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Identification of Responsible Region for the Polymerization of Plasmid pEC-3

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Plamid pEC-3의 중합에 필요한 부위의 동정

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ABSTRACT

In order to find specific acting site of Rec A protein in plasmic polymerization in *E. coli*, we randomly deleted various part of pEC-3 (a derivative of pBR322) with Sl nuclease treatment. Self-ligated plasmids were introduced into *E. coli* WA802 (Rec A$^+$). A number of colonies were analyzed if they contained monomeric or polymeric plasmids by gel electrophoresis. The plasmid (pEC-43), which was deleted the region of tetracycline gene, revealed only monomeric form in Rec A$^+$ *E. coli*. When two plasmids, pEC-3 and pEC-43, were co-transformed in the same *E. coli*, the original pEC-3 showed polymerization but pEC-43 revealed monomeric form only. These results suggest that Rec A protein requires the specific site for polymerization.

INTRODUCTION

The Rec A protein plays a central role in homologous genetic recombination in *Escherichia coli*(Radding, 1978; Radding, 1981), and it is a key element in a regulatory pathway mediating cellular responses to DNA damage(Gottesman, 1981). Single stranded DNA(ssDNA) governs all the activities of Rec A protein: ssDNA is a cofactor for the ATPase activity, the protease activity, the binding of duplex DNA, and the unwinding of duplex DNA(Wu *et al.*, 1982).

The Rec A protein is also involved in the conversion of monomeric plasmids to different oligomeric forms. It is, therefore, plausible the polymerization(a recombination) of plasmids in *Escherichia coli* Rec A$^+$ strain. Recently, James *et al.* (1983) isolated the DNA sequences which were necessary for the formation or maintenance of circular oligomeric plasmid. And Klysik *et al.* (1981) reported that the plasmids containing the dC-dG showed the shift of monomeric plasmid to a mixture of monomer, dimer, and higher forms.

Here we tried to identify if a specific region were involved in the polymerization of plasmid pEC-3 existing as monomer or as different oligomers in *Escherichia coli* strain WA802(Rec A$^+$). We induced deletions in this plasmid with Sl nuclease which reacts primarily on ssDNA. After the introduction of the plasmid into WA 802, the conversion of the form of plasmid was investigated. We found out the plasmid(pEC-43), showing no oligomer in WA802, and determined the deleted region with restriction mapping technique. We revealed, furthermore, that the recombinogenic segment of DNA served as *cis*-acting element when both pEC-3 and pEC-43 were present in the same cell.

MATERIALS AND METHODS

Plasmid pEC-3, a pBR322 derivative containing 4430 base pairs, was purified as described previously by Birnboim and Doly (1979). T4 ligase and restriction enzymes were purchased from Bethesda Research Laboratories Inc.. Sl nuclease was purified from *Aspergillus oryzae* crude α-amylase powder as described by Vogt (1973), and all the other chemicals used were reagent grade.

Electron Microscopy of DNA:

For the visualization of plasmid DNA, the basic protein film technique, developed by Kleinschmidt *et al.* (1968), was used with a slight modification. Thirty μl of spreading solution (0.5 μg/ml of DNA, 0.1mg/ml of cytochrome c, 0.5M NaAc(pH 7.5), and 1 mM Na_3EDTA) was appiled on the hypophase (0.25M NaAc (pH 7.5)), and then the film was picked up at once onto the grid. The grid was dipped for 30 second into the uranyl stain solution(uranyl acetate of 5×10^{-5}M in 90% EtOH), then rinsed for 10 second in 90% EtOH. When EtOH was dried out completely, the sample was applied on a electron microscope.

Induction of Deletion in pEC-3 and Transformation of *E. coli*:

Plasmid pEC-3 monomeric DNA, purified from Rec A$^-$ *E. coli* strain HB101, was treated with Sl nuclease under the condition of buffer containing 0.03M NaAc(pH 4.6), 1 mM $ZnSO_4$, 0.05 M NaCl, 35 μg/ml of pEC-3 DNA, and 2.5 units/μl of Sl nuclease. The reaction mixture was incubated for 5 min at 42°C, for 10 min at 50°C, and for 10 min at 30°C in a row. The reaction was stopped by adding 20 μl of stopping buffer(0.03% bromophenol blue, 20% sucrose, and 0.025M EDTA), and then the mixture was loaded on low melting point(LMP) agarose gel electrophoresis was carried out for 14 hrs at 35V as described by Parker and Seed (1980). The gel portion corresponding to pEC-3 linear DNA band(3mm×2mm×10mm) was cut out, and then soaked in distilled water for 30 min. The gel fragment was remelted at 65°C, and then the solution was mixed with 30 μl of prewarmed ligation buffer solution(0.1 M Tris/HCl (pH 7.5), 0.06M KCl, 0.1M $MgCl_2$, and 0.01M DTT), 30 μl of ligation addition solution (10mg/ml of BSA, 0.1M DTT, and 0.01M ATP), and 180 μl of distilled water. Ligation was performed for 10 hrs at 25°C by 4 units of T4 ligase.

The introduction of plasmids into *Escherichia coli* strain WA802(Rec A$^+$) was carried out in the ligation solution as described by Cohen *et al.* (1972). Transformed cells were selectively grown on the LB plate containing 20 μg/ml of tetracycline(Tc) or 50 μg/ml of ampicillin(Ap). The cells were transferred onto new agar plate containing Tc or Ap respectively, and into 2ml of liquid LB media, The cells grown in liquid were harvested in Eppendorf tubes, and then 100 μl of cracking buffer(0.05M Tris/HCl(pH 6.8), 1% SDS, 0.02 M EDTA, 0.4 M sucrose, and 0.01% bromophenol blue) was poured into each of them. After being incubated for 10 min at room temperature, the reaction mixtures were centrifuged in an Eppendorf centrifuge. The forms of plasmids were analyzed on a 1% agarose slab gel by applying 30 μl of the supernatant. The colony showing no polymer DNA band was selected.

Treatment of Restriction Enzymes and Gel Electrophoresis:

E. Coli cells(WA802) with plasmid pEC-3 or pEC-43 were grown in liquid LB broth containing 50 μg/ml of Ap, and then the plasmid DNA was isolated by means of alkaline lysis method as described by Birnboim and Doly (1979).

The plasmid DNA was digested in common digestion buffer(33 mM Tris/HCl(pH 7.0), 66 mM potassium acetate, 10 mM magnesium ace-

tate, and 0.5 mM DTT) by various restriction enzymes(Eco RI, Hin II, Hinf I, Sau 3A, Hpa II, Ava I, BamH I, Pvu II+Pst I, and Hin II+Pvu II). The digested DNAs were analyzed on a 1.5% agarose gel or a 7% acrylamide gel.

Co-transformation of *E. coli* WA802 by pEC-3 and pEC-43:

Calcium chloride method described by Cohen *et al.* (1972) was used to introduce pEC-3 into *E. coli* WA802 which contained pEC-43. The transformant were screened for the resistance to Ap and Tc. The transformants were then analyzed for the presence of oligomeric forms of the input plasmid DNAs by electrophoresis on an 0.8% agarose gel.

RESULTS

Electron Microscopy of Plasmid DNA:

Plasmid pEC-3 purified from WA802 was visualized by Electron Microscope with aqueous technique(Fig. 1). Supercoiled, linear and open circular DNAs were observed and oligomeric plasmids were covalently closed circular DNAs (cccDNA). Only open circular DNAs were shown in this paper in order to interpret easily the conformation of plasmid.

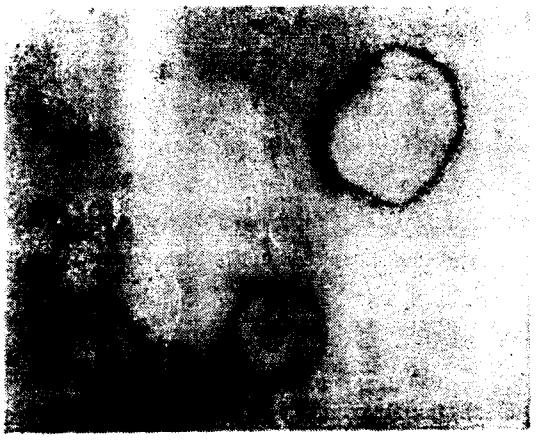


Fig. 1. Electron microscopic picture of the plasmids (pEC-3) replicated in *E. coli* WA802. DNA was prepared by the aqueous basic protein film technique and stained with uranyl acetate. Open circular monomers and covalently linked dimer were shown.

Identification of Responsible Region for the Polymerization of pEC-3:

The plasmid pEC-3 exists in *E. coli* Rec A+ strains(WA802 and RRI) as monomer, dimer, trimer, tetramer, and other oligomers (Fig.2, lane M_1). In order to identify the existence of DNA sequences which were responsible for the polymerization of plasmids in *E. coli* Rec A+

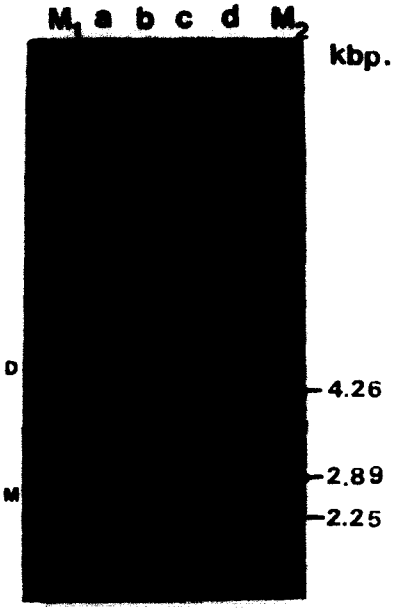


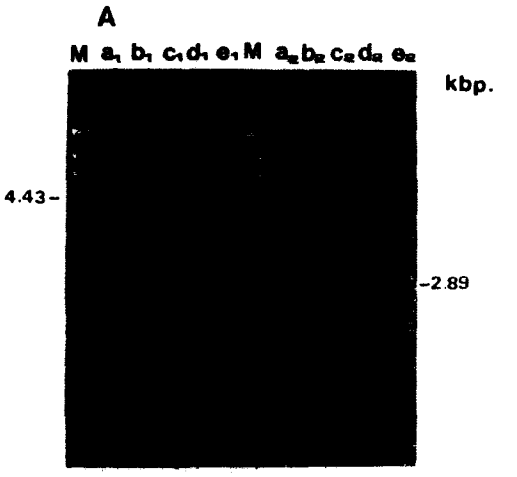
Fig. 2. Electrophoretic analysis of the polymerization of pEC-3 and pEC-43. Plasmid from various transformants were purified from WA802, and then analyzed on a 0.8% agarose gel. Artificial pEC-43 dimer was made as follows: Monomeric pEC-43 was digested with Pst I and was rejoined by T_4 ligase under the inter-ligation condition. The ligated DNA was introduced into WA802, and the transformants containing dimeric pEC-43 were screened by cracking method, pEC-3 in WA802 showed monomer(M), dimer(D), trimer, tetramer, and other polymers from bottom to top(lane M_1), but pEC-43 in WA802 showed only supercoiled monomer(lane a). Monomeric and dimeric form of pEC-43 digested by Pst I appeared at the same position(lanes b, c). Dimeric pEC-43 showed supercoiled dimer and thin open circular dimer or tetramer (lane d). Lane M_2 represented linear DNA size marker prepared by digesting λ DNA with Hind III.

strain, we induced deletions in pEC-3 with Sl nuclease and investigated the ability of polymerization in WA802. We found out, by cracking method, the clone which contained pEC-43 showing no oligomeric DNA band. Plasmid pEC-43 remained primarily monomeric form through 30 generations or more (Fig.2. lane a). This result implies the existence of specific DNA segment which facilitates the recombination of plasmid DNA.

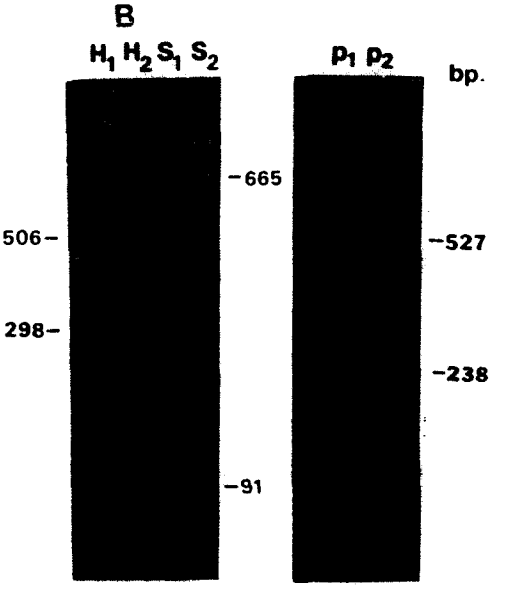
Whether the size of plasmid influence the polymerization or not was tested by investigating the conversion of the form of artificial dimeric pEC-43 in WA802. Original supercoiled dimer and thin open circular dimer (or tetramer) DNA bands were shown after the transformation of WA802 by the artificial dimeric pEC-43 (Fig. 2, lane d). This result indicates that the size of DNA has little influence on the polymerization of plasmid.

Determination of Deletion Site:

In order to identify the deletion site, we compared the band patterns of pEC-3 DNA digested by restriction enzymes and that of pEC-43



A. pEC-3 DNA was digested by Pvu II and Pst I (lane a_1), Hind II (lane b_1), Hind II and Pvu II (lane c_1), Ava I (lane d_1). And pEC-43 DNA was digested respectively by Pvu II and Pst I (lane a_2), Hin II (lane b_2), Hin II and Pvu II (lane c_2), Ava I (lane d_2), and BamH I. The electrophoresis was carried out on 1.5% agarose gel for 13 hrs at 20V. Lane M represented λ-Hind III size marker.



B. pEC-3 DNA was digested by Hinf I (lane H_2), Sau 3A (lane S_2), and Hpa II (lane p_1). And pEC-43 DNA was digested by Hinf I (lane H_1), Sau 3A (lane S_1), and Hpa II (lane p_2). The electrophoresis was carried out on 7% acrylamide gel. The DNA bands exhibited in pEC-3 but in pEC-43 correspond to the deleted region.

Fig. 3. Electrophoretic patterns of pEC-3 and pEC-43 after the treatment of restriction enzymes.

(Fig. 3 and Fig. 5). The results indicated that about 1550 base pairs, locating between the 50±20th base and the 1600±20th base, were deleted by Sl nuclease. The gene for Tc resistance of pEC-3 is located in this region (Sutcliffe, 1979).

Co-transformation of *E. coli* WA802 by pEC-3 and pEC-43:

By introducing pEC-3 into WA802 which contained pEC-43, we tried to find out the role of the element responsible for the pEC-3 polymerization. The plasmid pEC-3 was converted to a mixture of circular oligomers in a Rec A^+-dependent reaction both in a absence (Fig. 4, lane b) and presence (Fig. 4, lane c) of pEC-43. Plasmid pEC-43, however, exists as primarily monomer in either cases of absence (Fig. 4, lane a) or presence (Fig. 4, lane c) of pEC-3. These results indicate that the recombinogenic element present in pEC-3 can only act in *cis*.

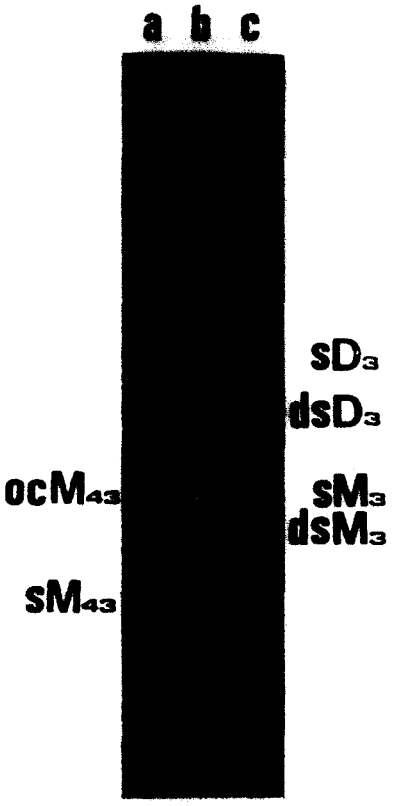


Fig. 4. Electrophoretic analysis of the stimulation of plasmid recombination in *cis*. The procedure for the cotransformation was described in Materials and Methods. Lane a, pEC-43 purified from WA802. Lane b, pEC-3 purified from WA802. Lane c, plasmid DNAs purified from WA802 transformed by both pEC-3 and pEC-43 monomeric DNA. Abbreviations stand for followings: s, supercoiled; ds, denatured supercoiled; oc, open circular; M_{43}, monomeric pEC-43; M_3, monomeric pEC-3; D_3, dimeric pEC-3.

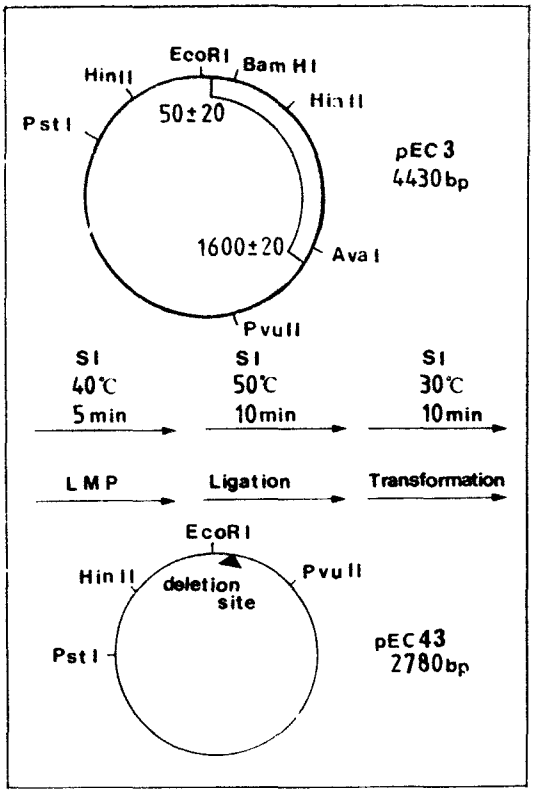


Fig. 5. Schematic diagram presenting the procedure for the induction of deletion in pEC-3. Detail procedure was described in Materials and Methods.

DISCUSSION

In this paper, we tried to find if the specific acting site of Rec A protein exists. We induced deletion in pEC-3 randomly with S1 nuclease, and then introduced the self-ligated plasmid into *E. coli* WA802(Rec A^+). The colony, containing the plasmid which revealed only monomeric form in Rec A^+ *E. coli*, was selected(Fig. 5). We determined the deleted region of pEC-43, responsible for the polymerization of plasmid, with restriction mapping technique. This recombinogenic segment was located inside of the Tc gene, and identified as *cis*-acting element by the experiment of co-transformation of pEC-3 and pEC-43 into *E. coli* WA802.

These results suggest that certain DNA segment can dramatically facilitate the recombination of certain plasmid DNA when the plasmid contains this DNA segment. It seems likely that this recombinogenic element increase either the level of plasmid recombination or the recovery of recombinant DNA molecule.

It is tempting to think this *cis*-acting elements are hotspots for recombination. Such types of DNA sequences, lac promoter-operator region (Hardies *et al.*, 1979) dC·dG tracks (Wells *et al.*, 1983), and *cis*-acting element in plasmid

pACYC184(James *et al.*, 1983), are known to be present in plasmid DNA and has been characterized. Among these examples, the lac promoter-operator region and B-Z junction of DNA following the dC-dG track are good candidates for the sequences which remain in the state of single stranded DNA long enough to interact with Rec A protein. As an indirect evidence of this assumption, Wells *et al.* (1983) reported that single stranded DNA was exposed on the junction between right-handed and left-handed segments of the plasmid pRW751. It is plausible, therefore, to think that the *cis*-acting element in pEC-3 serves as the initiation site of the recombination with the easiness of the transient unwinding of DNA *in vivo* condition. However, the precise explanation of the role of the *cis*-acting recombinogenic element will have to be reserved until the exact sequence of this element and the homology of the different *cis*-acting element should be determined.

적 요

대장균 세포에서의 plasmid 중합현상(polymerization)은 Rec A protein이 관여하여 일어난다고 알려져 있다. 본 실험에서는 Rec A protein의 작용부위를 알기 위하여 plasmid pEC-3(pBR322유도체)의 일부를 S1 nuclease를 처리하여 무작위로 제거하고 대장균주 WA802(Rec A$^+$)에 형질전환시키고 중합현상이 일어나는 지의 여부를 관찰하였다. 대조구인 pEC-3는 중합현상이 일어나지만 tetracycline gene부위가 제거된 plasmid(pEC-43)에서는 중합현상이 일어나지 않았다. 또한 이 두가지의 plasmid를 한세포내에 동시에 형질전환 시켰더니 pEC-3는 중합현상을 일으켰으나 pEC-43는 중합현상이 일어나지 않았다. 이 결과는 Rec A protein이 작용하는 특정부위가 존재함을 나타낸다.

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