An F factor based cloning system for large DNA fragments

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ABSTRACT

An effective technique using an *Escherichia coli* plasmid system was developed to clone fragments of exogenous DNA of as large as 100 kilobase pairs. The characteristic features of this technique are the use of a low copy number (one to two) mini-F based plasmid vector and the introduction of artificial lambda cosR ends into the termini of DNA sources and then of the cosL ends into those of linearized vector molecules. This terminal modification greatly facilitated the formation of active large recombinant molecules, which was rarely achieved when the modification was omitted. The efficiency with which large recombinant clones can be generated is high enough to allow construction of a comprehensive library of higher organisms. All analyses of the plasmids recovered have revealed that the inserts were faithful replicas of the human DNAs used as sources.

INTRODUCTION

The developments of techniques for molecular cloning and DNA sequencing have promoted great advances in analyses of genes and gene complexes in many organisms. Most single genes, ranging in size from a few kilobases (kb) to several dozen kb, can be covered with a single lambda or cosmid clone. However, many genes in higher eukaryotes have been shown to be surprisingly large and complex, and their analyses require laborious, large scale chromosome walking. Burke et al.(1) developed a new vector system based on the construction of yeast artificial chromosomes (YAC clones) for cloning large DNAs. With this system very large DNA fragments of up to several hundred kb can be cloned. However, molecular analysis and manipulation of the yeast chromosome is more difficult than those of bacterial plasmids, because no convenient method is available for separation of YACs from host chromosomes.

We developed a new technique for cloning large DNA fragments in an *E. coli* system. The technique involves a combination of the use of a low copy number mini-F based vector that allows increase in size of clonable DNA, and the introduction of artificial lambda cohesive ends (cos) into the termini of DNA sources and vector molecules to enhance the step of circularization of large chimeric molecules. So for, 103 kb human DNA fragments have been cloned by this method. With progress in technology for handling large DNA molecules, it will be possible to examine larger fragments, because of the open-ended capacity of this system.

MATERIALS AND METHODS

Bacterial strains

The *E. coli* strains used were SC109 and SC1451. SC109 is an F^- derivative of JM109 (2) obtained by acridine orange treatment. SC1451 is an F^-*recA*^{del} *mcrA*$^-$*B*$^-$ strain derived from ER1451 (3). ER1451 was treated with acridine orange to cure the F′ factor. Introduction of a *recA*^{del} mutation into the resulting F^- derivative of ER1451 was carried out by transduction with a P1 lysate grown on JC10289(del(*srl*−*recA*)306::Tn*10*) (4). $RecA^-$ transductants were selected from colonies resistant to tetracycline (15 μg/ml) and nalidixic acid (40 μg/ml) and UV super-sensitivity was checked by a reported method (2).

Construction of mini-F based plasmid vector

Plasmid pOF216 was constructed from a mini-F plasmid pKP1013 (5) via the intermediate plasmids pOF100 and pOF206. pOF100 is a derivative of pKP1013 with a deletion of the non-essential 0.75 kb *Sal*I−*Bam*HI fragment. For construction of a full length-*lacZ* gene containing a multi-cloning site within the coding frame, a 230 bp *Pvu*II−*Eco*RI fragment containing *lacP*, *O* and the N-terminal sequence of pUC13−N2 (a derivative of pUC13 (6) with a modified multi-cloning sequence) was inserted into a unique *Eco*RI site of a gene fusion vector pMC1403 (7). The resulting plasmid, designated as *lacP*−pMC1403, creates enzymatically active β-galactosidase. The 3.8kb *Dra*I fragment, which covers the region containing the *lacP*, *O* and *Z* genes, was recovered from *lacP*−pMC1403 and introduced between the *Hind*III and *Eco*RI sites of pOF100, both of which sites were blunt ended by end-filling before ligation. This construct, designated as pOF206, contains the multi-cloning site derived from pUC13−N2. The multi-cloning site sequence was replaced by a newly synthesized oligonucleotide in pOF216, as shown in Fig.1.

Plasmid pOF202 was essentially the same as pOF216 except that the positions of the *lacZ* gene and *spc* gene were reversed.

Preparation of DNA

Supercoiled DNAs of multi-copy number plasmids were prepared by alkaline lysis of host *E. coli* cells and purified by banding in an ethidium bromide/CsCl density gradient. Supercoiled DNA of mini-F based plasmids was purified in the same way, except that cleared lysates were prepared with lysozyme-EDTA-Triton X-100 by a reported method (5). High molecular weight DNA from cultured human HeLa or Namalwa cells was prepared by a reported method (8). Size fractionation of *Hind*III partial digests of HeLa DNA by sucrose density gradient centrifugation was carried out as described (9). For pulsed field gel (PFG) electrophoretic analysis, DNA was prepared in agarose plugs as described (10).

Molecular cloning strategy in a mini-F plasmid vector

Human Namalwa cell DNA in 5 pieces of agarose plug (1×10^7cells/ml, about 50 μg) was digested completely *in situ* with *Nar*I. CosR-adapter (dCGCCCGGGAGGTCGCCGCCC) or cosL-adapter (dCGCCCGGGGGGCGGCGACCT) and *Sma*I linker (dCCCGGG) were synthesized chemically by the phosphoramidite method. A mixture of 500 pmoles of ^{32}P-labeled cosR-adapter and 2,500 pmoles of unphosphorylated *Sma*I linker was heated at 65 C for 10 min, and then added to ligation buffer containing agarose plugs at a molar ratio of 1,000 : 5,000 : 1 (cosR-adapter : *Sma*I linker : insert DNA). The mixture was gently shaken at room temperature for several hours, and then ligated in a minimal volume of reaction mixture (700−800 μl) with 4,000 units of T4 DNA ligase (NEB) at 4°C. The agarose plugs were treated with ES (0.5 M EDTA-1% N-lauroyl sarcosine) and ESP (ES + 1 mg/ml proteinase K) at 50°C, and subjected to PFG electrophoresis using hexagonal electrodes on 1% low-gelling temperature (LGT) agarose (FMC, SeaPlaque) at CV 170 V, 25 sec pulse time for 24 hrs. After electrophoresis, the part of the LGT agarose gel containing DNA of the desired size was cut out, equilibrated in 5 mM EDTA−100 mM NaCl and melted by heating at 65°C for 10 min. The molten agarose was liquefied with 50 units/ml of agarase (Calibiochem) (11).

The vector pOF216 was also attached to the cosL-adapter. The ^{32}P- labeled cosL-adapter and unphosphorylated *Sma*I linker were added to *Nar*I digests of pOF216 in a molar ratio of 100 : 500 : 1. The mixture was heated at 65°C for 10 min and cooled to room temperature, and the ligation reaction was carried out of a final concentration of 100 μg/ml at 4°C. Agarose gel electrophoresis was used for removal of excess oligonucleotides and purification of the adapter ligated product. The recessed 5′ ends of the product recovered from the agarose gel were phosphorylated with T4 polynucleotide kinase.

The pOF216 vector DNA with attached adapter and human DNA was mixed at a DNA concentration of 0.2−0.5 μg/ml at various molar ratios of vector DNA and source DNA. The mixtures were annealed at the cos-12 mer sequence in 10 mM $MgCl_2$ at 42°C for 1 hr and then ligated overnight at 15°C.

Bacterial transformation

Competent cells were prepared by the method of Hanahan (12). Transformants harboring a recombinant pOF plasmid were selected on LB agar plates containing 15 μg/ml of spectinomycin and 40 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Spc^R and LacZ$^-$ colonies were rescreened by colony hybridization with a human repetitive Alu-2 probe (13). The DNA probe was labeled with α-^{32}P-dCTP by the random hexamer priming method and colony hybridization was carried out by the usual procedure (9).

PFG electrophoresis and Southern hybridization

PFG electrophoretic analyses of cloned human DNAs were carried out in 1% agarose with Pulsaphor (Pharmacia LKB) using a λ DNA ladder and *Hind*III digests of λ DNA as size markers. Electrophoresis was carried out with point electrodes at 300 V with a pulse time of 15 sec for 40 hrs at 9°C. Southern blot analysis was carried out with Hybond-N according to the instructions of the manufacturer (Amersham Int), except that gels were treated with 0.25 N HCl for 8 min before denaturation. For detection of *Not*I fragments containing human specific DNA or pOF216 vector specific DNA, hybridization was performed first with the ^{32}P- labeled Alu-2 probe and then with the ^{32}P-labeled *spc* gene probe. The *Bam*HI-*Hind*III-2.4Kb fragment from pKP1013 (5) was used as a DNA fragment containing the spectinomycin-resistance gene.

Genomic Southern hybridization

*Hind*III digests of cloned *E. coli* DNA or human DNA were subjected to 0.6% agarose gel electrophoresis. Cloned *E. coli* DNA was prepared in agarose plugs and after *Hind*III digestion, a piece of agarose was melted at 65°C for use. The DNA was transferred to a nylon membrane and hybridized to an isolated plasmid DNA in a mixture of 2.5% skimmed milk, 6×SSC, 50% formamide, 10% dextran sulfate, 0.1 mg/ml herring sperm DNA, ^{32}P-probe and competitor DNA at 42°C for 2 days. Isolated plasmid DNA was highly labeled (2×10^9 cpm/μg) with α-^{32}P-dCTP by the random hexamer priming method. The ^{32}P-plasmid DNA probe (10 ng/ml) was preincubated with 400 μg/ml of human placental DNA and 50 μg/ml of *E.coli* DNA at 42°C for 2 hrs before incubation with blotted DNA. The membrane was washed several times for 30 min periods first with 2× SSC-0.1% SDS at room temperature and then with 0.1×SSC−0.1% SDS at 65°C.

Densitometric measurement

DNA samples fractionated by pulsed field gel electrophoresis were stained with ethidium bromide (0.5 μg/ml) and photographed with chart film (Master Co., Tokyo). The film was then scanned with a densitometer (Bio-Rad, Model 1650).

RESULTS AND DISCUSSION

The pOF series vectors that we constructed were composed of three parts; a multiple cloning site inserted in frame within the *lacZ* gene, a selective spectinomycin (spc)-resistance gene and *rep* and *par* regions derived from the F factor. Transformants carrying inserts were distinguishable as white colonies on X-Gal agar plates. The *rep* and *par* regions are involved in the replication and partition of the F factor (14,15) and ensure stable maintenance of the resulting large plasmids at a copy number of one to two as intact F factor. The vector pOF216 is designed to clone fragments restricted with rare cutter enzymes and contains the sequences for *Nar*I, *Sal*I, *Not*I and *Sac*II.

In a pilot experiment, we examined the cloning capacity of the pOF vectors without terminal modification. Size-fractionated, partial *Hind*III digests of human DNA were cloned into the

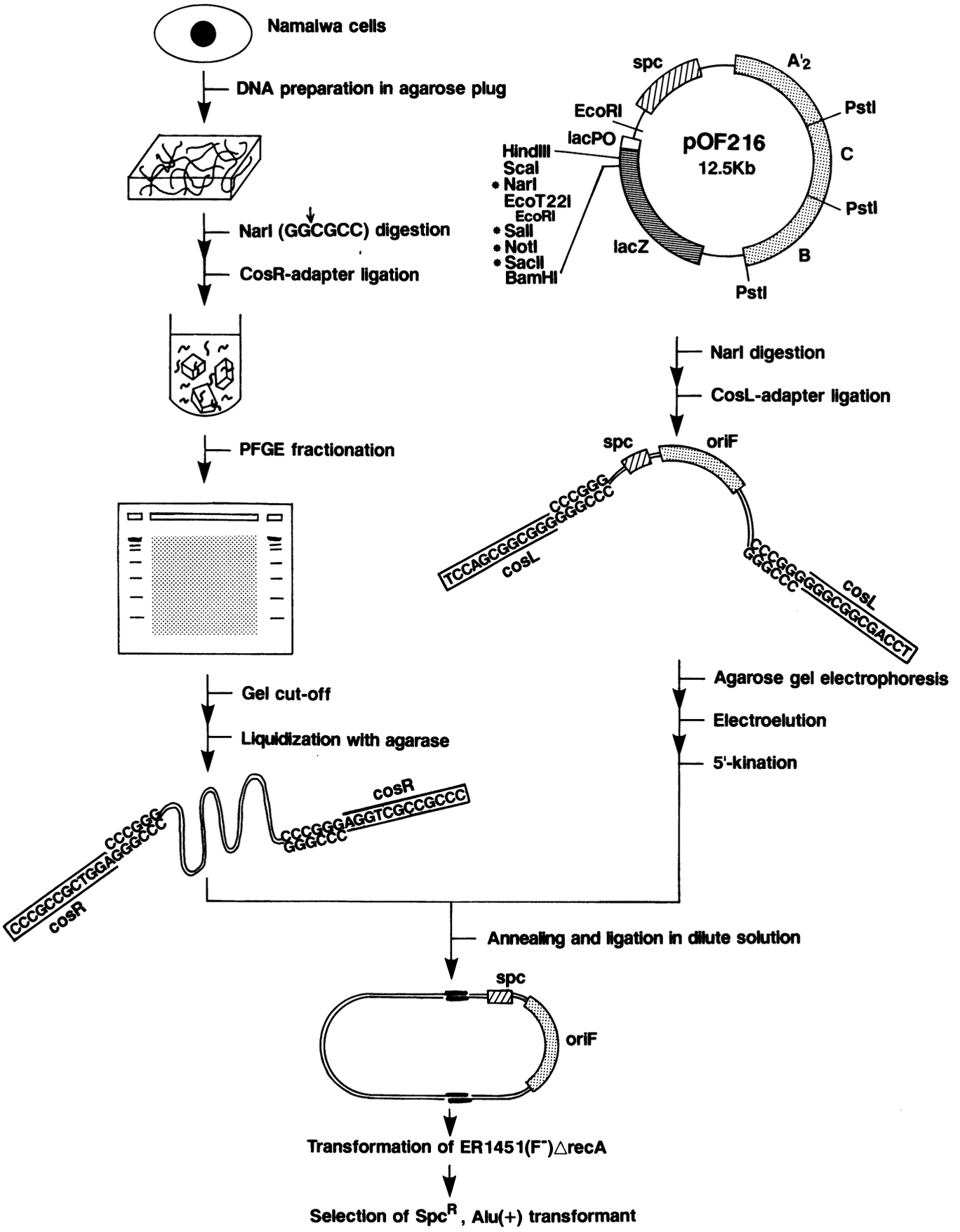

Figure 1. Molecular cloning strategy in a mini-F plasmid vector. Details of the procedure for preparation of the human source DNA and pOF216 DNA are described in MATERIALS AND METHODS. Asterisks (*) in the diagram of the multi-cloning site in pOF216 represent recognition sites of rare-cutter enzymes in mammalian genomes.

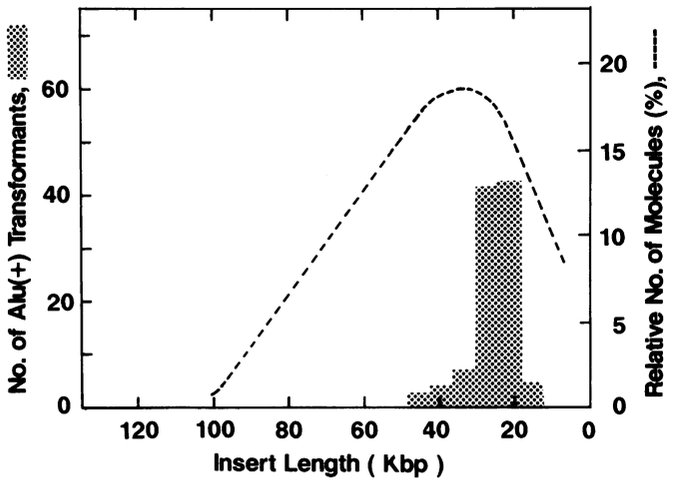

Figure 2. Length distribution of insert DNAs in Spc^R, Alu^+ transformants obtained by conventional ligation. HeLa DNA partially digested with *Hind*III was size-fractionated by sucrose density gradient centrifugation as described in the MATERIALS AND METHODS. The DNA source (fraction III) was ligated into the *Hind*III site of pOF202 at a DNA concentration of 1 μg/ml. Transformation was carried out with SC109. The DNA source was subjected to PFG electrophoresis and stained with ethidium bromide. The relative number of molecules (dotted line) was calculated from the staining intensity determined by densitometric tracing.

*Hind*III site within the multi-cloning site. By this conventional ligation reaction, Alu^+ transformants were generated at frequencies of 5.5×10^4, 1.0×10^4, and 1.1×10^3 per μg of insert DNA with fraction I (DNA source : average 10 kb, range 5 to 20 kb), fraction II (average 20 kb : range 10 to 40 kb), and fraction III (average 40 kb, range 15 to 70 kb), respectively. All three Alu^- transformants examined obtained with fraction I contained exogenous inserts. Therefore, the values represent minimal estimates. *Not*I fragments generated from the *E. coli* chromosome, which range in size from 40 to 1,000 kb, can be separated by pulsed field gel (PFG) electrophoresis (16). The plasmid vector pOF216 contains a unique *Not*I site within a multi-cloning site (Fig. 1). Therefore, plasmid DNA can be directly detected on PFG electrophoretic gel by analyzing *Not*I digests of whole cell DNA. This allows rapid and exact determination of insert sizes in individual clones. Fig. 2 shows the distributions of the lengths of the DNA source (dotted line) and cloned insert (shaded bars) in 108 clones randomly chosen from the library constructed with fraction III. The largest cloned insert was 48 kb, most being 18 to 30 kb (83 clones) and the distribution was heavily biased toward smaller molecules, regardless of the presence of a large quantity of molecules of more than 50 kb. This bias is thought to be mainly because the step of circle formation of chimeric molecules is rate limiting in generation of large recombinant molecules. Another possibility that competent cells selectively incorporate smaller molecules seems less likely, because the difference between the transformation efficiencies of 30 and 60 kb plasmids has been shown to be within a factor of 2 (12).

It has been shown that tailing DNA with asymmetric long linkers is very effective for the construction of large DNA fragment libraries (17). To overcome the technical difficulty mentioned above, we examined the effect of introduction of artificial cohesive ends *in vitro* into the termini of the insert DNA fragments and those of the linearized vector molecules (Fig. 1).

A synthetic 5'-phosphorylated 20 mer adapter (pCGCCCGGG-AGGTCGCCGCCC) and an unphosphorylated *Sma*I linker (CCCGGG) were annealed to form a small DNA duplex with a protruding λ cosR sequence and *Nar*I-end sequence at each end as follows :

*Nar*I-end *Sma*I cosR
pCGCCCGGGAGGTCGCCGCCC
GGGCCC

This mixture was added to an agarose plug containing human DNA digested *in situ* with *Nar*I, and the sticky small duplex fragments were fused *in situ* by ligation to the *Nar*I ends of human DNA pieces. The resulting modified human DNA fragments were size-fractionated by PFG electrophoresis using a low-gelling temperature agarose. DNA was recovered from gel slices by agarase digestion. The terminally modified vector molecules were prepared by ligating small DNA pieces composed of a 5'-phosphorylated 20 mer (pCGCCCGGGGGGCGGCGACCT) and the unphosphorylated *Sma*I linker having the structure as follows ;

*Nar*I-end *Sma*I cosL
pCGCCCGGGGGGCGGCGACCT
GGGCCC

to *Nar*I-digested pOF216 plasmid DNA. The pOF216 vector molecules having artificial λ cosL termini were then purified by electrophoresis to remove free oligomers and the termini were 5'-phosphorylated before use.

Human DNA fragments with λ cosR termini and the vector with λ cosL termini thus obtained were ligated in solution at a dilution of below 0.5 μg per ml. Transformation was carried out using competent cells of SC1451. This new ligation system gave 5×10^3 and 1×10^3 Spc^R Alu^+ transformants per μg of insert DNA with DNA source IV (size range, 20 to 50 kb ; averaged size, 30 kb) and source V (30 to 100 kb ; averaged, 60 kb), respectively. Fig. 3(A) shows the ethidium bromide-stained profiles of *Not*I digests of seven clones selected from transformants obtained with the latter DNA source. *Not*I digestion of transformant DNA generated one extra band, as shown in lanes 2, 3, 4, and 7, the sizes of these bands being 68, 76, 88, and 91 kb, respectively. Some of these extra bands (lanes 4 and 7) overlapped the 91 kb *E. coli* band, but their presence was easily detected by their difference in staining intensity. Lanes 5, 6 and 8 show multiple extra bands, indicating that the inserts contained one or two endogenous *Not*I sites ; the sizes of the fragments were 48, 35 and 17 kb in lane 5, 75 and 20 kb in lane 6, and 43, 35 and 27 kb in lane 8. Densitometric measurements revealed that the staining intensities of these extra bands were 1.5 to 2.0 times those of the adjacent host chromosome bands. The gel was probed with the human Alu sequence and a spc-resistance gene fragment (Fig. 3(B) and (C)). All autoradiographic signals exactly overlapped the bands detected by staining, except the 17 kb band in lane 5, indicating that this fragment had a unique sequence derived from the human genome. These results, together with the fact that the copy number of the F factor is one to two per host chromosome, indicate that recombinant molecules replicated as extra chromosomal elements without any interaction with host chromosomal DNA.

The histogram in Fig. 4 shows the length distribution of cloned inserts in 68 Alu^+ Spc^R clones chosen at random from a library constructed with DNA source V. The insert length was widely distributed from 23 kb to 103 kb and was roughly parallel with

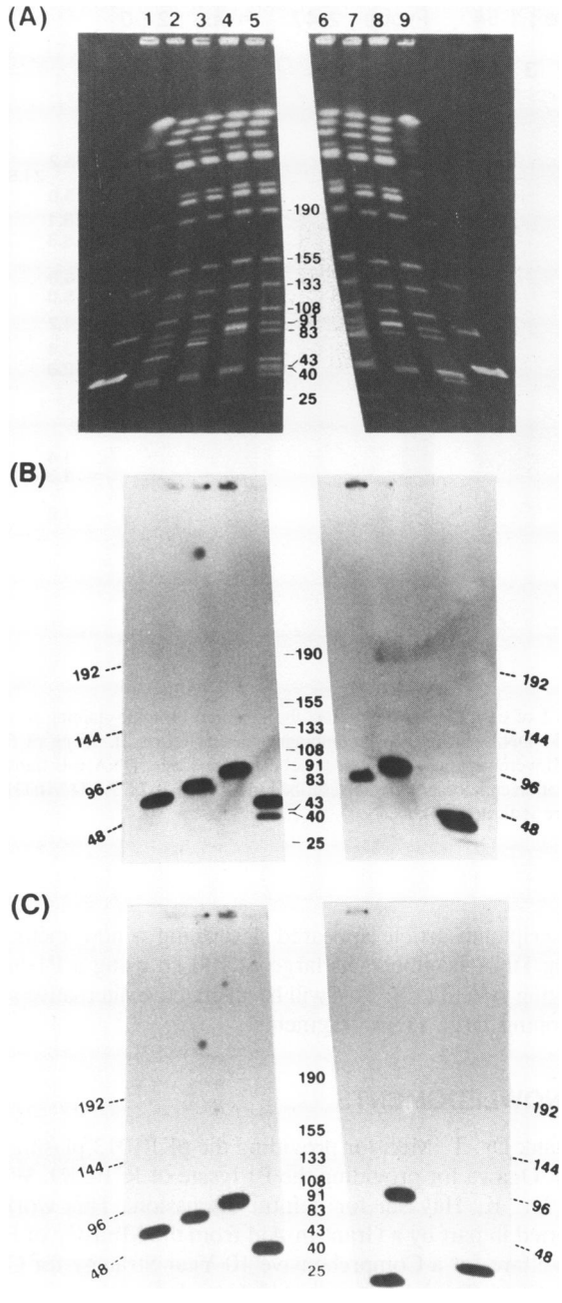

Figure 3. PFG electrophoretic analysis of recombinant plasmid clones. (A), ethidium bromide-staining pattern; (B), Southern hybridization probed with Alu; (C), Southern hybridization probed with the spc-resistance gene. Lanes 1 and 9; multimers of λ phage DNA. Lanes 2 to 8; *Not*I digestion patterns of independent *E. coli* transformant DNAs. The sizes of *E. coli Not*I fragments are indicated in kb.

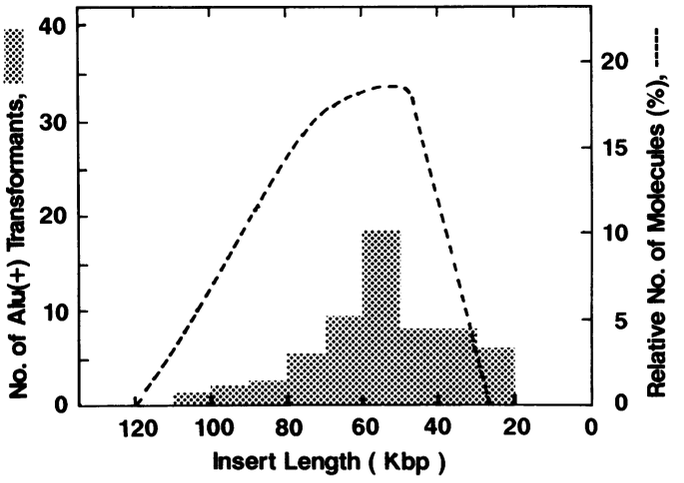

Figure 4. Length distribution of insert DNAs in Spc^R, Alu^+ transformants obtained by cos-adapter mediated ligation. The DNA source V was ligated to the adapter-attached pOF216 at a DNA concentration of 0.2 μg/ml. Transformation was carried out with SC1451.

that of the DNA fragments used as sources. The results do not show the heavy bias observed in the previous experiment, indicating that the introduction of artificial lambda cos ends is very effective for cloning large DNA molecules. The slight bias indicating more frequent cloning of shorter fragments is thought to be due to bias in the efficiency of transformation, because the gradual decrease in cloning efficiency with increase in insert size observed in Fig. 4, coincided well with the bias in transformation efficiency observed with F-based recombinant plasmids ranging in size from 50 kb to 105 kb : The transformation efficiencies were 4.5×10^6, 1.3×10^6, 1.9×10^5 and 1.7×10^5 transformants/μg with 50 kb, 75 kb, 95 kb and 105 kb plasmids, respectively. Although systematic studies have not been done with DNA sources larger than those discussed here, preliminary analysis of recombinant clones obtained from 70 to 150 kb DNA indicated that a significant fraction of transformants harbored inserts larger than 100 kb. We therefore have no evidence that there exists a strict limitation for cloning larger molecules than 100 kb.

An important problem was whether the insert fragments were faithfully cloned in this cloning system. To examine this problem, we compared the restriction patterns of the recombinant DNAs recovered with those of genomic DNAs. Of the 68 clones, we chose the six shown in Fig. 3 with insert sizes of 32 to 82 kb. Lane 1 of each column in Fig. 5 shows the ethidium bromide-stained pattern of the *Hind*III digest of the recombinant plasmid DNA. The sums of the lengths of the fragments generated were in good accord with the sizes estimated by PFG electrophoretic analyses. Autoradiograms show hybridization of *Hind*III digests of clonal DNA (lanes 2) and human DNA (lanes 4) with the corresponding recombinant plasmid probes. Human genomic fragments migrated slower than the corresponding clonal fragments as seen by comparison of lanes 2 and 4. However, this difference in migration rates was due to the difference in the amounts of DNA loaded because clonal fragments mixed in equimolar amount with genomic DNA also migrated more slowly concomitant with genomic fragments (lanes 3). A similar effect of loading has been observed in Southern hybridization to detect particular fragments in YAC clones and human DNA (18). The autoradiograms were over-exposed to detect signals for smaller fragments. Slowly migrating fragments were analyzed separately. Within the limits of resolution on agarose gel electrophoresis and genomic Southern hybridization, all the *Hind*III fragments derived from cloned DNA were detected in the genome, except junctional fragments representing sites of cloning of human DNA into the vector molecule : one end fragment is composed of a *Hind*III-*Nar*I fragment plus a 12.5 kb vector moiety, and the other is composed of a *Nar*I-*Hind*III fragment plus a 33 bp multi-cloning

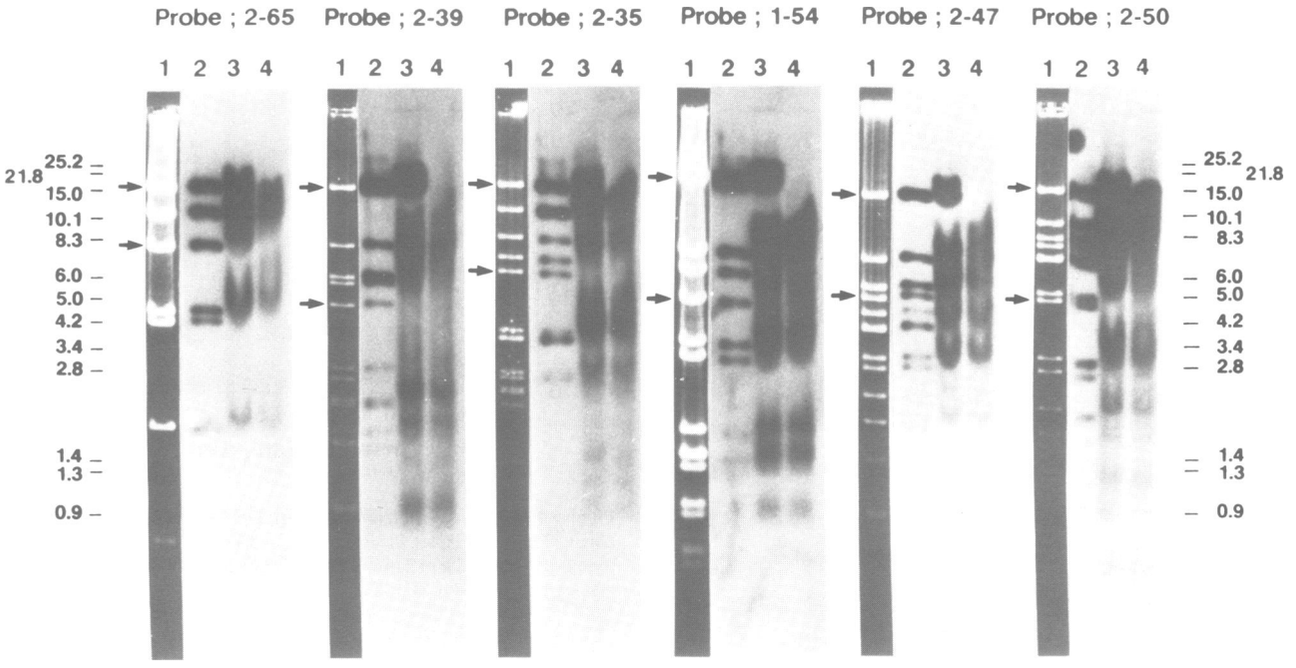

Figure 5. Comparison of the structures of cloned inserts and human genomic DNA. Lanes 1 of each column represents the ethidium bromide-staining pattern of the *Hind*III digest of purified plasmid DNA. Samples of 15 ng of *Hind*III digests of SC1451 DNA harboring a recombinant plasmid (lanes 2), 15 μg of *Hind*III digests of Namalwa DNA (lanes 4) and mixtures of the samples in lane 2 and lane 4 (lanes 3) were separated by agarose gel electrophoresis. DNA was transferred to a nylon membrane and the blot was probed with the corresponding plasmid DNA. Hybridization conditions were as described in MATERIALS AND METHODS. Arrows show the positions of DNA fragments containing a vector moiety. Size markers are indicated in kb.

site sequence. Southern hybridization analysis of *Hind*III digests of 8 other clones also showed no structural alteration (data not shown).

To test the stability of DNA, we isolated DNA from two transformants and from 5 single colony isolates of the same transformants after 20 generations of growth. The same insert sizes were found in all these clones ; no deletion or rearrangement was observed.

During evaluation of the transformation efficiencies of various size classes of F-based plasmids, we found that electroporation was 10 times more efficient than with competent cells for all the plasmids ranging in size up to 110 kb. Transformation efficiencies increased as following : 4.5×10^6 to 3.4×10^7 / μg with 50 kb plasmid, 1.3×10^6 to 8.9×10^6 / μg with 75 kb plasmid, 1.9×10^5 to 4.5×10^6 / μg with 95 kb plasmid and 1.7×10^5 to 2.6×10^6 / μg with 105 kb plasmid. These findings indicate that electroporation is also usable as a potent method to introduce large DNA molecules into *E. coli* cells as well as small plasmids. PFG electrophoretic analysis of transformants with 95 kb or 105 kb plasmid obtained by electroporation revealed no structural aberration compared with those of the parental DNAs. In the present study, we found a way to overcome the difficulty in the step of circle formation of large chimeric molecules. As we discussed, there is another difficulty in constructing a library containing large inserts ; namely the transformation efficiency decreases exponentially with increase in insert size. This difficulty might be overcome on the basis of the finding that polyamines can eliminate this size-dependent bias and also enhance the efficiency of transformation (Sternberg, N., personal communication). We are now attempting to up-grade our cloning system on the basis of this finding. During preparation of this manuscript, an article appeared describing a new method for cloning DNA fragments as large as 100 kb using a P1 *in vitro* packaging system (19). This will be an effective alternative system for cloning large DNA fragments.

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