0273-2289/94/4401—0119$03.00

Improved Procedure for the Preparation of DNA Restriction Fragments Suitable for Sequencing

PIERGIORGIO PIETTA,*[,1]
PIERLUIGI MAURI,[2] VALENTINA APPIERTO,[2]
MARINA MOSTARDINI,[2] AND IDA BIUNNO[2]

[1]Dipartimento Scienze e Tecnologie Biomediche, Via Celoria 2-20133, Milan, Italy; and [2]Istituto Tecnologie Biomediche Avanzate, CNR Via Amperé 56-20131, Milan, Italy

Received May 17, 1993; Accepted July 29, 1993

ABSTRACT

A rapid and integrated procedure was developed for the preparation of small DNA restriction fragments (≤ 1000 bp) starting from a large cosmid (35,000 bp) containing exogenous DNA.

The process is based on restriction enzymatic digestion followed by HPLC separation and fractions collection. All DNA fragments are separated in a single run, detected "on-line" by UV absorption, and straightforward collected with very high recovery.

Small fragments can be directly subjected to the sequence procedure, whereas those larger than 1000 bp are redigested with a second enzyme, the fractionated subfragments are separated, ligated to plasmid vector, and sequenced. A human genomic cosmid of 35,000 bp (26H7) has been chosen as a model.

Index Entries: DNA restriction fragments; preparation of; HPLC.

INTRODUCTION

The major task in the Human Genome Project is to map and sequence large portions of the genome, taking advantage of cosmid and YAC libraries obtained from specific chromosomes (*1*). These large clones are usually

*Author to whom all correspondence and reprint requests should be addressed.

converted into smaller fragments that are suitable for sequencing reactions. DNA fragments of ≈500 bp are considered to be ideal sequencing size both for single- and double-stranded DNA. However, no efficient means of converting large DNA into a set of ≈500 bp clones suitable for automated sequencing are available, and the manner in which these smaller fragments are obtained from the larger ones remains a crucial point for the overall sequencing strategy. Electrophoresis has been an important technique for the separation and size determination of DNA fragments (*2*). However, DNA extraction and recovery from agarose or polyacrylamide gels often occurs in low yields, and contaminants arising from the gel may interfere with subsequent manipulation. The resolving power and speed of capillary gel electrophoresis has been shown to be better than traditional slab gel techniques (*3*). Nevertheless, the procedure has some drawbacks, such as the need of derivatization, the lack of tested and commercially available gel filled capillaries, and the difficulty of collecting the separated fragments. On the other hand, high-performance liquid chromatography (HPLC) in the ion-exchange mode allows routinary separation, off- or on-line ultraviolet detection of the eluates and their easy collection (*4–6*).

In the context of our studies on more rapid and powerful methods for biomolecule analysis (*7*), a multistage integrated process has been developed to prepare small DNA fragments (≤1000 bp) suitable for sequencing starting from large cosmids (≈ 35,000 bp).

MATERIALS AND METHODS

Materials

The cosmid 26H7 was obtained from a human X chromosome Library (I.B., ITBA, Milan). A bacterial culture of the cosmid clone was grown overnight and DNA extraction was performed using the Magic MiniPrep Kit (Promega, Madison, WI). 20 μg of 26H7 was digested to completion with the restriction enzyme *Eco*R1 according to the manufacturer specification (New England Biolabs, Beverly, MA). Peak 3 was redigested with the restriction enzyme Pst1 after HPLC purification.

Apparatus

The HPLC system consisted of an automated gradient controller, two model 510 pumps, equipped with a Model U6K universal injector (Waters Assoc., Milford, MA). For detection of the peaks a Model Lambda Max 480 IV detector (Waters) connected to a CR3A integrator (Shimadzu, Kyoto, Japan) was used. Peak collection and reinjection was done by means of a Model 232 automatic sample processor and injector with a Model 401 dilutor (Gilson Medical Electronics, Villiers le Bel, France) equipped with a Rheodyne 7010 injector.

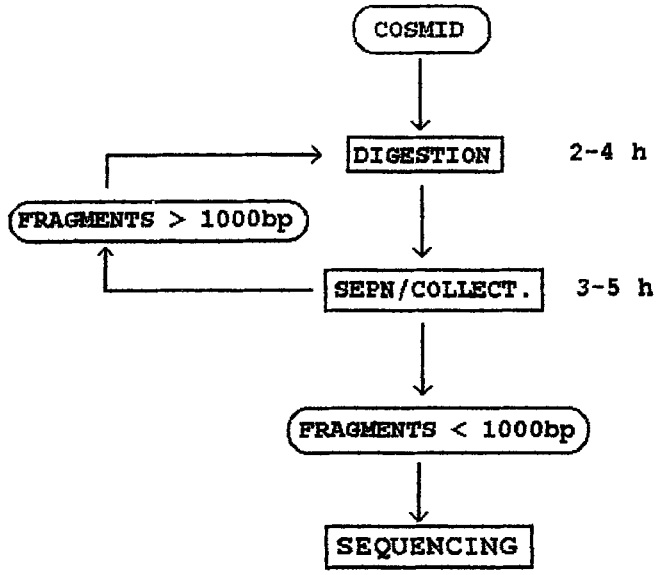

Fig. 1. Enzymatic digestion of cosmid 26H7, separation by HPLC and collection of the fragments, sequencing of fragments lower than 1000 bp, subsequent processing of fragments larger than 1000 bp.

Methods

1. All HPLC runs were performed on a Waters Gen-Pak FAX columns (100 × 4.6 mm, id). Eluent A was 25 m*M* sodium phosphate (pH 7.0) and eluent B was the same buffer containing 1*M* sodium chloride. Phosphate buffer was made using the monobasic salt and the pH was adjusted using sodium hydroxide. Eluents were filtered through a 0.45-μm membrane. The gradient profile was 40–70% eluent B in 20 min (curve 5). Flowrate was 0.8 mL/min. The peaks were detected at 260 nm.

2. Gel electrophoresis was carried out on 0.7% agarose (*8*) using 0.08*M* Tris-phosphate, pH 8.0, 2 m*M* EDTA.

Desalting and concentration were performed using Ultrafree MC filters 10,000 NMWL (Millipore).

Each eluted HPLC fragment was ligated to pBlueScript IISK$^+$ (Promega) as described in Maniatis (*8*) and X-L1 Blue competent cells were transformed with the recombinant plasmid (*6*). Recombinants were subjected to polymerase chain reaction (PCR) using T7 and T3 primers.

All the fragments obtained by this approach were sequenced (G. De Bellis, ITBA), and the results will be published elsewhere.

RESULTS AND DISCUSSION

The overall process to obtain fragments smaller than 1000 bp and suitable for sequencing is shown in Fig. 1.

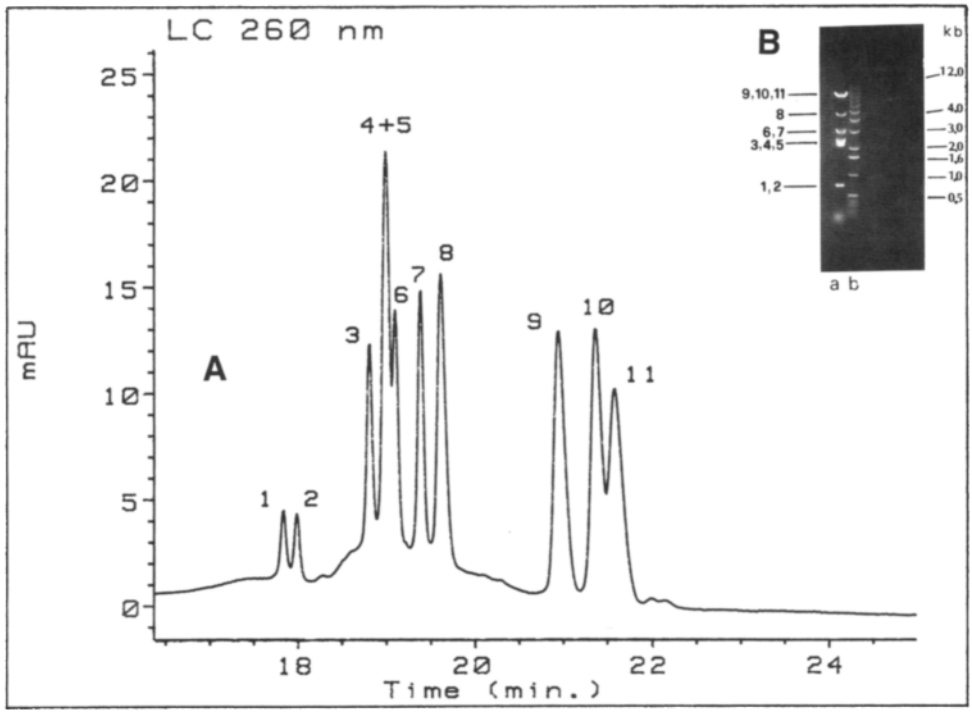

Fig. 2. (A) HPLC profile of cosmid clone 26H7 digested with *Eco*R1, and (B) the respective agarose gel electrophoresis. Lane a, cosmid 26H7 digested with *Eco*R1. Lane b, 1 kb ladder.

As a model the human genomic cosmid 26H7 of 35,000 bp cloned in pWe 15 (8200 bp) cosmid vector was digested with *Eco*R1 and the digested DNA mixture was separated by HPLC. Twenty micrograms of cosmid sample were fractionated in a single run and eluted from the column with about 90% recovery. Eleven peaks were obtained in less than 25 min (Fig. 2A), where peak 9 represents the vector as confirmed by cochromatography. The Kb values of each peak was obtained by means of the relation previously described (*6*), and are reported in Table 1.

The same DNA fragments mixture was separated by gel-electrophoresis and a poorer resolution was achieved, as shown in Fig. 2B. Indeed, gel-electrophoresis was unable to separate the fragments 1 and 2 of similar size (800 and 817 bp, respectively) into individuals bands as well as fragments 6, 7, and 9–11.

Peaks 1–3 and 6–11 were collected separately, whereas peaks 4 and 5 were collected as a mixture. The small fragments 1 and 2 were in the 0.5–1 μg range suitable for sequencing. The other fragments larger than 1000 bp, beside the vector (peak 9), needed to be further processed. As an example, peak 3 (2300 bp) was desalted by ultrafiltration, ligated to a plasmid vector, and redigested with Pst1. The resulting mixture was separated by HPLC (Fig. 3A), and the subfragments were evaluated for their Kb values (Table 1).

Table 1
Kb Values of Peaks 1–11 from 26H7 Digested with *Eco*R1 and Subfragments a–f from Peak 3 Digested with *Pst*1[a]

Peaks from 26H7	Kb	Subfragments from peak 3	Kb
1	0.80	a	0.056
2	0.82	b	0.107
3	2.3	c	0.140
4 + 5	4.9	d	0.160
6	2.7	e	0.844
7	3.0	f	3.74
8	4.8		
9	8.8		
10	9.0		
11	8.0		

[a]Peak 9 and subfragment f correspond to the vectors.

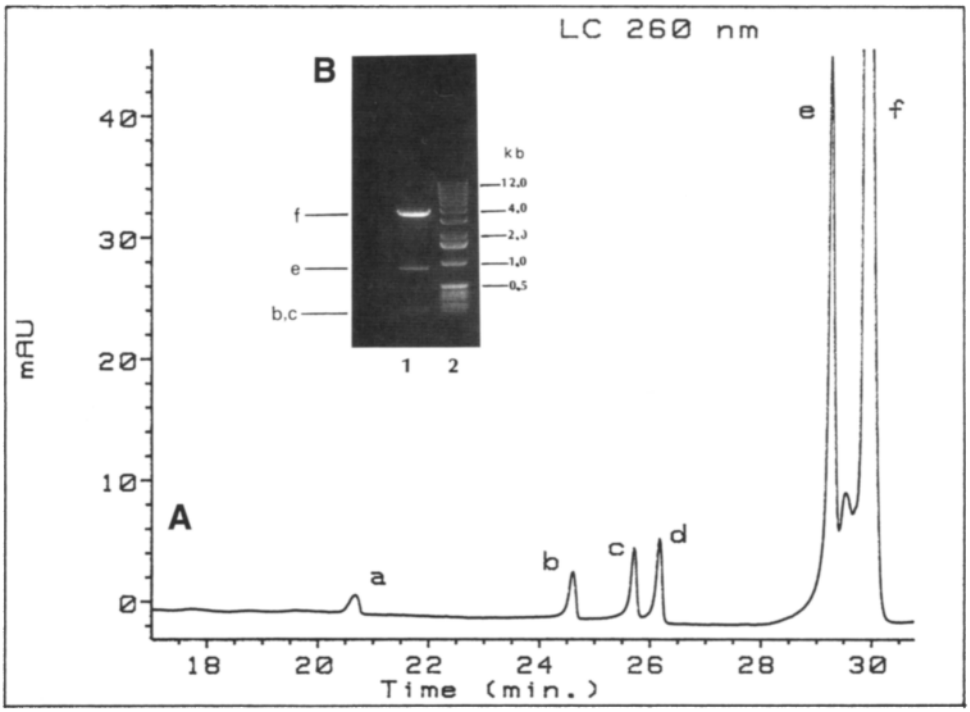

Fig. 3. (A) HPLC profile of fragment 3 digested with Pst1 and (B) the respective agarose gel electrophoresis. Lane 1, fragment 3 digested with Pst1. Lane 2, 1 kb ladder.

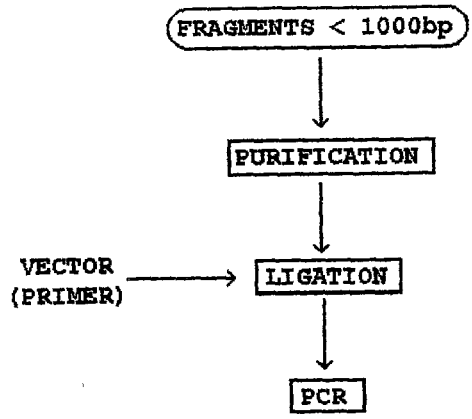

Fig. 4. Purification of the subfragments by ultrafiltration, ligation to the vector, and subsequent polymerase chain reaction (PCR).

Also in this case, gel-electrophoresis failed in evidencing the shortest pieces (Fig. 3B). The subfragments a–e were collected, and processed as shown in Fig. 4. prior their sequencing. Desalting and ligation to primers were always necessary and, in the case of the low amount available, a further amplification step by PCR was needed. On the other side, the subfragment f (3740 bp) was redigested with *Eco*R1 to yield the vector (2900 bp) and a further subfragment (840 bp), as confirmed by HPLC.

These results confirm that restriction fragments (500–1000 bp) can be separated by HPLC in a single run and with better resolution than by gel-electrophoresis, or detected online at low level (10 ng) and easily evaluated for their size. The high recovery collection combined with the amenable desalting and amplification represents an additional precious feature of the method.

The automation of the entire process is possible and under study in our laboratory.

REFERENCES

1. Dulbecco, R. (1990), *Genomics* **7,** 294.
2. Thompson, J. A. (1986), *Biochromatography* **1,** 162.
3. Trainor, G. L. (1990), *Anal. Chem.* **62,** 418.
4. Thompson, J. A. (1987), *Biochromatography* **2,** 4.
5. Stowers, D. J., Keim, J. M. B., Paul, F. S., Dyoo, Y. S., Merion, M., and Benbow, R. M. (1988), *J. Chromatogr.* **44,** 47.
6. Mauri, P. L., Pietta, P. G., and Pace, M. (1991), *J. Chromatogr.* **548,** 281.
7. Biunno, I., Mauri, P. L., Susani, L., and Pietta, P. G. (1992), *Genomics* **13,** 1566.
8. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982), in *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.