

In vivo site-specific genetic recombination promoted by the *EcoRI* restriction endonuclease

(DNA/plasmid/cloning)

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ABSTRACT Site-specific genetic recombination promoted *in vivo* by the *EcoRI* endonuclease has been demonstrated by using constructed hybrid plasmids in which the chloramphenicol resistance gene was inactivated by insertion of DNA fragments at an *EcoRI* site within the gene. Such recombination can involve either the joining of intracellularly generated cohesive termini of the same DNA fragment or intermolecular ligation of different DNA fragments. DNA cleavage and ligation *in vivo* are precise: recombinant DNA molecules show functional continuity of the gene sequence cleaved by the enzyme and regeneration of nucleotide recognition sites for both the *EcoRI* endonuclease and the *EcoRI* DNA methylase. In other experiments, *EcoRI*-generated fragments of eukaryotic DNA that had not been modified by the *Escherichia coli* K methylase were shown to be taken up by bacterial cells and to undergo intracellular ligation to segments of bacterial plasmid DNA.

The ability of restriction endonucleases to cleave DNA *in vitro* at specific sites and the capacity of DNA ligases to join endonuclease-generated DNA segments to plasmid and viral replicons have been important elements in the development of DNA cloning methods (1). Although methods for cleaving and joining DNA segments in cell-free systems have enabled construction of a wide variety of recombinant DNA molecules that have not been observed in nature, there is reason to suspect that restriction endonuclease-promoted genetic recombination may also occur intracellularly as a natural biological process. Endonucleases similar to those used for *in vitro* recombination experiments are found in a wide variety of bacterial species (2, 3); although site-specific endonucleases are commonly called "restriction enzymes," some bacterial species that contain the enzymes show no detectable restriction of foreign DNA *in vivo*, and it has been speculated that the primary function of such endonucleases may be DNA recombination (2, 4, 5). DNA ligases are also widespread in nature (6) and potentially could play a role in site-specific recombinational events.

In early DNA cloning experiments, separate fragments of R6-5 plasmid DNA generated *in vitro* by the *EcoRI* endonuclease were found to be ligated together *in vivo* after their introduction into *Escherichia coli* by transformation (7). Furthermore, the biological characteristics of restriction/modification systems have suggested that DNA cleavage by restriction endonucleases occurs also *in vivo*: the plaque-forming ability of infecting phage DNA is restricted by several orders of magnitude in cells that produce the *EcoRI* enzyme (8, 9), implying that most entering DNA molecules are cleaved *in vivo* before they can be methylated by the modification enzyme associated with the *EcoRI* restriction system.

The experiments reported here demonstrate that site-specific genetic recombination is promoted *in vivo* by the *EcoRI* re-

striction endonuclease. The recombinant DNA molecules formed by the *in vivo* ligation of DNA fragments that have been cleaved intracellularly by the *EcoRI* enzyme are structurally and genetically indistinguishable from those constructed *in vitro* by the use of recombinant DNA methods. In addition, *EcoRI*-generated fragments of eukaryotic DNA unmodified by the *E. coli* methylase have been shown to undergo ligation *in vivo* to segments of bacterial plasmid DNA.

MATERIALS AND METHODS

E. coli K-12 strain C600 and its derivative C600 $r_{\bar{c}}m_{\bar{c}}$ (SC181) have been described (10). The ligase-overproducing strain SF8 (*lop*-11, *recB*⁻, *recC*⁻) was kindly provided by F. Schachat. The sources and relevant properties of plasmids used are listed in Table 1.

L-broth (17) and Penassay agar (Difco) plates were used for bacterial growth. Antibiotic concentrations used were 20 μ g/ml for ampicillin (Ap), kanamycin (Km), and chloramphenicol (Cm) and 10 μ g/ml for tetracycline (Tc). The general procedures used for preparing covalently closed circular plasmid DNA (18, 19), electrophoresis in 0.7% agarose gels, staining of gels with ethidium bromide (20, 21), and ligation of *EcoRI* endonuclease-generated fragments *in vitro* (22) have been described. Digestions of plasmid DNA with restriction enzymes *EcoRI*, *HindIII* (Miles Laboratories), and *Sal I* (BioLabs) were carried out in 10 mM Tris-HCl, pH 7.9 (at 37°)/60 mM NaCl/7 mM MgCl₂/6 mM 2-mercaptoethanol. Transformation of *E. coli* by plasmid DNA was performed essentially as described previously (23) with minor modifications: competent cells resuspended in 1/25 of the original volume in 30 mM CaCl₂ were mixed with plasmid DNA in a 17 × 100 mm culture tube; after 20-min incubation on ice and 2-min heat pulse (42°), 5 ml of L-broth was added and growth was then allowed for 2 hr at 37°. When necessary, portions or all of the cells in the transformation mixture were pelleted and resuspended in a small volume of broth before plating on selective agar plates.

P2 containment conditions were used for the propagation of plasmids containing purified fragments of previously cloned mouse mitochondrial DNA and for the cloning and purification of *Bacillus pumilus* plasmid DNA.

RESULTS

Ligation of *EcoRI*-Generated DNA Fragments *In Vivo*. Earlier experiments have shown that an *EcoRI* endonuclease cleavage site splits the Cm gene of the R6-5 plasmid (K. Timmis, F. Cabello and S. N. Cohen, unpublished data). Plasmid pFC001 (see Table 1) carries one segment of this gene; the pCR1 plasmid, which contains a Km-resistance segment de-

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Abbreviations: Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline.

Table 1. Relevant properties of plasmids used

Plasmid	Relevant genes	Derivation of plasmid	Ref.
pFC001	None	<i>EcoRI</i> fragment 1 of R6-5 inserted into <i>ColE1</i>	*
pACYC 111	<i>Ap, Tc</i>	From pMM22 (11) by translocation of Tn3 to <i>EcoRI</i> fragment of mouse mitochondrial DNA	†
pACYC 189	<i>Km, Cm</i>	<i>ColE1</i> derivative containing single <i>EcoRI</i> site in <i>Cm</i> gene from R6-5	†
pMB4	<i>Ap</i>	Codes for <i>EcoRI</i> restriction and modification phenotype	(12)
pACYC 184	<i>Tc, Cm</i>	p15A derivative containing single <i>EcoRI</i> site in <i>Cm</i> gene from R6-5	†
pML21	<i>Km</i>	Mini- <i>ColE1</i> derivative contains <i>Km</i> -resistance fragment from R6-5 via pSC105	(7, 13)
pCR1	<i>Km</i>	Contains <i>ColE1</i> plus <i>Km</i> -resistance fragment from R6-5 via pSC105	(7, 14, 15)
pPL10	None	<i>Bacillus pumilus</i> plasmid with two clustered <i>EcoRI</i> sites	(16)

* K. Timmis, F. Cabello, and S. N. Cohen, unpublished data.

† A. Chang and S. N. Cohen, unpublished data.

rived originally from R6-5 (7, 14, 15), carries a single *EcoRI* cleavage site and the remainder of the *Cm* gene. Transformation of bacterial cells with a mixture of *EcoRI*-cleaved pFC001 and pCR1 DNA should result in *Cm*-resistant bacteria only when the *Cm* resistance gene is reconstructed by the precise *in vivo* ligation of its two components.

E. coli C600 bacterial cells were transformed with equal amounts (1.25 $\mu\text{g}/\text{ml}$ of each) of *EcoRI*-digested pFC001 and pCR1 plasmid DNA, and *Cm*-resistant transformants (80 transformants per μg of DNA) were selected. Twenty-five randomly chosen *Cm*-resistant transformants were all found to be concurrently resistant to *Km*, as expected, confirming that the DNA segment of pCR1 that is required for reconstruction of the *Cm*-resistance gene also carries the genetic determinant for *Km* resistance. Agarose gel electrophoresis of one of these clones (designated pSC350) (Fig. 1A) verified the conclusion from genetic evidence that reconstruction of a functional *Cm* resistance gene involves ligation of the pCR1 plasmid to the fragment of pFC001 derived from R6-5.

In vivo ligation in *E. coli* of *EcoRI*-cleaved plasmid DNA to similarly cleaved fragments of eukaryotic origin was also observed by using the following assay system. Plasmid pACYC111 contains pSC101 plus two *EcoRI* fragments derived from mouse mitochondrial DNA (11); the Tn3 transposon, which includes *Ap* resistance gene derived originally from pSC50 (24) was introduced as a marker onto the larger of the two eukaryotic DNA fragments, by the selected translocation procedure (25). pACYC189 is a constructed plasmid that carries both the *Cm*- and *Km*-resistance genes from R6-5 (A. C. Y. Chang and S. N. Cohen, unpublished data). Introduction of a foreign DNA fragment into the *EcoRI* site of pACYC189 leads to insertional inactivation (26) of the *Cm*-resistance gene of the plasmid.

EcoRI digests of pACYC111 DNA, isolated from $r\bar{c}m\bar{k}$ host (*E. coli* strain SC181) and pACYC189 plasmid DNA obtained

from *E. coli* strain C600 were mixed in a 1:3 ratio (0.25 μg of pACYC111 DNA, 0.75 μg of pACYC189 DNA) and used to transform C600 cells as described in *Materials and Methods*. Two *Ap*-*Km*-resistant transformants were isolated and tested for *Cm* sensitivity to distinguish bacteria carrying recombinant plasmids from doubly transformed cells. Both of the *Ap*-*Km*-resistant clones were *Cm*-sensitive, suggesting that the mouse mitochondrial DNA fragment carrying the Tn3 transposon had been introduced into the *EcoRI* site within the *Cm*-resistance gene of pACYC189; agarose gel analysis of *EcoRI*-treated plasmid DNA (e.g., pSC351, Fig. 1A) isolated from these clones showed a digestion pattern consistent with the above interpretation. Digestion of the pACYC111 and pSC351 plasmids with combinations of *EcoRI*, *Bam* I and *Hind*III endonucleases and electron microscope analyses of heteroduplexes formed between these plasmids (data not shown) provided further confirmation of the *in vivo* linkage of mouse cell mitochondrial DNA to the pACYC189 plasmid. We conclude from these results that *in vivo* ligation of unmodified eukaryotic DNA fragments to prokaryotic plasmid DNA can occur in bacterial cells. However, the observed frequency of *Ap*-resistant transformants was low, as would be expected from the introduction of unmodified DNA into a strain carrying a competent *E. coli* *K* restriction system (27).

Site-Specific Recombination Promoted *In Vivo* by the *EcoRI* Endonuclease. In order to determine whether the combined actions of the *EcoRI* endonuclease and DNA ligase can accomplish site-specific genetic recombination *in vivo*, we used a series of plasmids derived from pACYC184 (Fig. 2); all of the plasmids were constructed and cloned by previously described procedures (see *Materials and Methods*), and each contained an *EcoRI*-generated DNA fragment that had been introduced *in vitro* into the *EcoRI* cleavage site within the *Cm* gene of pACYC184. In each case the introduced fragment caused insertional inactivation of the *Cm*-resistance gene. Expression of *Cm* resistance requires both precise excision of the introduced *EcoRI* fragment and ligation of the resulting termini of the pACYC184 segment of these plasmids.

Cm-resistant clones were obtained after transformation of CaCl_2 -treated *E. coli* C600 by pSC352 plasmid DNA that was subjected to *in vitro* treatment with the *EcoRI* endonuclease (Table 2). Transformation of the same strain by untreated pSC352 DNA yielded no *Cm*-resistant transformants during experiments that produced a total of 2×10^7 *Tc*-resistant transformants. Transformation of pSC352 DNA into strain C600 carrying the *EcoRI* endonuclease-producing plasmid pMB4 resulted in a reduced number of *Tc*-resistant transformants, consistent with the expected restriction of incoming DNA by the endonuclease product of pMB4. A small fraction of the *Tc*-resistant transformants (6×10^{-5}) were concurrently resistant to *Cm*, suggesting that the DNA segment that had been introduced into the *EcoRI* endonuclease cleavage site had been excised and that a functional *Cm* gene had been reconstituted.

Several *Cm*-resistant derivatives of pSC352 were selected for further study by agarose gel electrophoresis. Because these plasmids were isolated from cells that concurrently carried the pMB4 plasmid, their *EcoRI* cleavage sites were not susceptible to *in vitro* digestion by the enzyme as a result of the action of the DNA methylase specified by the pMB4 plasmid. The two plasmids were separated by a cycle of transformation (28) to produce recognition sequences that could be cleaved by the *EcoRI* enzyme. Analysis of a representative *Cm*-resistant pSC352-derived plasmid [i.e., pSC353 (Fig. 1B and C)] showed *EcoRI* cleavage and *EcoI*-*Sal* I double digestion patterns indistinguishable from those of plasmid pACYC184. These results

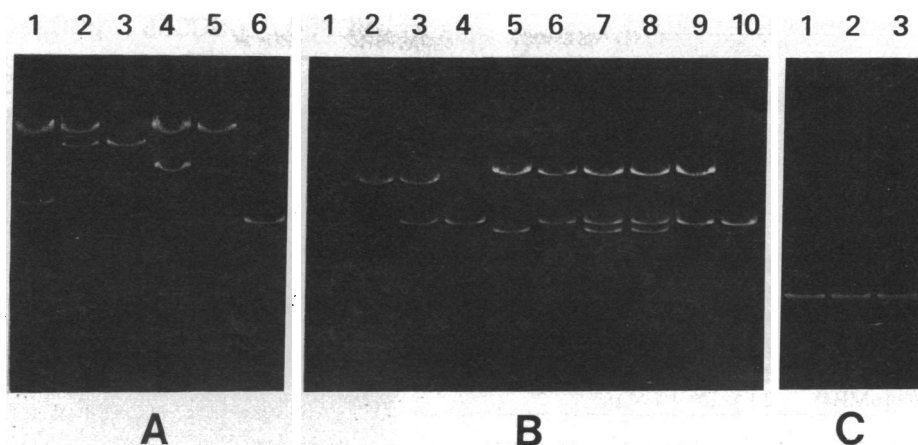


FIG. 1. Agarose gel electrophoresis patterns. Electrophoresis was carried out in a 0.7% agarose slab gel (Tris-borate buffer, pH 8.5) for 1.5 hr at a constant 150 V at room temperature. The gel was stained with ethidium bromide and photographed under UV light as described (20). (A) *Eco*RI digests of plasmid DNA: (1) pFC001, (2) pSC350, (3) pCR1, (4) pACYC111, (5) pSC351, and (6) pACYC189. (B) *Eco*RI digests of plasmid DNA: (1) pACYC184, (2) pPL10, (3) pSC352, (4) pSC353, (5) pML21, (6) pSC354, (7) pSC355, (8) pSC356, (9) pSC357, and (10) pSC358. An additional fast-moving band representing a DNA fragment of approximately 0.6 kb base was present in the *Eco*RI digest of pPL10 plasmid but was visible only when analyzed in 1% agarose gel. (C) *Eco*RI-*Sal*I digests of plasmid DNA: (1) pACYC184, (2) pSC353, and (3) pSC358. Note that each pattern contains two barely resolved DNA bands.

indicate that: (i) the *Eco*RI endonuclease acting *in vivo* in C600(pMB4) bacterial cells is capable of cleaving incoming pSC352 plasmid DNA at *Eco*RI sites bracketing an inserted DNA fragment, and (ii) the linear pACYC184 plasmid DNA generated by excision of the foreign DNA fragment is recircularized and ligated precisely *in vivo* to form a biologically functional replicon. These results also demonstrate that the nucleotide recognition sequence for the *Eco*RI methylase is reconstituted accurately by the *in vivo* action of the *Eco*RI endonuclease and DNA ligase and that methylation of the re-constructed sequence occurs intracellularly.

*Eco*RI-promoted site-specific *in vivo* recombination involving multiple and physically separate fragments of plasmid DNA was also observed (Table 2). The pSC355 and pSC356 plasmids were both constructed *in vitro* by joining pACYC184 to another plasmid [i.e., pML21, (13)] containing mini-ColE1 and the Km-resistance fragment from R6-5; however, the orientation of the component fragments of pSC355 and pSC356 was different (Figs. 2 and 3). Excision of both the mini-ColE1 and Km-resistant fragments from either of these plasmids would be expected to result in Cm- Tc-resistant (but Km-sensitive) plasmids indistinguishable from pACYC184. Formation of a

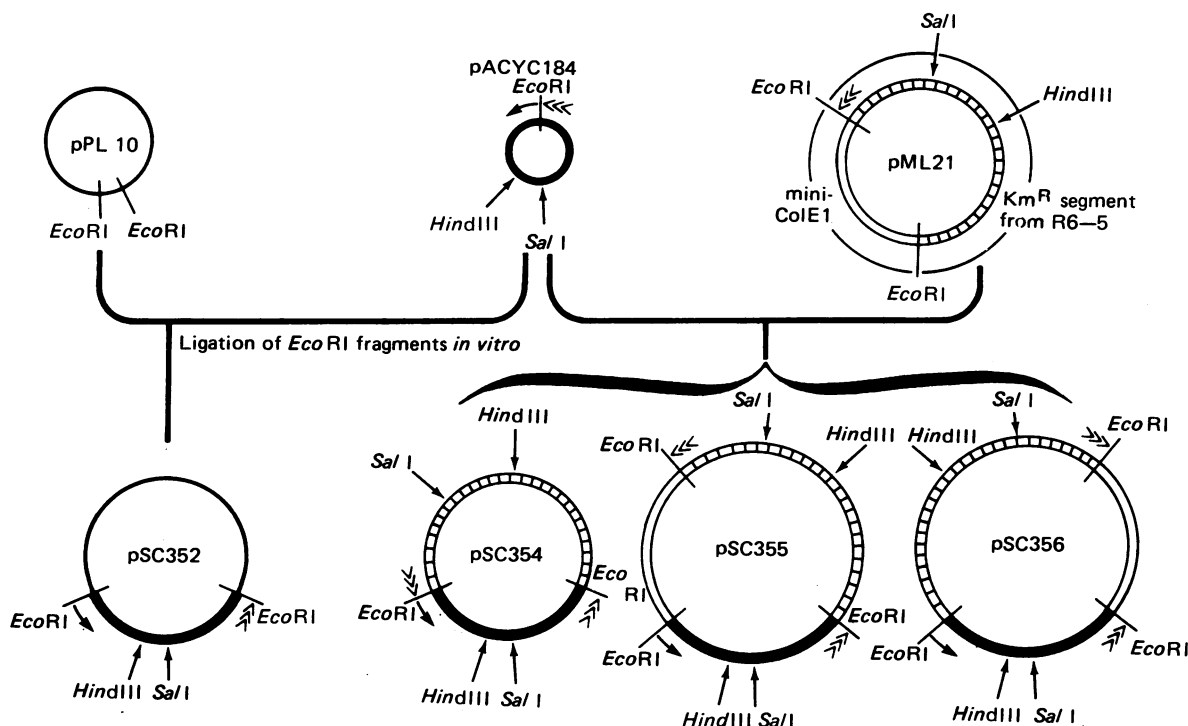


FIG. 2. Schematic presentation of the genealogy and structure of the plasmid chimeras used in the experiments reported. The endonuclease cleavage maps of the constructed plasmids were determined from agarose gel electrophoretic analyses of plasmid DNA digested with *Eco*RI, *Sal*I, and *Hind*III endonucleases (see Figs. 1 and 3). The locations of the relevant *Eco*RI, *Sal*I, and *Hind*III sites are indicated. The arrow (→) indicates continuity or discontinuity of the *Cm* gene: it does not indicate the size of the transcript or the direction of transcription. The initial cloning of the pPL10 plasmid was carried out in *E. coli* strain χ 1776.

Table 2. Transformation of plasmid DNA*

Plasmid DNA	CaCl ₂ -treated cells	Transformants, [†] no./μg of DNA		
		Tc ^R	Km ^R	Cm ^R ‡
A pSC352 (<i>Eco</i> RI-digested <i>in vitro</i>)	C600	6.6 × 10 ³	—	5.6 × 10 ³
B pSC352	C600	3.7 × 10 ⁶	—	0
	C600(pMB4)	2.5 × 10 ⁵	—	16
C pSC355	C600	3.5 × 10 ⁶	2.8 × 10 ⁶	0
	C600(pMB4)	2.2 × 10 ⁵	1.9 × 10 ⁵	9
D pSC356	C600	3.5 × 10 ⁶	2.7 × 10 ⁶	0
	C600(pMB4)	2.1 × 10 ⁵	1.7 × 10 ⁵	7

* Transformations were carried out as described in *Materials and Methods* with 1 μg of plasmid DNA in a total volume of 0.4 ml of transformation mixture (final DNA concentration, 2.5 μg/ml). Plasmid DNA digested *in vitro* by the *Eco*RI endonuclease was used in experiment A; closed circular plasmid DNA was used in the other transformation assays.

† Tc^R, tetracycline-resistant; Km^R, kanamycin-resistant; Cm^R, chloramphenicol-resistant.

‡ Additional Cm^R transformants were obtained from C600(pMB4) bacteria in separate experiments. The total number of Cm^R transformants obtained from such cells transformed with plasmids pSC352, pSC355, and pSC356 at various DNA concentrations was 56, 74, and 94, respectively. No Cm^R transformants were ever obtained from transformed C600 cells in the respective control experiments which yielded greater than 2 × 10⁷ Tc^R transformants.

Cm- Tc- Km-resistant plasmid from pSC355 could occur by simple excision of the mini-ColE1 fragment, which allows reformation of an intact Cm-resistance gene by linkage of the complementary components located on pML21 and pACYC184 (Fig. 2); in contrast, formation of a Cm- Tc- Km-resistant plasmid from pSC356 would require endonuclease cleavage at all three *Eco*RI sites of the plasmid followed by reinsertion of the separated Km-resistant fragment in an orientation opposite from the original one.

Of 74 Cm-resistant clones isolated from *E. coli* C600(pMB4) cells transformed with pSC355 DNA, 63 were also Tc-resistant but Km-sensitive and 11 were triply resistant, indicating that the intracellular action of the *Eco*RI endonuclease can accomplish recombination at either adjacent or nonadjacent enzyme recognition sites. This interpretation was confirmed by electrophoretic analysis of DNA derived from separate Km-Cm-resistant or from Km-sensitive Cm-resistant clones; in each case, the plasmid DNA was passed through a cycle of transformation in C600 cells to separate the pSC355 derivatives from pMB4 and from coexisting plasmids derived from the mini-ColE1 replicon.

Additional digestion of plasmid DNA with the *Sal* I and *Hind*III endonucleases permitted assignment of the direction of orientation of *Eco*RI fragments. From the gel patterns of *Eco*RI digests (Fig. 1B), it is evident that the Km- Cm-resistant plasmids (e.g., pSC357 in Fig. 1B) deleted only the mini-ColE1 fragment and are identical in structure to pSC354. The Km-sensitive Cm-resistant plasmids (e.g., pSC358 in Fig. 1B and C) deleted both the mini-ColE1 and Km fragments and were indistinguishable from pACYC184. Analysis of four Km-Cm-resistant derivatives [e.g., pSC359 (Fig. 3)] of pSC356 generated *in vivo* in C600(pMB4) cells shows gel electrophoresis patterns identical to the one observed for the pSC354 plasmid (Figs. 2 and 3). These findings indicate that physically separate DNA fragments generated intracellularly by the action of the *Eco*RI endonuclease can be recombined *in vivo* by intermolecular ligation.

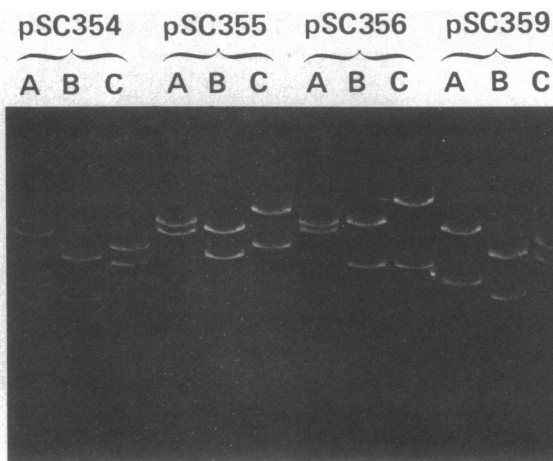


FIG. 3. Agarose gel electrophoresis patterns of plasmid DNA analyzed after digestion with *Sal* I (A); *Sal* I and *Hind*III (B); or *Hind*III (C). The source of each DNA is indicated. Each plasmid contained two *Sal* I and two *Hind*III cleavage sites. A low molecular weight (~0.57 kbase) band present in all of the *Sal* I-*Hind*III digests ran off this 0.7% gel. From the *Sal* I-*Hind*III cleavage patterns of pSC354, pSC355, and pSC359, it is concluded that a single *Sal* I site is present in each of the *Hind*III fragments, and that one *Hind*III site occurs in each *Sal* I fragment. The cleavage pattern of pSC356 DNA shows that the small *Hind*III fragment of this plasmid contains no *Sal* I site (see Fig. 2).

Potentially, the Cm-resistant clones obtained in the above experiments might have resulted from transformation of bacterial cells by DNA fragments cleaved extracellularly by endonuclease that might have been released by CaCl₂-treated bacteria. To test this possibility, we used a mixture of equal amounts of CaCl₂-treated C600 and C600(pMB4) cells as recipients in transformation. With either pSC352 or pSC355 plasmid DNA, a total of 34 Cm-resistant transformants were isolated; the presence of the pMB4 plasmid in each of these clones was demonstrated by the expression of Ap-resistance, a property conferred upon the cell by the pMB4 plasmid (12). These findings confirm the conclusion that the *Eco*RI-dependent recombinational events described here result from *intracellular* action of the endonuclease coded by the pMB4 plasmid.

***Eco*RI Endonuclease-Promoted Site-Specific Recombination during Normal Bacterial Cell Growth.** When C600 cells harboring the pSC352 plasmid were grown under normal culture conditions (i.e., in L-broth), no Cm-resistant clones were observed in a total of 10¹¹ cells tested. Assuming that the pSC352 plasmid exists in approximately the same copy number as the parent pACYC184 replicon (20 copies/chromosome equivalent) (A. C. Y. Chang and S. N. Cohen, unpublished data), we conclude that, in the absence of the pMB4 *Eco*RI endonuclease-producing plasmid, the frequency of precise excision of an inserted DNA fragment and ligation of the resulting termini to yield a functional *Cm* gene is less than 5 × 10⁻¹³. However, in *E. coli* C600 cells carrying pMB4, genetic recombination at the *Eco*RI sites of the pSC352 plasmid was observed under normal growth conditions. In these experiments, eight separate clones of C600 carrying both the pMB4 and pSC352 plasmids were grown to stationary phase in a liquid culture containing Tc, and 2 × 10⁹ bacteria from each culture were plated onto solid media containing Cm. A total of 22 Cm-resistant colonies were isolated from six of the eight cultures, and one clone from each culture was analyzed by gel electrophoresis. All six of the spontaneously produced Cm-resistant plasmids showed an endonuclease fragmentation pattern indistinguishable from the pattern depicted in Fig. 3

for the pACYC184 parent. These findings suggest that some of the pSC352 DNA molecules that coexisted with the pMB4 plasmid in "restrictive" cells escaped *EcoRI* modification under normal bacterial growth conditions long enough to be cleaved by the *EcoRI* endonuclease.

DISCUSSION

The experiments reported here indicate that the *EcoRI* endonuclease can function *in vivo* in conjunction with the *E. coli* DNA ligase to accomplish site-specific genetic recombination. Such recombination can involve either intracellularly generated termini on the same DNA molecule or the intermolecular ligation of different DNA fragments. Excision and ligation *in vivo* are precise and lead to: (i) functional continuity of the DNA sequences cleaved by the enzyme, and (ii) regeneration of the recognition sequence for both the *EcoRI* endonuclease and the *EcoRI* DNA methylase. Although we expected that the intracellular action of the *EcoRI* endonuclease would be most evident on incoming DNA, we observed that *EcoRI*-promoted recombination also occurred during the normal growth of bacterial cells. Although the current experiments concern only genes located on plasmids, it seems reasonable to expect that the *EcoRI* endonuclease also accomplishes *in vivo* cleavage, rearrangement, and reassortment of chromosomal genes in bacterial cells.

Previous work from our laboratory demonstrated that *E. coli* plasmid DNA fragments generated *in vitro* by the activity of the *EcoRI* endonuclease are ligated *in vivo* (7). Such ligation appears to be increased 2-fold in an *E. coli* mutant strain (SF8, see *Materials and Methods*) that is an overproducer of the DNA ligase (S. Chang and S. N. Cohen, unpublished data). The findings reported here indicate that fragments of eukaryotic DNA that have not been modified by the *E. coli* K restriction system can also be taken up by bacterial cells, can be ligated *in vivo* to bacterial plasmid DNA, and can be propagated in *E. coli* by the plasmid. Ordinarily, such *in vivo* joining of eukaryotic to prokaryotic segments of DNA would remain undetected experimentally; however, the intracellular formation of a chimeric plasmid can be shown by using a readily selectable genetic determinant carried by a transposable genetic element that has been introduced onto the eukaryotic DNA segment.

Detection of the *in vivo* recombinational events reported here also has been facilitated by the availability of an easily selectable gene (i.e., Cm resistance) carrying an *EcoRI* cleavage site. Insertion of a DNA fragment at this site inactivates Cm resistance; precise excision of the inserted fragment and ligation of the resulting *EcoRI*-generated termini leads to reconstruction of the Cm gene. Moreover, availability of a separately cloned R6-5 DNA fragment carrying both a portion of the Cm-resistance gene plus an additional genetic marker (i.e., Km resistance) has permitted a simple assay for detection of intermolecular recombinational events that occur at low frequency. All of the components of the recombinational event are introduced as part of a single DNA molecule; site-specific excision of a fragment of the molecule and precise reinsertion of the fragment in the opposite orientation results in Cm resistance.

Endonuclease activity functionally similar to the *EcoRI* enzyme is present in a wide variety of microorganisms and perhaps in most bacterial species (2). The findings reported here raise the possibility that at least some of the other "restriction" enzymes can also function *in vivo* in conjunction with DNA ligases to promote site-specific genetic recombination. Such recombination could involve incoming (unmodified) DNA species introduced by transduction, conjugation, or transfor-

mation or the rearrangement of segments of genomes already present in vegetatively growing cells.

In the continuing process of gene interchange among different bacterial species in nature (29), plasmids can be passed through a series of microorganisms that potentially are producers of different restriction endonucleases. Thus, plasmids may be subjected to a series of site-specific recombinational events that bring about structural reorganization of their genes. It seems reasonable to speculate from our findings that restriction endonucleases may play a major role in the natural evolution of plasmid, and perhaps chromosomal, genomes.

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