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Positive-selection Cloning Vehicle Useful for Overproduction of Hybrid Proteins

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Plasmid pSCC31 contains the *Eco*RI endonuclease gene downstream from lambda p_L. It does not yield transformants upon introduction into *Escherichia coli* unless the structural integrity of the endonuclease is destroyed. This makes it useful as a positive-selection cloning vehicle which can be employed for regulated overproduction of hybrid proteins.

The structural genes for *Eco*RI restriction and modification enzymes have been localized to a 2,200-base pair segment of DNA, and the sequence of this region has been determined (4, 11). We recently constructed several new plasmids carrying the *Eco*RI genes, as well as derivatives which contain large deletions within the gene for methylase. We describe here the construction and properties of one such r^+ m^- plasmid in which expression of the endonuclease gene is under control of the bacteriophage λ p_L promoter. The properties of this plasmid indicate that it may prove useful as a positive-selection cloning vehicle.

The construction and structure of the *Eco*RI r^+ m^- plasmid pSCC31 is presented in Fig. 1 to 3. As shown in Table 1, the transformation efficiency of this plasmid into a λ lysogen was about five orders of magnitude less than that for pAN4, an r^+ m^+ derivative of pBR322 (11). However, transformation efficiency was restored to high levels if the host contained a functional *Eco*RI methylase gene or if the integrity of the endonuclease gene within pSCC31 was destroyed by an insertion, as in the case of pSCC5076 and pSCC5077. As described below, these latter plasmids contain an 0.5-kilobase segment inserted in the two possible orientations into the *Bgl*II site of pSCC31. These properties suggest that failure to obtain transformants of *Eco*RI m^- hosts with pSCC31 is due to lethal effects of endonuclease production upon cell entry. The expression of endonuclease under such conditions could reflect low-level transcription from p_L in the repressing host used, transcription from an *Eco*RI promoter within the insert, or both.

The nature of transformants obtained upon introduction of pSCC31 or *Bgl*II linears of the plasmid into *Eco*RI m^- hosts (Table 1) was also examined. Only one Ap^r clone was recovered from several experiments with intact pSCC31. Plasmid preparations derived from this strain indicated it to be a rare double transformant containing both r^+ m^- pSCC31 and r^- m^+ pJC1, the latter being a contaminant due to method of isolation of pSCC31 (see legend to Fig. 3). A total of 15 Ap^r transformants were recovered from four experiments with *Bgl*II linears of pSCC31. In all cases, these strains contained pSCC31 derivatives which had lost the *Bgl*II site and contained deletions of variable size (data not shown).

As in the case of the *Bgl*II site, the *Hin*dIII site of pSCC31 can be employed for positive selection of plasmids containing inserts within the endonuclease gene, with the background being on the order of 1 to 2%. Although not shown, these experiments also demonstrated stable maintenance of a 3.19-kilobase insert within the *Hin*dIII site of the plasmid.

We also tested the possibility that in-phase insertions would yield hybrid proteins in model experiments in which a 507-base pair segment from phage f1 gene 3 was inserted into the endonuclease gene of pSCC31. This segment, corresponding to nucleotides 1,716 through 2,222 of the f1 sequence (1), was generated by *Dpn*I cleavage of viral replicative form I and coupled to d(CpCpGpGpApTpCpCpGpG) *Bam*HI linkers with T4 DNA ligase. After cleavage with *Bam*HI, the segment was inserted into the *Bgl*II site of pSCC31 (Fig. 3). Inspection of *Eco*RI (4, 11) and f1 gene 3 (1) DNA sequences indicated that in one orientation the inserted gene 3 segment would be in phase with the amino-terminal sequence of the endonuclease gene to yield a 319-residue polypeptide with an M_r of 35,000. In this protein, amino acids 1 through 146 are expected to be identical to the amino-terminal sequence of the endonuclease, whereas residues 148 through 316 are from the f1 gene 3 product (corresponding to amino acids 47 through 215 of the gene 3 polypeptide). The

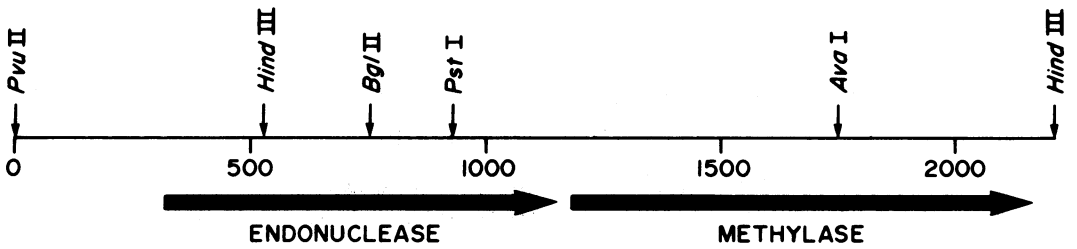

FIG. 1. Restriction map of *Eco*RI structural gene region. Adapted from Newman et al. (11) and Greene et al. (4).

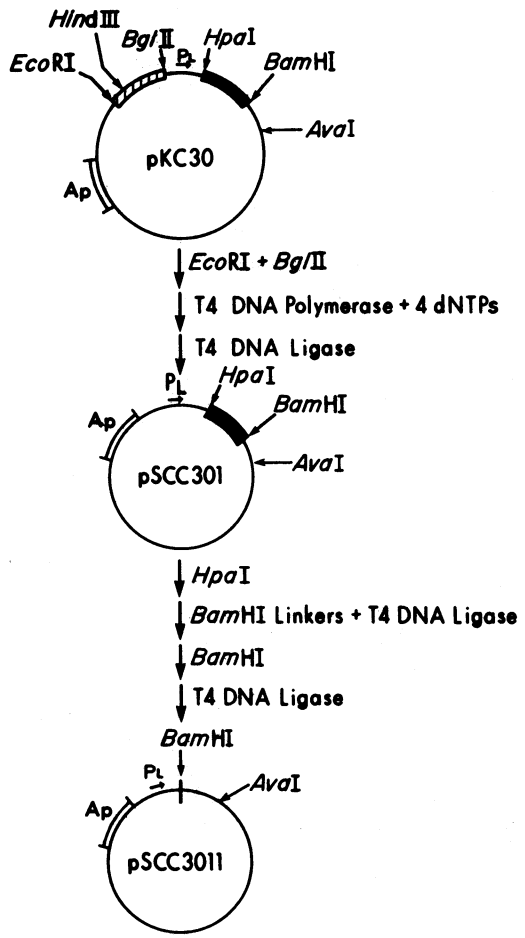

FIG. 2. Construction of pSCC301 and pSCC3011. The lambda-pBR322 hybrid plasmid pKC30 (14) was cleaved with *Bgl*II and *Eco*RI, and DNA termini were repaired with T4 DNA polymerase. After closure with T4 DNA ligase, plasmid pSCC301 was obtained upon transformation (6). Plasmid pSCC3011 was derived from pSCC301 by enzymatic deletion of the small *Hpa*I-*Bam*HI fragment, attachment of *Bam*HI linkers to the blunt *Hpa*I termini (8), and covalent closure with T4 ligase. Both plasmids were propagated in *E. coli* N99 (Sm^r λ^+). The aim of these modifications of pKC30 was to facilitate insertion downstream from o_L p_L as well as elimination of *Hin*dIII and *Bgl*II sites so that, upon insertion of *Eco*RI structural genes (Fig. 1), the *Hin*dIII and *Bgl*II sites within the endonuclease gene would be unique. Sequences deleted from pKC30 by these manipulations are indicated by crosshatching and bold face. Crosshatched sequences between the *Hin*dIII and *Bgl*II sites and bold-faced sequences between *Hpa*I and *Bam*HI sites of pKC30 are derived from bacteriophage lambda (14). Crosshatched sequences between *Eco*RI and *Hin*dIII sites are derived from pBR322, which was used to construct pKC30.

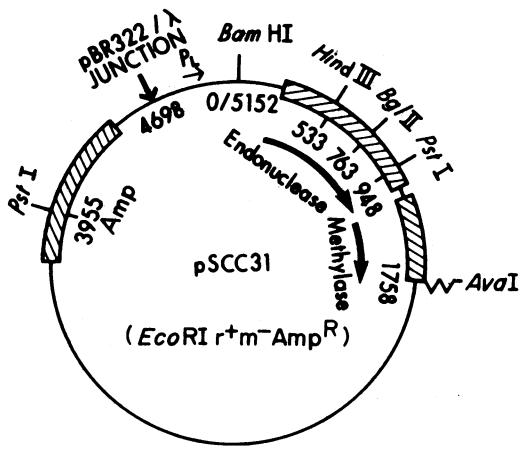

FIG. 3. Structure of *Eco*RI r^+ m^- plasmid pSCC31. The *Eco*RI structural gene region (Fig. 1) was isolated from plasmid pAN4 (11) as a product of *Pvu*II cleavage and partial *Hin*dIII hydrolysis. After repair of *Hin*dIII termini with T4 DNA polymerase and attachment of *Bam*HI linkers, the DNA segment was inserted into the *Bam*HI site of pSCC3011 (Fig. 2). Resulting plasmids were screened for orientation of the insert, and a plasmid with the desired orientation relative to p_L was isolated. Hydrolysis with *Ava*I and closure with T4 ligase yielded pSCC31, which is deleted for the COOH-terminal portion of the methylase gene rightward from the *Ava*I site (Fig. 1). This Ap^r, *Eco*RI r^+ m^- plasmid was propagated in *E. coli* JC4588 (*endA recA56 gal his322 thi* λ^+) (provided by A. J. Clark, University of California, Berkeley) harboring the compatible Cm^r, *Eco*RI, r^- m^+ plasmid pJC1. The latter plasmid was constructed by transfering the *Hin*dIII fragment spanning the methylase gene (Fig. 1) into the *Hin*dIII site of pACYC184 (2). Total plasmid DNA was isolated from this strain (10), and pJC1 was eliminted by exhaustive hydrolysis with *Sal*I endonuclease followed by banding in cesium chloride-ethidium bromide gradients.

TABLE 1. Transformation efficiency of pSCC31 and derivatives

DNA	Host[a]	No. of Ap^r transformants per μg
pAN4 r^+ m^+	N99(λ$^+$)	1.4×10^5
pSCC31 r^+ m^-	N99(λ$^+$)	3[b]
*Bgl*II linear of pSCC31	N99(λ$^+$)	40[c]
pSCC31 r^+ m^-	N99(λ$^+$ pJC1)	0.7×10^5
pSCC5076 r^- m^-	N99(λ$^+$)	4.6×10^5[d]
pSCC5077 r^- m^-	N99(λ$^+$)	5.5×10^5[d]

[a] As described in the legend to Fig. 3, plasmid pJC1 specifies a functional *Eco*RI methylase and is compatible with pSCC31, which is a derivative of pBR322 (14).

[b] One transformant was recovered from four experiments with a total of 0.37 μg of DNA. Since this isolate was resistant to both ampicillin and chloramphenicol and was *Eco*RI r^+, it evidently had been transformed by both pSCC31 and pJC1, the latter being a contaminant due to the method of isolation of pSCC31 (see the legend to Fig. 3).

[c] A total of 15 transformants were recovered from four experiments with a total of 0.37 μg of DNA. Plasmid DNA recovered from these isolates was resistant to *Bgl*II and contained deletions of variable size.

[d] Plasmids pSCC5076 and pSCC5077 were derived from pSCC31 by insertion of a segment of f1 gene 3 into the *Bgl*II site of pSCC31. Gene 3 and *Eco*RI sequences are in phase in pSCC5077, resulting in production of a hybrid protein. In pSCC5076, f1 sequences are present in the opposite orientation, resulting in production of a truncated *Eco*RI polypeptide.

opposite orientation should result in the production of a 149-amino acid polypeptide with an M_r of 17,000 of which the first 146 residues are identical to the amino terminus of the endonuclease.

Figure 4 shows immunological blot analysis of a sodium dodecyl sulfate gel loaded with total lysates derived from cells harboring plasmids containing the f1 insert in either orientation. The host employed, M5248, harbors a defective λ *cI857* lysogen permitting thermal induction of the p_L promoter of the pSCC31 vector. Results obtained with anti-*Eco*RI endonuclease immunoglobulin G (IgG) are shown in the left panel. Extracts of thermally induced cells contained a cross-reacting protein of M_r 39,000 in the case of the in-phase f1 insert, whereas a 17,000 species was found when the insert was in the opposite orientation (Fig. 4, lanes B and D). No detectable cross-reacting material was observed in either case in the absence of thermal induction (Fig. 4, lanes A and C). The right panel shows results of an identical experiment, except that in this case, blotting was performed with anti-f1 gene 3 protein IgG. Although cross-reaction of this antiserum with two cellular components was observed in all cases, thermal induction of the plasmid containing the in-phase insert yielded a major component of M_r 39,000 and two minor components of M_r 35,000 and 28,000. (The apparent M_r 39,000, of the major cross-reacting component is somewhat higher than the expected value of 35,000. This does not reflect the presence of multiple *Bam*HI linkers of the f1 insert, since plasmid pSCC5077 contains only one *Bam*HI site corresponding to that in the pSCC31 vector [Fig. 3]. It seems likely that the anomalous mobility of the hybrid protein reflects the hydrophobic nature of the f1 gene 3 product, which is a structural component of the filamentous phage. Previous analyses of the intact gene 3 polypeptide by sodium dodecyl

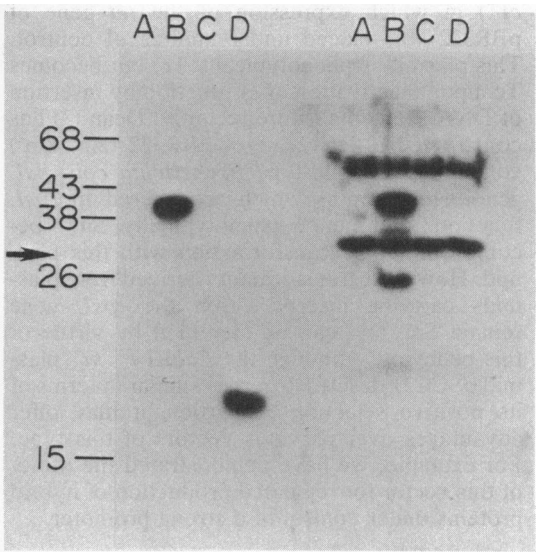

FIG. 4. Immune blot analysis of proteins produced upon insertion of f1 gene 3 sequences into the *Eco*RI endonuclease gene of pSCC31. Plasmid pSCC5077 contains the f1 insert in phase with the endonuclease gene, whereas in pSCC5076, the insert is present in the incorrect orientation. These plasmids were introduced into M5248 λ *bio275 cI857 ΔH1* (provided by M. Rosenberg, National Institutes of Health, Bethesda, Md.). Cells were propagated at 30°C in Luria broth (9) to an absorbance at 590 nm of 1 and were then shifted to 42°C for 4 h. Extracts were prepared (18) and subjected to sodium dodecyl sulfate gel electrophoresis (7, 15). Proteins were transferred to nitrocellulose strips (16) which were probed with the indicated IgG and staphylococcal ^{125}I-labeled protein A (17). Lanes A and B were derived from M5248(pSCC5077), uninduced and thermally induced by p_L expression, respectively. Lanes C and D contained comparable samples derived from M5248(pSCC5076). The immune blot shown in the left panel was prepared with anti-*Eco*RI endonuclease IgG (13), whereas that in the right panel was prepared with anti-gene 3 protein IgG (generously provided by R. Webster, Duke University Medical Center, Durham, N.C.). The ordinate shows apparent M_r, with the arrow indicating the position of migration of intact *Eco*RI endonuclease.

sulfate gel electrophoresis have indicated M_r values 36 to 61% greater than that predicted from the gene sequence [5].) The latter proteins may represent proteolytic products of the 39,000 M_r form since extensive hydrolysis of this polypeptide was observed upon storage of cell lysates at 0°C for several days (data not shown). In contrast, no new species appeared upon thermal induction of cells harboring the f1 insert in the wrong orientation. These results have led us to conclude that pSCC31 can be employed for regulated synthesis of hybrid proteins.

Two positive-selection cloning vectors have been previously described. Roberts et al. (12) have described the plasmid pTR262 (Ap^r Tc^r cI^+) in which expression of the *tet* gene of pBR322 was placed under lambda *c*I control. This plasmid is phenotypically Tc^s but becomes Tc^r upon inactivation of *c*I function by insertion of DNA segments. More recently, Dean (3) has constructed a derivative of pBR322 (Ap^r Sm^s) containing the wild-type *Escherichia coli rpsL* gene. Host strains which are altered in *rpsL* function, and hence phenotypically Sm^r, become Sm^s upon transformation with this plasmid. However, transformants derived from plasmids carrying inserts within the *rpsL* gene remain Sm^r and can be identified by virtue of this behavior. Although the *Eco*RI r^+ m^- plasmid pSCC31 described here is similar in terms of its positive selection properties, it may offer advantages over previous vectors of this type. For example, we have demonstrated the utility of this vector for regulated production of hybrid proteins under control of a strong promoter.

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