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CHEMICAL SYNTHESIS OF VERSATILE ADAPTORS FOR MOLECULAR CLONING

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SUMMARY

In this communication we report the chemical synthesis of two types of oligodeoxynucleotides to be used as adaptors in molecular cloning. The first type is used to create specific cohesive end sequences at the termini of a blunt-end DNA molecule without the use of restriction enzymes. The second type of adaptor is used to convert one kind of restriction-enzyme-generated specific cohesive end to another. This includes both the conversion of one type 5'-protruding end to another 5'-protruding end, and of a 3'- to a 5'-protruding end and vice versa.

Recently, a general method was introduced for inserting DNA sequences into cloning vehicles (1,2). In this method, a chemically synthesized oligonucleotide duplex containing a specific restriction enzyme recognition sequence is joined by blunt-end ligation, using T_4 DNA ligase, to both ends of the DNA to be cloned. The resulting DNA is cleaved by the same restriction enzyme to generate specific cohesive ends which are then joined to the same sites of the cloning vehicle. This method has wide applicability in molecular cloning experiments (1,2). However, it cannot be applied in certain cases. Firstly, if the DNA fragment to be cloned already contains internally a certain restriction enzyme recognition sequence, that enzyme cannot be used for creating cohesive ends on the ligated adaptors without also cleaving the DNA internally. Although adaptors with different restriction enzyme recognition sequences can be chosen, a large DNA fragment is likely to contain many res-

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triction sites and therefore cannot be cloned this way. Secondly, any cohesive ends present in the DNA fragment inappropriate for joining to a specific cloning vehicle must be removed, either by digesting away the single strands by a singlestrand-specific nuclease (3) or by repairing the cohesive ends by a DNA polymerase (4,5). In either case, the restriction enzyme site present at the termini of the DNA fragment is lost and the cloned DNA cannot be re-isolated by using the same restriction enzyme. To overcome these limitations, we have synthesized two new types of oligodeoxynucleotides to be used as adaptors in molecular cloning. They include (a) preformed (ready-made) adaptors which do not require cleavage with restriction enzymes for creating cohesive ends, and (b) conversion adaptors which can be used for converting one kind of cohesive end into another. The chemical synthesis and applications of these adaptors in molecular cloning will be presented.

MATERIALS AND METHODS

Chemical Synthesis of Deoxyribooligonucleotides. The chemical synthesis of deoxyribooligonucleotides was carried out by the modified triester method developed in our laboratory (6,7,8). The reaction conditions are summarized in Table 1. The fully protected deoxymononucleotide was treated with an excess of anhydrous triethylamine-pyridine (9) at room temperature for 2-4 hr to remove selectively the β-cyanoethyl group. Excess triethylamine, pyridine, and acrylonitrile were removed by evaporation in vacuo to a foam. The coupling reaction was performed in situ with the incoming component containing 5'-hydroxyl group, and mesitylenesulfonyl (MS) tetrazole (6) in anhydrous pyridine as the condensing agent. After completion of the reaction an excess of bis(triazolyl)-p-chlorophenyl phosphate (7) was added to phosphorylate any unreacted starting material containing free 5'-hydroxyl group. After the usual work-up (7) the crude mixture was applied to silica-gel thin layer plates which were developed with 10% methanol-chloroform solvent. The desired product was isolated from the plates by two elutions with 60 ml of 20% methanol-chloroform solvent.

Methods for deblocking of the fully protected deoxyribooligonucleotides have been reported (7). The oligodeoxynucleotide compounds containing phosphodiester bonds were purified on PEI-Cellulose thin layer plates and developed at 60° with 0.5 M lithium chloride-7 M urea solvents (10). Each of the purified compounds (200 pmol) was phosphorylated with T_4-polynucleotide kinase and [γ-^{32}P]ATP (10,11). Their sequences were determined using the mobility-shift analysis (11) (Figure 1).

Construction of BamI and EcoRI Preformed Adaptors. The EcoRI preformed adaptor was constructed by annealing 40 pmol of 5'd(HO-A-A-T-T-C-C-C-G-G-G) and 100 pmol of 5'd(pC-C-C-G-G-G) at 70°C for 30 min, followed by slow cooling to room temperature. The BamI preformed adaptor was constructed in a similar way (Fig. 2, structure I) using d(HO-G-A-T-C-C-C-C-G-G-G) and d(pC-C-C-G-G-G).

The preformed adaptor (20 pmol) was joined to a blunt-ended DNA molecule (2 pmol) by incubation with 1-2 units of T_4 ligase (Figure 2, step a) in 100 µl of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 10 mM $MgCl_2$ and 30 µM ATP at 20°C for 6 hr. The solution was heated to 70° for 5 min and cooled slowly to room temperature whereupon 25 µl of 5 M NaCl and 300 µl of ethanol were added. The contents were kept at -20° overnight and centrifuged at 10,000 RPM for 30 min. The resulting DNA-adaptor hybrid was then phosphorylated at the 5' ends (12) (Figure 2, step b), joined to the BamI-enzyme cut pBR322 DNA (Step c), and cloned (1,12).

Construction of BamI-EcoRI Conversion Adaptor. The BamI-EcoRI conversion adaptor was constructed by annealing 100 pmol each of the single-stranded BamI decamer, 5'd(G-A-T-C-C-C-C-G-G-G), and the EcoRI decamer, 5'd(A-A-T-T-C-C-C-G-G-G) at 70° for 30 min and slowly cooling to room temperature. The resulting duplex molecule contained the desired product with 5'd(A-A-T-T-) as one protruding end and 5'd(G-A-T-C) as the other protruding end (Figure 3a, structure I). The mixture was used without purification since side products would not be cloned (Figure 3b).

An alternative method was also used to prepare the conversion adaptors. For example, 500 pmol each of double-stranded HindIII and BamI double-stranded adaptor (1) were incubated with 5 units of T_4 ligase in 140 µl of a solution containing 20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol and 35 µM ATP. The mixture was incubated overnight at 20° (16 hr), then heated at 70° for 5 min, and slowly cooled to 4°. The total ligation mixture was digested with restriction enzymes HindIII and BamI (1) to produce HindIII-BamI conversion adaptor. Similar conversion adaptors were also prepared by ligating EcoRI and BamI or EcoRI and HindIII adaptors and digesting these with the appropriate restriction enzymes.

Cloning of a DNA fragment with EcoRI ends at the BamI site of pBR322 DNA by the use of BamI-EcoRI conversion adaptor. The DNA fragment (DNA-X in Figure 3a) with EcoRI end was first ligated to the BamI-EcoRI converison adaptor (Structure I). To avoid polymerization of the adaptor, the 5'd(HO-G-A-T-C) end on the conversion adaptor was kept dephosphorylated. The mixture was purified on a Sephadex G-75 column to remove unreacted conversion adaptor (1) and the product phosphorylated with ATP and polynucleotide kinase. In step (b) the DNA-X with BamI cohesive ends was ligated to BamI-cut pBR322 DNA (13). The reaction was worked up as usual and then used for transformation (1).

RESULTS AND DISCUSSION

Synthetic oligodeoxynuceotides of defined sequence can serve as useful tools in solving various problems in molecular biology (14). In order to extend the usefulness of the earlier method (1,2) we have synthesized several new types of adaptors.

Preformed Adaptors. The preformed adaptor has a blunt and a single-stranded cohesive end corresponding to one of the restriction enzyme recognition sites. Since the cohesive end is already present, the need for its creation with a restriction enzyme (1,14) is eliminated. As shown in Figure 2 (Step a), the preformed adaptor (Structure I) was used for cloning by blunt-end ligation (15)

Table I. Reaction conditions and the yields of various deoxyoligonucleotides

5'-Protected[a] Component (μmole)	5'-Hydroxyl Component (μmole)	Mesitylenesulfonyl Tetrazole (μmole)	Reaction Time (hrs)	Fully Protected Products & Yield (%)
		I Ready-made BamI Adaptor 5' G-A-T-C-C-C-C-G-G-G 3' G-G-G-C-C-C 5'		
[(MeO)$_2$Tr]dbzC-bzC-bzC-ClPh (40)	dIsoG-IsoG-IsoG(OAc) (28)	120	2 hr	[(MeO)$_2$Tr]dbzC-bzC-bzC-IsoG-IsoG-IsoG(OAc) (60)
[(MeO)$_2$Tr]dIsoG-bzA-T-bzC-ClPh (10)	dbzC-bzC-bzC-IsoG-IsoG-IsoG(OAc) (5)	30	4 hr	[(MeO)$_2$Tr]dIsoG-bzA-T-bzC-bzC-bzC-bzC-IsoG-IsoG-IsoG(OAc) (62)
		II Ready-made EcoRI Adaptor 5' A-A-T-T-C-C-C-G-G-G 3' G-G-G-C-C-C 5'		
[(MeO)$_2$Tr]dbzA-bzA-T-T-T-ClPh (11)	dbzC-bzC-bzC-IsoG-IsoG-IsoG(OAc) (9)	30	4 hr	[(MeO)$_2$Tr]dbzA-bzA-T-T-T-bzC-bzC-bzC-IsoG-IsoG-IsoG(OAc) (55)
		III Single-strand EcoRI-Pst I Adaptor 5' A-A-T-T-C-C-T-G-C-A 3'		
[(MeO)$_2$Tr]dbzA-bzA-T-T-T-bzC-ClPh (12)	dbzC-T-IsoG-bzC-bzA(OAc) (14)	80		[(MeO)$_2$Tr]dbzA-bzA-T-T-T-bzC-bzC-T-IsoG-bzC-bzA(OAc) (68)
		IV Single-strand EcoRI-Hae II Adaptor 5' A-A-T-T-C-G-G-C-G-C 3'		
[(MeO)$_2$Tr]dbzA-bzA-T-T-T-bzC-ClPh (12)	dIsoG-IsoG-bzC-IsoG-bzC(OAc) (12)	36	4 hr	[(MeO)$_2$Tr]dbzA-bzA-T-T-T-bzC-IsoG-IsoG-bzC-IsoG-bzC(OAc) (57)

[a] Triethylammonium salt was used in each coupling.

[b] Abbreviations are as suggested by the IUPAC-IUB, Biochemistry 9, 4022 (1970). A phosphodiester linkage is represented by hyphen and phosphotriester linkage is represented by (-) symbol. Each internal internucleotidic phosphate is protected with p-chlorophenyl group (ClPh).

to the DNA to be cloned (Structure II, in this example, a chemically synthesized lac operator duplex (1)). The adaptor can only join the DNA fragment at the blunt end since the BamI cohesive end (5'G-A-T-C) that lacks a 5' phosphate cannot be ligated. The DNA that carries a preformed BamI adaptor on each end (Structure III) was next phosphorylated (Structure IV) and joined to BamI-cut pBR322 DNA. The hybrid lac-pBR322 DNA (vehicle DNA-X hybrid) was used for transformation of E. coli cells (12). The insertion of lac operator into plasmids was confirmed by the appearance of blue colonies on X-Gal plates (12), and by quantitative β-galactosidase assay (16). As shown in Table 2, in experiment 2, E. coli carrying a hybrid lac-pBR322 plasmid produced a high level of β-galactosidase (0.22), equal to that reported earlier (16) for cloned hybrid lac-pMB9 (experiment 4). Another advantage of this adaptor is that its

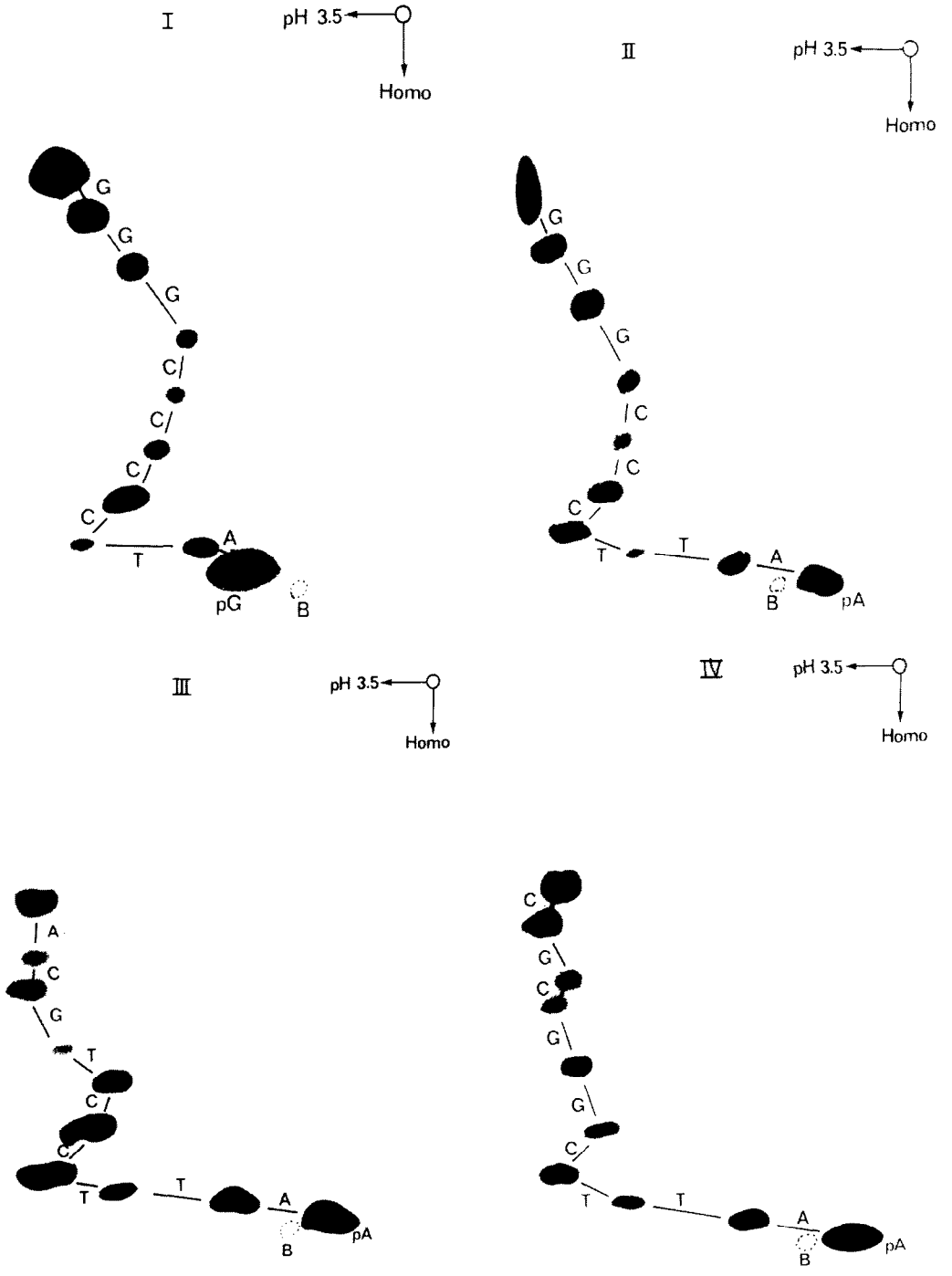

Figure 1. Two dimensional homochromatography fingerprint of synthetic adaptors after partial snake venom phosphodiesterase digestion of (I) 5'd (^{32}pG-A-T-C-C-C-C-G-G-G) and (II) d (^{32}pA-A-T-T-C-C-C-G-G-G), (III) d (^{32}pA-A-T-T-C-C-T-G-C-A) and (IV) d (^{32}pA-A-T-T-C-G-G-C-G-C). The first dimension is electrophoresis on cellulose acetate strip at pH 3.5, and the second dimension is homochromatography on 20 x 20 cm DEAE-cellulose thin-layer plate (11). B represents the location of the Xylene cyanol FF dye marker.

Table 2 β-galactosidase specific activity in *E. coli* cells carrying plasmid DNA or hybrid *lac* operator-plasmid DNA

Experiment	*E. coli* carrying plasmid§	Size of Cloned *lac* Operator	Adaptor Used	Color of Colony+	β-galactosidase specific activity*
1	pBR322	-	-	cream	0.004
2	*lac*-pBR322	21	Preformed *Bam*I	dark blue	0.220
3	*lac*-pBR322	21	*Bam*I-*Eco*-RI conversion	dark blue	0.210
4	*lac*-PMB9	21	None	dark blue	0.240

§ *E. coli* cells carrying pMB9 plasmid or pBR322 plasmid were gifts from H. Boyer. *E. coli* cells carrying *lac*-pMB9 plasmid were as described (12). *E coli* strain CGSC 5346 (also known as M94 of M. Meselson) was from J. Calvo; cells from this strain were used as recipients for *lac*-pBR322 hybrid plasmids.

+ Plasmids pMB9 and pBR322 are present in 10-20 copies per cell. Because there are only about 10 molecules of *lac* repressor per cell, *E. coli* clones containing the *lac* operator ligated to either plasmid pMB9 or pBR322 should have all *lac* repressor molecules bound to the *lac* operator on plasmids, leaving the *E. coli* *lac* operon derepressed (12). The large amounts of β-galactosidase produced in the cell will break down X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in the nutrient plate and release the blue dye 5-bromo-4-chloro-indigo.

* The β-galactosidase specific activity (Z/B) was as defined by Jobe et al. (18). In the presence of inducer (1 mM isopropylthiogalactoside), the β-galactosidase specific activity was approximately 2.0 in all experiments.

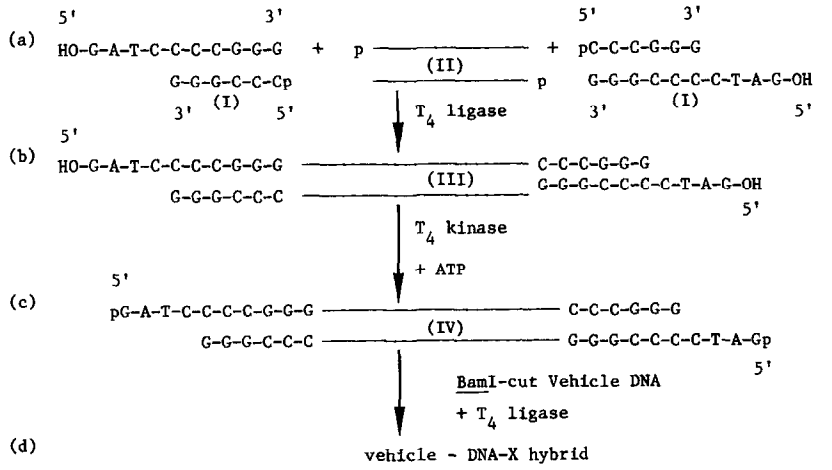

Figure 2. A scheme for insertion of blunt-end DNA into cloning vehicle by preformed adaptors (for details, see text and reference 1).

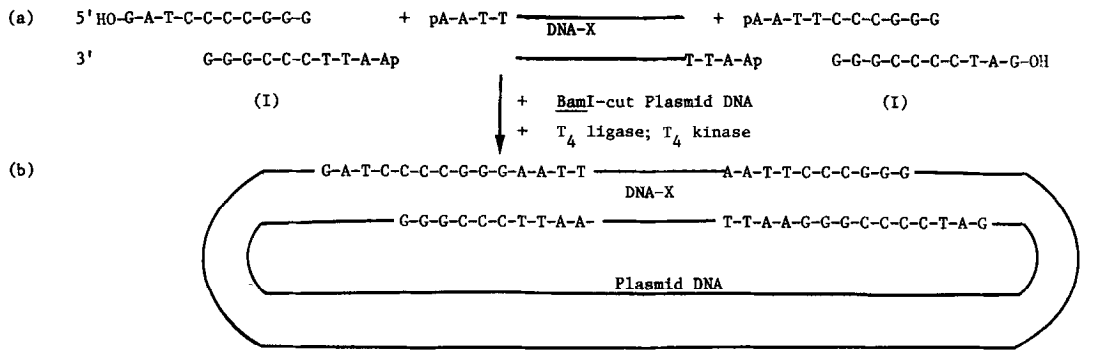

Figure 3. A scheme for conversion of one type of cohesive end to another by the use of adaptors.

six-nucleotide-long duplex region has been designed to contain the recognition sequence for XmaI and SmaI (17) so that an inserted fragment can be cleaved and recovered intact by either enzyme.

Conversion Adaptors. A second type of adaptor has been designed for cases in which a DNA fragment obtained by digestion with one restriction endonuclease is to be cloned at a different restriction site on the vehicle DNA. For example, for cloning an EcoRI enzyme-digested DNA fragment at the BamI site of the cloning vehicle, a BamI-EcoRI double-headed conversion adaptor was synthesized. This adaptor may be prepared in two ways. The first involves blunt-end ligation (15) of two different types of adaptors (blunt end or preformed) and have been described under Materials and Methods. The second method involves the synthesis of two different single-stranded decadeoxynucleotides (Table I, the upper strands of duplex I and II), each containing a sequence of four nucleotides at the 5' end corresponding to the central part of the recognition sequence of two restriction enzymes and a six-nucleotide-long d(C-C-C-G-G-G) sequence self-complementary) at the 3' end. When the two decanucleotides are annealed together, a double-headed conversion adaptor with a BamI cohesive end on one side and an EcoRI cohesive end on the other is formed (Figure 3a, Structure I). To test the usefulness of this adaptor for cloning, a BamI-EcoRI conversion adaptor was ligated to the lac operator fragment carrying EcoRI cohesive ends.

Then, the lac operator (DNA-X) now carrying BamI cohesive ends was joined to the BamI-enzyme-cut pBR322 plasmid DNA and cloned (1,12). Successful cloning was shown by the high level of β-galactosidase activity (Table 2, experiment 3) produced in the E. coli carrying the cloned lac operator (at BamI site)-pBR322 plasmid. In control experiments, with plasmid alone (experiment 1), or with the ligase omitted, only 0.004 units of β-galactosidase was produced. Thus, when the lac operator (21-nucleotides long) with EcoRI cohesive ends was converted to BamI ends and cloned with the plasmid, the E. coli now produced 50 times more β-galactosidase (experiment 3). Other methods in characterizing the cloned lac operator, including the excision of the lac operator from the hybrid lac-pBR322 plasmid and determining its size by gel electrophoresis, have been reported previously (1,12). This procedure may also be used for cloning a DNA fragment which has an EcoRI recognition sequence at one end and a BamI sequence at the other end, at either the BamI site or the EcoRI site of the plasmid. Conversion adaptors containing other restriction enzyme recognition sequences are now being synthesized to make the approach more widely applicable.

Conversion adaptors for 3' protruding ends. So far, we have discussed only the conversion adaptor involving restriction enzymes which give 5' protruding ends. However, there are several restriction enzymes which digest DNA to give 3'-protruding ends (see 17). The best way to clone a DNA fragment with 3'-protruding ends (e.g. from digestion with PstI enzyme) at the EcoRI or BamI site on the cloning vehicle is to use single-stranded conversion adaptors. These adaptors are single-stranded decadeoxynucleotides containing two five-nucleotide sequences, one of which is complementary to part of the recognition sequence of a 3'-protruding end, and the other to the 5'-protruding sequence of a restriction enzyme. Two such adaptors, the EcoRI-PstI and the EcoRI-HaeII adaptors (Table I, Structure III and IV) have been synthesized. For cloning, an EcoRI-HaeII adaptor (5'A-A-T-T-C-G-G-C-G-C) can be inserted by ligation in between a DNA fragment containing 3' protruding ends (e.g. G-C-G-C ends produced by HaeII

enzyme) and a cloning vehicle with 5' protruding ends (e.g. A-A-T-T ends produced by EcoRI enzyme). The two-nucleotide gap at each end can then be filled by repair synthesis *in vitro* (12), or can be filled *in vivo* after transformation.

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