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A novel procedure for selective cloning of *Not*I linking fragments from mammalian genomes

Takashi Ito[1] and Yoshiyuki Sakaki[1,2]

[1]Department of Preventive Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852 and [2]Research Laboratory for Genetic Information, Kyushu University, Fukuoka 812, Japan

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ABSTRACT

A novel procedure has been developed for selective cloning of *Not*I linking fragments from mammalian genomes. Since the majority of the *Not*I sites in mammalian genomes are considered to be localized in so-called HTF (*Hpa*II tiny fragment) islands, an HTF library was constructed as an initial step to enrich the *Not*I sites. The plasmid DNAs were isolated *en masse* from the HTF library and digested with *Not*I. Linearized plasmid DNAs derived from *Not*I linking clones were efficiently separated from undigested circular DNAs by an unique pulsed field polyacrylamide gel electrophoresis (PF-PAGE). The linear DNAs were eluted from the gel, recircularized with T4 DNA ligase and introduced into *E.coli* cells. About 95% of the transformants were found to contain *Not*I linking fragments. The procedure will thus provide a simple and useful way of collecting *Not*I linking fragments for long range physical mapping of mammalian genomes.

INTRODUCTION

Restriction endonucleases with rare cutting sites, called rare cutters, are of great use for long range physical mapping of the genomes of complex organisms. For mammalian genomes, eight-base cutters (*Not*I and *Sfi*I) and certain enzymes with the recognition sites containing CpG dinucleotides (e.g. *Mlu*I) have been proved to be very informative : their cutting sites can act as milestones or landmarks of the genome map.

DNA fragments bearing such rare cutter sites, termed rare cutter linking fragments, are used in combination with jumping clones for long range physical mapping (1-4). The linking clones can be also utilized as probes for external mapping of large DNA fragments (2) which is performed by the pulsed field gel electrophoretic analysis of partially-digested genomic DNA. Furthermore, they provide indirect end-labeling probes for Smith-Birnstiel type restriction mapping of large DNA fragments generated by rare cutter digestion (1-3). To develop an efficient method for cloning of rare cutter linking fragments will thus contribute toward the physical mapping of the genome.

Although several procedures for cloning of linking fragments have been proposed (1-3), they include a step for insertion of foreign DNA fragments, such as

selectable markers or vectors, into the corresponding rare cutter sites. Since intermolecular ligation is generally less efficient than intramolecular ligation, it is favorable to avoid such intermolecular ligation steps (e.g. marker insertion) if possible.

For this reason, we have devised a simple method for cloning of linking fragments without insertion of selectable makers. This method is based on a novel application of pulsed field gel electrophoresis (PFGE) as a means of separating heterogeneously-sized linear and circular DNAs each other from their mixture. This paper describes the details of the novel method for efficient physical isolation of *Not*I linking clones from an HTF (*Hpa*II tiny fragment (5)) library, which may contain the majority of the *Not*I sites of mammalian genomes (6).

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase and bacterial alkaline phosphatase were purchased from Takara Shuzo (Kyoto, Japan) and Nippon Gene (Toyama, Japan). Low gelling temperature agarose was obtained from Nippon Gene (Toyama, Japan). High molecular weight DNA was isolated from a human placenta as described previously (7).

General DNA techniques

General DNA manipulations were carried out according to Maniatis *et al.* (8) with some modifications.

Preparation of an HTF library

An HTF library was constructed by a simplified version of the original method described by Bird *et al.* (5). Briefly, human placental DNA was digested with *Hpa*II, and subjected to gel electrophoresis using 1% low gelling temperature agarose. DNA fragments less than 500 bp were recovered by melting the gel, and ligated with *Acc*I-digested and phosphatased pUC19 (9). DNAs were introduced into competent cells (JM83 (10)), and transformants were grown up in LB-broth or on LB-agar plates containing 100 μg/ml ampicillin. The HTF plasmids were isolated *en masse* from the overnight culture of the library or numerous colonies on the plates.

PFGE using polyacrylamide gels

Gels (9cm x 12cm x 0.8cm) were composed of 4% polyacrylamide (acrylamide : N, N'-methylene bisacrylamide = 30:1) dissolved in 0.5 x TBE (1 x TBE : 89 mM Tris, 89 mM boric acid, 2 mM EDTA), and polymerized on glass plates in disposable plastic trays with wells formed by appropriate combs. The gels were run at 6.7 V/cm (initial current, about 230 mA) with a switching interval of 15 seconds for 16-20

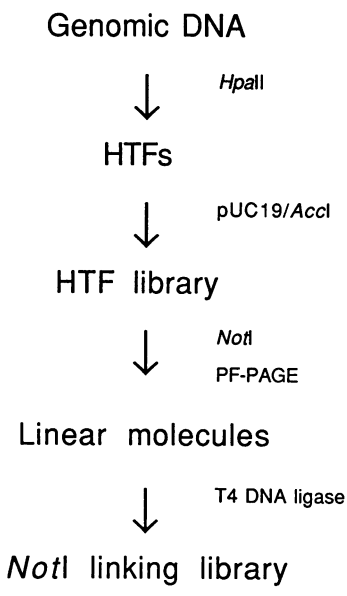

Figure 1. Summary of the strategy for selective cloning of *Not*I linking fragments from mammlian genomes. HTFs : *Hpa*II tiny fragments (5), PF-PAGE : pulsed field polyacrylamide gel electrophoresis.

hours at 10-15°C in a simplified hexagonal apparatus for CHEF (contour-clamped homogeneous electric field) gel electrophoresis (11). DNAs were visualized by staining with ethidium bromide dissolved in 0.5 x TBE.

Cloning of *Not*I linking fragments

A few μg of plasmid DNAs from the HTF library were digested with *Not*I, and subjected to the PFGE using a polyacrylamide gel described above. Linear DNAs were electrophoretically eluted from the gel, and purified by phenol extraction and ethanol precipitation. Then, they were recircularized with T4 DNA ligase and introduced into competent cells of *E. coli* (JM83). Transformants were selected on LB-agar plates containing 100 μg/ml ampicillin.

RESULTS

Our procedure for selective cloning of *Not*I linking fragments from mammalian genomes is summarized in Fig. 1.

Construction of an HTF library

Since it has been considered that the majority (about 90%) of the *Not*I sites in mammalian genomes are located in so-called HTF (*Hpa*II tiny fragment) islands (6), we constructed an HTF library as an initial step to enrich *Not*I linking clones.

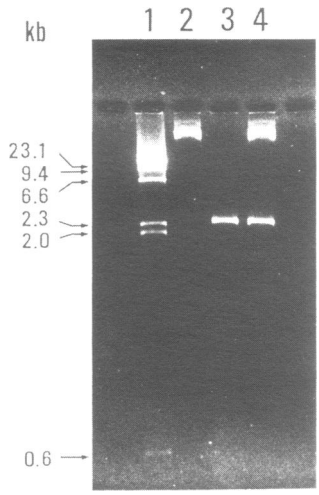

Figure 2. Migration of circular and linear DNAs in PF-PAGE. The gel was electrophoresed as described in MATERIALS AND METHODS and stained with ethidium bromide to visualize DNAs. Lane 1, lambda DNA digested with *Hin*dIII, as size markers; lane 2, non-digested, circular pUC19; lane 3, pUC19 linearized by *Hin*dIII digestion; lane 4, the mixture of circular and linear pUC19.

Human placental DNA was digested with *Hpa*II, and DNAs corresponding to HTFs were cloned into *Acc*I site of pUC19 to prepare a library (see MATERIALS AND METHODS). About 3-4x10^4 HTF clones were obtained from 100 μg of placental DNA. Plasmids were then isolated *en masse* from the HTF library and digested with *Not*I. As a result, a mixture of linearized DNAs from *Not*I linking clones and circular DNAs from non-linking clones was generated.

Separation of linear and circular DNAs by pulsed field polyacrylamide gel electrophoresis (PF-PAGE)

Pulsed field gel electrophoresis (PFGE) is an excellent technique for separating very large DNA molecules that cannot be analyzed by the conventional agarose gel electrophoresis (11-15). In addition, it has been noticed that circular (both open and closed) DNAs exhibit abnormal migration properties in PFGE (16-20). We intended to utilize such characteristics of PFGE systems as a means of separating linear and circular DNA molecules. We developed a PFGE system using polyacrylamide gels (see MATERIALS AND METHODS), in which linear DNAs migrated much faster than circular DNAs of the same size (Fig. 2), and thereby clear separation of linear and circular DNAs was achieved. This method has been proved to be able to separate heterogeneously-sized (at least, 3-23 kb) linear and

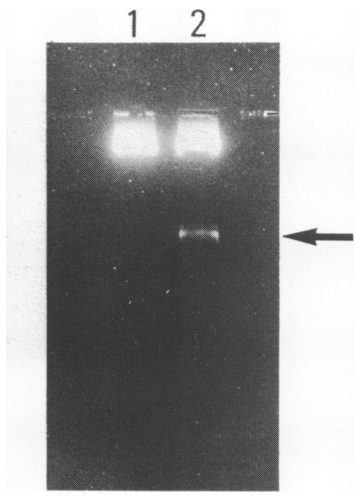

Figure 3. Separation of linearized *Not*I linking clone DNAs from circular non-linking clone DNAs by PF-PAGE. Plasmids isolated *en masse* from the HTF library were subjected to the PF-PAGE without (lane 1) or with (lane 2) *Not*I digestion. Arrow indicated the position of the linearized *Not*I linking clone DNAs.

circular DNAs each other from their mixture. For example, a 23 kb DNA fragments migrated faster than a supercoiled plasmid of about 3 kb long (Fig. 2).

We therefore utilize this novel PFGE system, termed pulsed field polyacrylamide gel electrophoresis (PF-PAGE), for selective separation of linearized *Not*I linking clone DNAs from circular non-linking clone DNAs. The mixture of linear and circular DNAs prepared from the HTF library as described above was subjected to the PF-PAGE. As shown in Fig. 3, the vast majority of the plasmid DNAs from the HTF library were not cut by *Not*I and retained their circular forms as expected. However, a small but distinct fraction of the HTF plasmids was linearized by *Not*I digestion and separated clearly from circular molecules by PF-PAGE (compare lanes 1 and 2 in Fig. 3).

Selective cloning of *Not*I linking fragments

The linear DNAs separated by PF-PAGE were electrophoretically eluted from the gel, recircularized with T4 DNA ligase and introduced into *E. coli* competent cells. Starting from a few μg of HTF plasmids, approximately 10 ng of linear DNA was recovered and finally about 1000 transformants were obtained.

To test the validity of our procedure, plasmids were isolated from the randomly picked-up transformants and digested with *Not*I. Twenty eight out of the thirty transformants tested were found to contain *Not*I linking fragments (see Fig. 4a, for

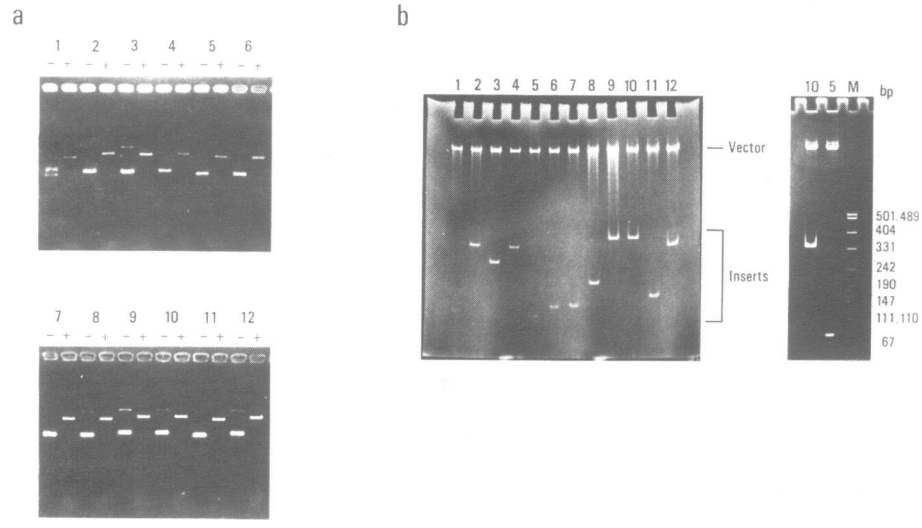

Figure 4. Characterization of the randomly picked-up transformants. (a) Plasmids isolated from 12 transformants (No.1-12) were digested with *Not*I and subjected to agarose gel electrophoresis. –: without *Not*I digestion, +: with *Not*I digestion. (b) The plasmids analyzed in (a) were digested with *Eco*RI and *Hin*dIII, and subjected to polyacrylamide gel elecrtophoresis (left). To estimate the size range of insert DNAs, plasmid DNAs containing the longest (No.10) and the shortest (No.5) inserts of the 12 transformants were digested as above and electrophoresed with *Hpa*II-digested pUC19 DNA (lane M) as size markers (right).

example). To know whether they were independent each other, twelve plasmid DNA samples shown in Fig. 4a were digested with *Eco*RI and *Hin*dIII and subjected to polyacrylamide gel electrophoresis (Fig. 4b). Comparison of the DNA fragment lengths revealed that, at least, eight out of these twelve clones were independent each other, suggesting that several hundreds out of 1000 transformants obtained above were independent clones. The sizes of the insert DNAs seemed to range approximately from 50bp to 300bp (Fig, 4b). Thus, the procedure described above has been proved to be a simple and efficient way of cloning *Not*I linking fragments.

DISCUSSION

A novel procedure has been developed for selective cloning of *Not*I linking fragments from mammalian genomes, as summarized in Fig. 1.

A critical point of our procedure is the use of an HTF library as a starting material. The total number of *Not*I sites in the human genome has been estimated to be $3\text{-}4\text{x}10^3$ (2,6). It is difficult to pick up such rare sites directly from the whole

genome. However, the majority (about 90%) of the *Not*I sites in mammalian genomes are considered to be localized in so-called HTF islands, which represent only 1% of the genome (5,6). We can thus enrich the *Not*I sites nearly up to 100-fold by constructing an HTF library as an initial step of the procedure.

Another critical point of our procedure is an efficient technique for separating heterogeneously-sized linear and circular DNAs each other. In usual, closed circular DNA is separated from linear DNA by the cesium chloride-ethidium bromide density equilibrium centrifugation (8). However, linear DNA fraction obtained by this method also contains open circular DNA. Linear DNA can be separated from open and closed circular DNAs by conventional gel electrophoresis, but this system does not work efficiently for heterogeneously-sized linear and circular DNA mixture. As demonstrated in this paper, an unique PFGE system using polyacrylamide gels (PF-PAGE : pulsed field polyacrylamide gel electrophoresis) can efficiently separate linear and both types of circular DNAs of considerable size heterogeneity (ranging, at least, from 3 to 23kb). It may be possible to employ agarose instead of polyacrylamide. Our preliminary results, however, showed that linearized plasmid DNA of about 3kb long could not be separated from its supercoiled circular form in agarose gels (1.5-2.0%) so clearly as in polyacrylamide gels. We thus recommend to use polyacrylamide gels. This technique has enabled us to isolate linearized *Not*I linking clone DNAs (plasmids linearized by *Not*I digestion) physically from circular non-linking clone DNAs. The isolated linear DNAs were then efficiently recircularized via intramolecular ligation (self-circularization) and introduced into *E. coli* cells to obtain linking clones. Thus, the PF-PAGE enabled us to skip an intermolecular ligation step, such as marker insertion or vector ligation at the corresponding rare cutter sites.

As described in RESULTS, several hundreds of independent *Not*I linking clones were obtained from $3\text{-}4\times10^4$ HTF clones. Assuming that the total number of *Not*I sites in the human genome is $3\text{-}4\times10^3$ (2,6), it will not be difficult to clone the majority of the *Not*I linking fragments by our procedure.

The procedure will not be effective to clone *Not*I sites which are not located in HTF islands. For this reason, source of DNA is crucial. We recommend to use placental DNA, which is considerably undermethylated (Hohjoh and Sakaki, unpublished results). Use of *Msp*I instead of *Hpa*II may also improve the library. *Not*I sites in non-GC rich regions, although the number of such sites is considered to be quite small, will not be cloned by this method. The library constructed by the procedure may contain very short linking fragments which are not suitable for long range physical mapping. The problem will be overcome by cloning of larger

DNA fragments from conventional genomic library using such short linking fragments as probes.

PF-PAGE technique developed in this study will be widely applicable for other purposes. For instance, we have successfully applied the technique to clone distinct members containing both *Bss*HII and *Nar*I sites from thousands of L1 repetitive sequences (Hohjoh and Sakaki, unpublished results).

For the collection of linking fragments on a specific chromosome, it is possible to use the chromosome specific genomic library or sorted chromosomes as a starting material for our procedure. Such trials are now in progress.

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