be separated from other DNA fragments which result from preceding preparatory steps. However, the widely used electrophoretic elution of DNAs from agarose gels mostly yields DNA probes contaminated with agarose materials, which hamper or completely inhibit enzyme reactions. Recently, it was shown that viroid

0021-9673/84/$03.00

RNA and other small RNAs can be rapidly purified by high-performance liquid chromatography (HPLC), based on steric exclusion[11,12], and this technique can also be used for DNA fragments[13]. We have applied an HPLC method to the purification of these cloning vector and linker molecules and obtained recombinant DNA of high biological activity in molecular cloning experiments.

EXPERIMENTAL

Materials

Restriction enzyme Hind III was purchased from New England BioLabs and Pst I and Hpa I were products of Boehringer Mannheim. Terminal transferase, *E. coli* DNA-ligase and RNase H, oligo(dA)- and oligo(dC)-cellulose (both type 7) were supplied by PL Biochemicals. *E. coli* DNA polymerase I was purchased from New England Nuclear. Reverse transcriptase (avian myeloblastosis, AMV-enzyme) and AMV-virus were obtained from J. Beard Life Sciences. Radioactive nucleotides were supplied by Amersham Buchler.

Methods

Preparation of vector and linker plasmid. The vector-primer and the linker plasmid were constructed from pBR322 and SV 40 DNA as described[10], and are called here PSV (*p*BR322-*S*V40-*v*ector) and PSL (*p*BR-*S*V40-*l*inker), respectively.

Preparation of dT-tailed vector-primer and dG-tailed linker. A 100-μg amount of PSV was digested with Kpn I endonuclease, 60–80 deoxythymidylate (dT) residues were added to the 3'-termini with terminal transferase and the DNA was digested with Hpa I endonuclease, as described[10]. For the preparation of the linker, 200 μg of PSL were digested with Pst I endonuclease, 12–15 deoxyguanosylate (dG) residues were added with terminal transferase and the DNA was digested with Hind III endonuclease. Vector and linker DNAs were further purified by affinity chromatography on oligo(dA) and oligo(dC)columns, as described[10]. The eluates were adjusted to 10 m*M* Tris (pH 7.4), 1 m*M* EDTA and 2 *M* ammonium acetate, and the DNA was precipitated with 2 volumes of ethanol at -20°C.

Separation of DNA mixtures. HPLC gel filtration was performed at room temperature with an LKB chromatography system, consisting of an UltroPac TSK G 4000 SW column (60 × 0.75 cm I.D.), an HPLC pump (LKB 2150), a SuperRac fraction collector and a UV monitor (LKB, VW-monitor). The chromatography buffer contained 10 m*M* Tris (pH 7.4), 0.5 m*M* EDTA and 50 m*M* NaCl. The DNA samples (100 μg) were dissolved in 50 μl of water, and up to 10 μl of the solution were loaded on the column through a Rheodyne injector. Elution was performed with a constant flow-rate of 0.1 ml/min, and 0.1-ml fractions were collected. The effluent was monitored by UV absorption at 260 nm. The peak fractions were pooled and the DNA was precipitated with 2.5 volumes of absolute ethanol at -20°C. The linker solution was concentrated by lyophilization and directly used in the cloning procedure.

Isolation of AMV-RNA. The AMV-RNA was purified from the virus pellets by phenol extraction and ethanol precipitation by standard techniques[14].

Cloning of mRNA. All enzymatic reactions were carried out as described by Okayama and Berg[10]. For transformation we used *E. coli* strain C 600. Individual

colonies were investigated by *in situ* colony hybridization[15] or by the method described by Birnboim and Doly[16].

Agarose gel electrophoresis. Gel electrophoresis was performed in 1% agarose gels in a buffer containing 36 m*M* Tris–HCl, 33 m*M* NaH_2PO_4 and 10 m*M* EDTA (pH 7.5). After electrophoresis (300 mA for 2 h) the gel was stained with ethidium bromide (1 μg/l) and the DNA bands were electrophoretically eluted into a buffer containing 50 m*M* Tris–HCl (pH 7.5), 5 m*M* EDTA and 50 m*M* NaCl. The DNA-containing solution was extracted twice with buffer-saturated butanol and twice with phenol–chloroform, and was finally precipitated with ethanol at −20°C. The pellet was dissolved in 50 μl of Tris–EDTA buffer (10 m*M* Tris–HCl, 1 m*M* EDTA; pH 7.5), 50 μl of 4 *M* ammonium acetate were added and the DNA was reprecipitated with 2 volumes of ethanol.

RESULTS

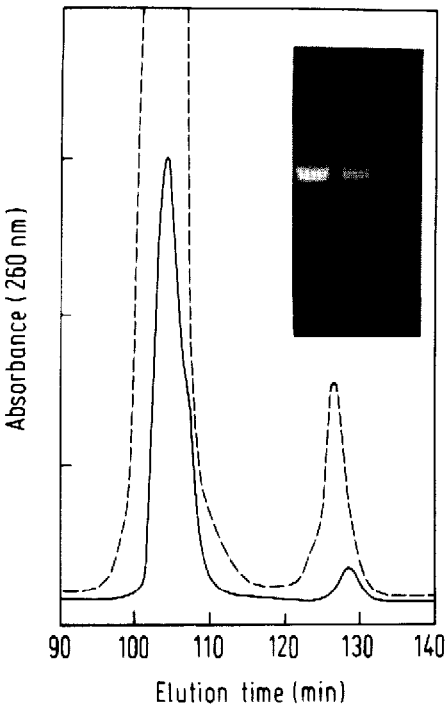
The vector-primer was prepared as described under *Methods* and purified through an oligo(dA) column to remove undigested plasmids and the PSV molecules without an oligo-dT tail. As shown earlier (LKB Application Note, German Edition, GF-15 A-D), DNA fragments from 50 to 500 base pairs length can be separated on an LKB/TSK G 4000 SW column, from which larger sized DNA fragments are eluted in the exclusion volume of the column. Fig. 1 shows two peaks in the chromatographic profile, demonstrating the clean separation of the DNA fragments. Investigations of aliquots of the pooled peak fractions by agarose gel electrophoresis established that finding (Fig. 1, insert). Because of peak broadening (mass overloead) not more than 20 μg of PSV-DNA can be applied to the column. To achieve a high resolution, the volume of sample injected should be as small as possible; it ranged from 5 to 10 μl in our experiments.

The oligo(dG)-tailed linker was prepared as described under *Methods* and was purified on an oligo(dC) column. Affinity chromatography is essential for removing highly supercoiled, undigested PSL plasmid. As steric exclusion chromatography separates on the basis of solution hydrodynamic volume, this species would be eluted together with the 280 bp linker and, therefore, would yield high *E. coli* transformation rates. In our cloning experiments, up to 50% of the colonies carried PSL plasmids if this step had been omitted (data not shown).

In our experience, only 50% of the (dG)-tailed DNAs are retained on the oligo(dC) column. As the maximum amount of PSL-DNA that can be applied to the HPLC column is 20 μg (mass overload), we recommend performing this purification step before the HPLC.

Chromatography of the above PSL preparation on the LKB/TSK G 4000 SW column results in two peaks (see Fig. 2). As mentioned above, the two large DNA fragments (3.8 and 0.78 kbp) are eluted in the exclusion volume. Analysis of aliquots of the pooled peak fractions confirms the clean purification of the desired 280 bp DNA fragment (Fig. 2, insert).

Concentrating the pooled linker fractions (400 μl) by lyophilization to half of the original volume yielded linker concentrations of approximately 2 ng/μl in buffer (20 m*M* Tris–HCl, 1 m*M* EDTA, 100 m*M* NaCl) when 20 μg of the above preparation were applied to the column. We standardized the cloning procedure (see below)



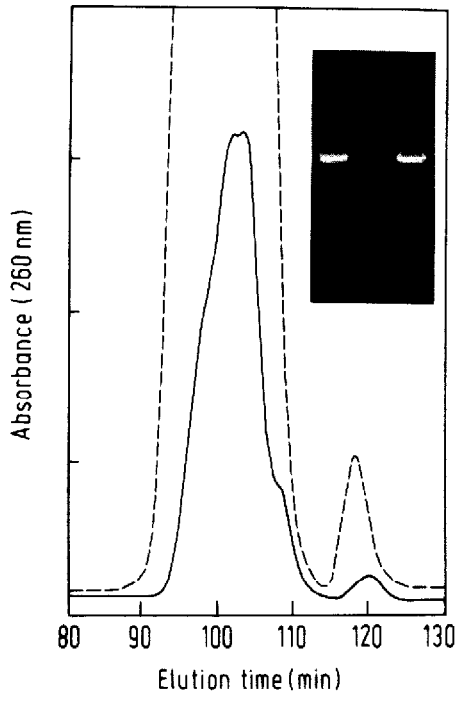


Fig. 1. Purification of the vector-primer: 10 μg of DNA were dissolved in 7 μl of water and applied to a TSK G 4000 SW column. Eluent: 10 m*M* Tris–HCl (pH 7.4)–0.5 m*M* EDTA–50 m*M* NaCl. Solid line, 0.4 a.u.f.s.; broken-line, 0.12 a.u.f.s. flow-rate, 0.1 ml/min; path length, 10 mm; chart speed, 2 mm/min. Insert: analysis of the peak fractions by 1% agarose gel electrophoresis. From left to right: vector-primer preparation before chromatography, first peak, second peak material.

Fig. 2. Purification of the oligo(dG)-tailed linker: 20 μg of DNA were dissolved in 10 μl of water and applied to a TSK G 4000 SW column. Solid line, 0.4 a.u.f.s.; broken line, 0.12 a.u.f.s. Conditions as in Fig. 1. Insert: analysis of the peak fraction by 1% agarose gel electrophoresis. From left to right: first peak, second peak, material before chromatography.

with 1 μg of the vector-primer, the linker being added to one tenth of the starting material. Thus, 10 μl of linker solution can be directly applied to achieve the prescribed conditions[10] for the cyclization of the recombinant DNA (*cf.*, Fig. 3, step 4).

Cloning of mRNA

A detailed description of the cloning procedure is given in Fig. 3. As we were interested in cloning nanogram amounts of a high-molecular-weight RNA (>30 S), we decided to standardize the cloning technique with AMV-RNA (35 S, 7500 bp), which was available in highly purified form. Reverse transcription (probably the most crucial step in any cloning technique) of AMV-RNA, performed with vector-primer purified by HPLC, results in a 5–10 times higher yield of cDNA compared with PSV eluted electrophoretically from agarose gels, and reached the yield obtained with a synthetic oligo-dT_{12-18} primer (see Table I).

The efficiency of the cloning procedure was monitored by the determination of the number of transformed bacteria carrying recombinant DNA. As shown in

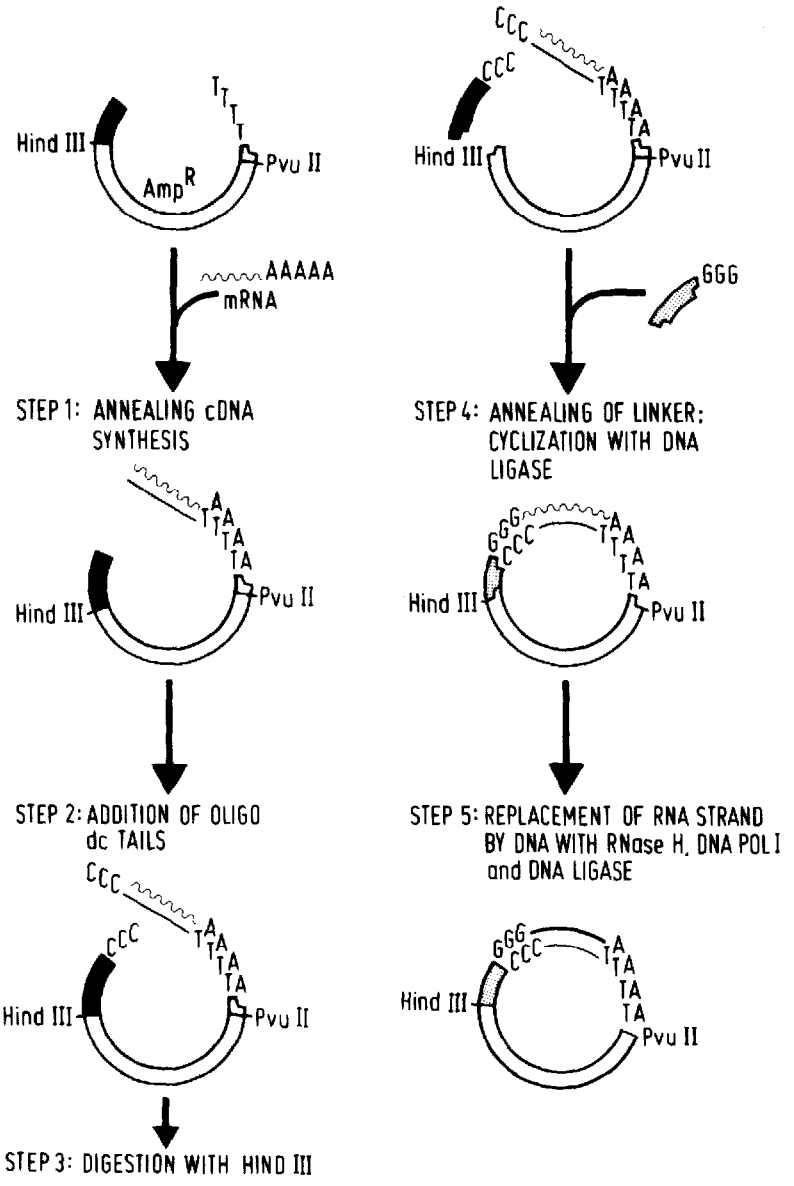


Fig. 3. Steps in the cloning of AMV-RNA, as described by Okayama and Berg[10]. cDNA synthesis is performed by use of the oligo(dT) tail of the cloning vector as primer. After addition of oligo(dC) tails and Hind III digestion, the recombinant DNA is covalently closed by the addition of an oligo(dG)-tailed linker.

Table II, we achieved high transformation rates with HPLC-purified vector and linker, whereas we did not succeed with vector and linker eluted electrophoretically from agarose gels. This failure may be due to contaminant agarose materials, which inhibit one or more of the performed enzyme reactions.

Table II also shows that cloning of 100 ng of AMV-RNA (corresponding to

TABLE I

EFFECT OF THE PURIFICATION PROCEDURE OF VECTOR-PRIMER ON cDNA SYNTHESIS

cDNA synthesis experiments performed with PSV, purified by HPLC or agarose gel electrophoresis. AMV-RNA was annealed to the oligo(dT) tail of the vector-primer and cDNA synthesis was performed as described earlier[10].

Material	*RNA transcribed into cDNA (%)*
AMV-RNA primed with PSV electrophoretically eluted from agarose gel	0.5–1%
AMV-RNA primed with HPLC-purified PSV	4–7%
AMV-RNA primed with oligo dT	4–7%

TABLE II

EFFECT OF PURIFICATION OF VECTOR-PRIMER AND LINKER ON CLONING EFFICIENCY OF RECOMBINANT DNA PLASMIDS

The cloning procedure was performed as shown schemmatically in Fig. 3. Vector-primer: 1 μg of PSV.

Conditions	*Amount of AMV-RNA (μg)*	*Molar ratio, vector:RNA*	*No. of transformants per μg vector*	*No. of insert pos. clones per 100***
PSV and linker purified with HPLC–*		–	30,000	–
PSV and linker purified with HPLC	1	1:1	12,000	30
PSV and linker purified with HPLC	0.5	2:1	12,000	10
PSV and linker purified with HPLC	0.1	10:1	11,000	1–3
PSV and linker electrophoretically eluted from agarose gels	1	1:1	50	–

* Cloning procedure started at step 2 without the prescribed addition of poly-rA to the terminal transferase reaction cocktail[10].

** Positive clones, detected by *in situ* colony hybridization[15].

less than 10 ng of an average.sized mRNA) was successful, although the yield of insert positive colonies was very low.

DISCUSSION

Our results clearly demonstrate that HPLC based on steric exclusion is also useful for the separation of DNA fragments used in molecular cloning experiments. The separation procedure is easy to handle and takes only a few hours. Further, this method does not result in an excessive loss of material. As very sensitive enzyme reactions can be performed on nanogram amounts of the nucleic acids purified by this method, we conclude that they are virtually free of any interfering contamination. The commonly used elution of DNAs and RNAs from agarose gels is time consuming and usually yields samples contaminated with agarose materials, which clearly inhibit enzyme reactions. Therefore, HPLC can be considered as a very suitable method for the purification and separation of nucleic acids.

Although larger pore size columns are currently available (G 5000 PW, G 6000 PW), the resolution of RNA and DNA fragments is still restricted by their size and, therefore, this method is at present limited in its application. Development of new steric exclusion matrices or ion exchangers should mitigate the shortcomings of this approach for the purification of DNA and RNA fragments.

REFERENCES

1 F. Rougeon, P. Kourilsky and B. Mach, *Nucleic Acids Res.*, 2 (1975) 2365.
2 T. H. Rabbitts, *Nature (London)*, 260 (1976) 221.
3 K. O. Wood and J. C. Lee, *Nucleic Acids Res.*, 3 (1976) 1961.
4 C. Brack, M. Hirama, R. Lenhard-Schuller and S. Tonegawa, *Cell*, 15 (1978) 1.
5 R. Breathnach, J. L. Mandel and P. Chambon, *Nature (London)*, 270 (1977) 314.
6 A. Efstratiadis, F. C. Kafatos and T. Maniatis, *Cell*, 10 (1977) 571.
7 S. M. Tilghman, P. J. Curtis, D. C. Tiemeir, J. G. Seidman, B. M. Peterlin, M. Sullivan, J. V. Maizel and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 1309.
8 A. J. Jeffreys and R. A. Flavell, *Cell*, 12 (1979) 1097.
9 B. Cordell, G. Bell, E. Tischer, F. DeNoto, A. Ulrich, R. Dictet, W. J. Rutter and H. M. Goodman, *Cell*, 18 (1979) 533.
10 H. Okayama and P. Berg, *Mol. Cell Biol.*, 2 (1982) 161.
11 L. Graeve, W. Goemann, P. Földi and J. Kruppa, *Biochem. Biophys. Res. Commun.*, 107 (1982) 1559.
12 L. Graeve, J. Kruppa and P. Földi, *J. Chromatogr.*, 268 (1983) 506.
13 Y. Kato, M. Sasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, *J. Chromatogr.*, 266 (1983) 341.
14 J. Maisel, F. Klement, M. Lai, W. Ostertag and P. Duesberg, *Proc. Natl. Acad. Sci. U.S.A.*, 70 (1973) 3536.
15 M. Grunstein and D. S. Hogness, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 3961.
16 H. C. Birnboim and J. Doly, *Nucleic Acids Res.*, 7 (1979) 1513.