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STUDY ON THE CLEAVAGE REACTION
OF BACTERIOPHAGE ϕ 80 CI REPRESSOR BY RecA PROTEIN

By
YUTAKA EGUCHI

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CHAPTER I
Introduction

Introduction

Escherichia coli displays a response to the treatments that damage DNA or inhibit DNA replication. This response, often termed the SOS response, includes phenomena such as enhanced capacity for DNA repair and mutagenesis, and inhibition of cell division (filamentation). Although many genes are involved in these phenomena, the expressions of them are regulated by a repressor, LexA protein. Treatments that induce these phenomena result in the activation of RecA protein to allow it to cleave the LexA repressor at Ala - Gly bond in the middle of the polypeptide (Horii et al., 1981). This cleavage inactivates the LexA repressor so that the genes hitherto repressed by the repressor begin to express constitutively. Similarly, prophage λ is induced with the same treatments which induce SOS response owing to the inactivation of λ CI repressor. This inactivation is also brought by the cleavage at Ala - Gly bond in the middle of the polypeptide chain analogous to LexA repressor.

Bacteriophage $\phi 80$ (Matsushiro, 1963) is one of the lambdoid phages. The genetic organization of phage $\phi 80$ is very similar to that of phage λ (Sato et al., 1968; Fianndt et al., 1971; Youderian & King, 1981; Ogawa et al., 1987a, 1987b). Replication of prophage $\phi 80$ can be induced by UV-irradiation as other lambdoid phages (Matsushiro, 1963). The induction of prophage $\phi 80$, however, has some characteristic differences from the induction of prophage λ as follows: 1) prophage $\phi 80$ can be induced with about one third lower dose of UV-light than that required for the induction of prophage λ under the same genetic background (T. Ogawa & H. Ogawa, personal communications), 2) prophage $\phi 80$ can be induced in a recA430 mutant by UV-irradiation or nalidixic acid treatment, while prophage λ

cannot be induced in that mutant (Devoret et al. ., 1983; T. Ogawa, personal communications), 3) prophage $\phi 80$ in permeabilized lysogen can be induced by addition of a specific deoxydinucleoside monophosphate, d(G-G) or d(A-G) without any inducing treatments, while prophage λ cannot be induced under the same condition (Irbe et al., 1981). Furthermore, Ogawa et al. (1987a) showed that $\phi 80$ CI repressor has no Ala - Gly bond which is known as the target sequence of cleavage by the RecA protein in other analogous repressors, LexA, λ CI and P22 C2. These observations prompted me to study on the mechanism of inactivation of $\phi 80$ CI repressor by RecA protein. Comparative study of the inactivation of $\phi 80$ CI repressor and other repressors should give us further detailed informations to elucidate the mechanism of the activation of RecA protein.

In this thesis, I will describe the analysis of in vitro cleavage of $\phi 80$ CI repressor, comparing with those of other repressors. Major results obtained are as follows. I purified the $\phi 80$ CI repressor to more than 98 % purity from cells that overproduce the repressor. The purified $\phi 80$ CI repressor can be cleaved by wild-type RecA protein and also, by RecA430 protein in the presence of single-stranded DNA and ATP or its analogues at the Cys¹¹⁰-Gly¹¹¹ bond in the middle of the polypeptide chain. This site is different from the common cleavage site of other repressors (Chapter II). Kinetic analysis of cleavage shows that the rate is very low in the first 1 hour and then increases sharply as the incubation is continued. The rate of cleavage is greatly increased by preincubating RecA protein and single-stranded DNA prior to addition of ATP- γ -S, which is subsequently added to ensure cleavage of repressor. This implies that the RecA protein and ssDNA form a certain complex effective for cleavage of the repressor during the

preincubation, and indicates that the two kinds of ternary complexes of RecA protein, ssDNA and ATP- γ -S are formed with or without the preincubation, which have different activities to cleave ϕ 80 CI repressor (Chapter III). Specific deoxydinucleoside monophosphate d(G-G) and d(A-G) greatly stimulate the cleavage reaction of ϕ 80 CI repressor by binding to the carboxyl-terminal domain of the repressor to increase the affinity between the repressor and RecA protein. The efficient cleavage of ϕ 80 CI repressor requires not only the activation of RecA protein but also the activation of the repressor (Chapter IV).

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Abbreviations used

Abbreviations used in this thesis are as follows:

UV, ultraviolet light; kb, 10^3 base-pairs; ATP- γ -S, adenosine 5'-O-(3-thiotriphosphate); ssDNA, single-stranded DNA; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetate; cpm, counts per minutes; $E_{280}^{1\%}$, absorbance per cm at 280 nm of 1 % solution.

CHAPTER II

Cleavage of Phage ϕ 80 CI Repressor by RecA Protein.

1. Summary

I have purified the CI repressor protein of phage $\phi 80$. Its N-terminal amino acid sequence and its amino acid composition agree with those predicted from the nucleotide sequence of the cI gene. The $\phi 80$ CI repressor was cleaved at a Cys - Gly bond by the wild-type RecA protein in the presence of single-stranded DNA and ATP or its analogues. This cleavage sequence is different from other repressors such as LexA, λ CI and P22 C2 which were cleaved at a Ala - Gly bond. The $\phi 80$ CI repressor was cleaved at the same site by the RecA430 protein, but was not cleaved by the RecA1 protein. This effect of the bacterial recA mutations on cleavage is consistent with the effects on inducibility of prophage $\phi 80$ in each recA mutant lysogen.

2. Introduction

Bacteriophage $\phi 80$ (Matsushiro, 1963) is one of the lambdoid phages. The genetic organization of phage $\phi 80$ is very similar to that of phage λ (Sato et al., 1968; Flandt et al., 1971; Youderian & King, 1981; Ogawa et al., 1987a, 1987b). Replication of prophage $\phi 80$ can be induced by UV-irradiation as other lambdoid phages (Matsushiro, 1963). However, the induction of prophage $\phi 80$ has some characteristic differences from that of prophage λ . For example, prophage $\phi 80$ can be induced in a recA430 mutant by UV-irradiation or nalidixic acid treatment, while prophage λ cannot (Devoret et al., 1983; T. Ogawa personal communications). The LexA, λ CI and P22 C2 repressors, which are known to be inactivated by the RecA protein, are all cleaved at a specific alanine - glycine sequence in the middle of the polypeptide chain (Horii et al., 1981a; Sauer et al., 1982a), while no alanine-glycine sequence is present in $\phi 80$ CI repressor (Ogawa et al., 1987a). Comparative study of the inactivation of these repressors should give us further information on the mechanism of inactivation of repressors by the RecA protein.

In this chapter, I show that the $\phi 80$ CI repressor is cleaved by the wild-type RecA protein at a cysteine - glycine sequence in the middle of the polypeptide chain. The RecA430 protein also cleaves the repressor at the same site.

3. Materials and Methods

(a) Bacteria and Plasmids

The Escherichia coli strains used in this study are listed in Table II-1. Plasmid pSW4 (Ogawa et al., 1987a) is a 6.7 kb pBR322-derived plasmid containing the 3 kb EcoRI-F segment of ϕ 80 DNA which carries the genes cI, 30, cII and a part of gene 15. Plasmid pMY16-6 (supplied by Dr. T. Tsurimoto) was made by replacing the shorter EcoRI-AvaI fragment of pBR322 with the fragment carrying the λ P_L promoter (from position 35,468 to 35,715 on λ DNA; Sanger et al., 1982). On this plasmid, the direction of the transcription from λ P_L is the same as that from the bla promoter. Plasmid pEG70, which carries the ϕ 80 cI gene (position 490 to 1542; Ogawa et al., 1987a) downstream of the λ P_L promoter on pMY16-6, was constructed as described below and used as a source of the ϕ 80 CI repressor. Plasmid pTM2430 and pTM2001, which carry the recA430 mutant gene and the recA1 mutant gene, respectively, were constructed as described below and used as a source of RecA protein coded by each recA mutant gene.

(b) Enzymes, DNA and Chemicals

Restriction enzyme BamHI was prepared as described by Wilson & Young (1975). Restriction enzyme HpaII and nuclease Ba131 were purchased from Takara-Shuzo, Japan, and Bethesda Research Laboratories, USA, respectively. T4 DNA ligase was prepared as described by Weiss (1971). Rabbit muscle creatine kinase, creatine phosphate and ATP- γ -S were purchased from Boehringer Mannheim, West Germany. ATP and dATP were

Table II-1. Bacterial Strains

Strains	Relevant genotype	Source, reference or derivation
Km723	<u>recA</u> 1	Matsubara (1974)
YE723	Km723, (F' <u>trp</u> 28*)	T. Tsurimoto (Osaka Univ.)
YE723(pEG70)	YE723, (pEG70)	transformed YE723 with pEG70
C600(pTM2)	<u>recA</u> ⁺ <u>lexA</u> ⁺ , (pTM2)	Ogawa <u>et al.</u> (1978)
KL16	<u>recA</u> ⁺ <u>lexA</u> ⁺ , (HfrPO45)	Low (1968)
GY6130	<u>recA</u> 430 <u>lexA</u> ⁺	Blanco <u>et al.</u> (1975)
KL16 <u>srl</u>	KL16, <u>srl</u> ::Mucts62	lysogenized Mucts62 phage in the <u>srl</u> gene of KL16
KL16 <u>recA</u> 430	KL16, <u>recA</u> 430	transduced KL16 <u>srl</u> with P1 grown on GY6130
DM1187	<u>recA</u> 441 <u>lexA</u> (Def) <u>sfiA</u> 11	Mount (1977)
STS <u>recA</u> 430	DM1187, <u>his</u> <u>recA</u> 430	mated DM1187 with KL16 <u>recA</u> 430
STS <u>recA</u> 430(pTM2430)	STS <u>recA</u> 430, (pTM2430)	transformed STS <u>recA</u> 430 with pTM2430
KL16-99	<u>recA</u> 1, (HfrPO45)	Low (1968)

Table II-1 (continued)

STS <u>recA</u> 1	DM1187, <u>his</u> <u>recA</u> 1	mated DM1187 with KL16-99
STS <u>recA</u> 1(pTM2001)	STS <u>recA</u> 1, (pTM2001)	transformed STS <u>recA</u> 1 with pTM2001
W3102 <u>recA</u> 3	<u>recA</u> 3 <u>lac</u>	Gottesman and Yarmolinsky, (1968)
W3102 <u>recA</u> 3(pKB252)	W3102 <u>recA</u> 3, (pKB252)	transformed W3102 <u>recA</u> 3 with pKB252 (Backman <u>et al</u> , 1976)
W3623(pTH227)	<u>lexA</u> <u>recA</u> , (pTH227)	Horii <u>et al</u> . (1981b)

(*) defective λ cI857 phage was integrated in the F' episome carrying trpA gene (Spiegelman, 1971).

obtained from Yamasa Shoyu, Japan. Calf thymus DNA was purchased from Sigma, USA.

(c) Construction of Plasmids

(i) Plasmid pEG70

As the repressor is expected to control the expression of its own gene, I replaced the operator-promoter region of the $\phi 80$ cI gene with the λ P_L promoter for production of a large amount of the protein. The HpaII fragment which carries the cI gene, the O_R operator and a part of gene 30 of $\phi 80$ (Ogawa et al., 1987a) was obtained from plasmid pSW4. About 200 base pairs were removed from each end of the HpaII fragment by digestion with nuclease Bal31. This treatment removed gene 30 and a part of the O_R operator, while the cI gene was kept intact. The resultant DNA fragments were inserted into the BamHI site of pMY16-6 using the BamHI linkers (Genex, USA). The BamHI site is located 120 base pairs downstream of the start site of transcription of the λ P_L promoter on the plasmid. The DNA mixture was used to transform YE723, which contains a F'trp plasmid carrying a defective λ cI857 prophage, by selecting for ampicillin resistance at 30°C. The transformants were examined at 30°C for resistance to $\phi 80$ cI phage by the cross streak method. Synthesis of the $\phi 80$ CI repressor at 42°C was examined for 16 clones of the resistant transformants by SDS(sodium dodecylsulfate)-polyacrylamide gel electrophoresis of the lysates (see Results). Plasmid pEG70 is one of four clones that produced the $\phi 80$ CI repressor. Nucleotide sequence analysis of the region joining the $\phi 80$ sequence with the λ P_L promoter in this plasmid showed that the $\phi 80$

cI gene with a part of the O_R operator had been linked with the λ P_L promoter in the proper orientation (data not shown).

(ii) Plasmids pTM2430 and pTM2001

These plasmids were constructed by Drs. T. Ogawa and H. Wabiko according to the method previously used for preparation of plasmid pTM2 carrying the wild-type recA gene (Ogawa et al., 1978) as follows. The transducing phages, λ precA430alaS⁺ and λ precAlalaS⁺, were selected from the phages obtained by the induction of GY6130 and KL16-99 (see Table 1) which had been lysogenized with λ precA⁺alaS⁺, respectively. The 3 kb BamHI fragment containing the recA mutant gene was obtained from each transducing phage DNA, and was inserted into the BamHI site of the plasmid ColEl::Tn3 (Ogawa et al., 1978) to generate pTM2430 and pTM2001, respectively.

(d) Purification of Proteins

(i) ϕ 80 CI repressor

YE723(pEG70) cells were grown in 4 liters of L-broth to 4×10^8 cells/ml at 32°C, and then the temperature was shifted to 42°C rapidly by adding the same volume of L-broth at 52°C. This treatment inactivates the λ CI857 repressor and consequently transcription from the λ P_L promoter upstream of the ϕ 80 cI gene is derepressed. After 1.5 hours at 42°C, cells were harvested by centrifugation and stored at -20°C. Subsequent operations were carried out at 2°C, and centrifugations were carried out at 12,000 x g for 20 minutes, unless otherwise indicated. Frozen cells (12 g) were thawed and suspended in 48 ml of buffer A (50 mM Tris-HCl, pH 8.0, 5

mM EDTA, 7 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 % glycerol) containing 0.1 M NaCl. 10 minutes after addition of 15 mg lysozyme, cells were disrupted by sonication (fraction I). To the suspension, 1.5 ml of 10 % (w/v) sodium deoxycholate was added, and the mixture was centrifuged at 60,000 x g for 60 minutes. After adjustment of the absorbance at 260 nm of the supernatant to 160 per cm by addition of buffer A containing 0.1 M NaCl, a 2 % solution of polyethyleneimine-HCl (pH 7.9; Miles, USA) was added with stirring to give a final concentration of 0.5 %. After stirring for an additional 15 minutes, the precipitate was collected by centrifugation. It was suspended in 60 ml of buffer A containing 0.5 M NaCl, and the suspension was stirred for 60 minutes. To the supernatant obtained by centrifugation, solid ammonium sulfate was added slowly with stirring to 40 % saturation, and the precipitate was removed by centrifugation. To the supernatant, ammonium sulfate was added to give a final concentration of 65 % saturation. The precipitate obtained by centrifugation was dissolved in 5 ml of buffer B (10 mM Tris-HCl, pH 7.6, 2 mM EDTA, 5 mM 2-mercaptoethanol, 5 % glycerol) containing 0.15 M NaCl, and dialyzed against the same buffer for 12 hours with 3 changes of the outer buffer solution. After clarification of the dialyzate by centrifugation, the supernatant (fraction II) was diluted 4-fold with buffer B containing 0.15 M NaCl, and applied to a 2.2 x 10 cm DEAE-cellulose (Brown, USA) column previously equilibrated with buffer B containing 0.18 M NaCl. The column was washed with 100 ml of the same buffer, and proteins were eluted with 100 ml of buffer B containing 0.26 M NaCl. A portion of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis, and the amount of the repressor was estimated from the density of its characteristic band after staining with Coomassie Brilliant Blue R-250. The fractions containing the repressor were pooled (fraction

III), and proteins were precipitated by dialyzing against saturated ammonium sulfate solution containing 50 mM potassium phosphate (pH 6.8) and 5 mM 2-mercaptoethanol for 12 hours. The precipitate was collected by centrifugation and dissolved in 5 ml of buffer C (10 mM potassium phosphate, pH 6.8, 0.1 mM EDTA, 2 mM 2-mercaptoethanol), and dialyzed against the same buffer for 12 hours. The dialyzate was diluted 3-fold with buffer C and applied to a 2.2 x 5 cm phospho-cellulose (P11 Whatman, USA) column previously equilibrated with buffer C, and the column was washed with 40 ml of the same buffer. The flow through fractions were directly applied to a 2.2 x 6.5 cm Affi-Gel Blue (Bio Rad, USA) column which was joined to the bottom of the phospho-cellulose column. The Affi-Gel Blue column was then separated from phospho-cellulose column, and was washed with 100 ml of buffer C containing 0.7 M NaCl. Proteins were eluted with 150 ml of buffer C containing 1.1 M NaCl (fraction IV). Fraction IV, containing the CI repressor at greater than 95 % purity, was then applied to a 2 ml hydroxyapatite (Seikagaku Kogyo, Japan) column previously equilibrated with buffer P1 (10 mM potassium phosphate, pH 6.8, 2 mM 2-mercaptoethanol) to concentrate the protein. The column was washed with 8 ml of buffer P2 (0.1 M potassium phosphate, pH 6.8, 2 mM 2-mercaptoethanol) and the proteins were eluted with 5 ml of buffer P3 (0.4 M potassium phosphate, pH 6.8, 2 mM 2-mercaptoethanol). The eluate was then applied to a 1.6 x 65 cm Sephacryl S-200 superfine (Pharmacia Fine Chemicals, USA) column previously equilibrated with buffer B containing 0.5 M NaCl but without EDTA, and filtrated with a flow rate of 14 ml/hour. ϕ 80 CI repressor was eluted at 1.2 x void volume and appeared to be greater than 98 % pure judged by densitometric scanning of the stained gel after electrophoresis of a portion of the fraction (fraction V). Fraction V was

concentrated with hydroxyapatite as above. The eluate was dialyzed against buffer B, and then against buffer G (10 mM Tris-HCl, pH7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 50 % glycerol) and stored at -20°C. This preparation did not show any nuclease activity against single- or double-stranded DNA.

(ii) φ80 CI repressor labelled with [³⁵S]-cysteine

YE723(pEG70) cells were grown to 3×10^8 cells/ml at 32°C in 500 ml of 3XD medium (Fraser & Jerrel, 1953), containing all amino acids (40 µg/ml each) except cysteine and methionine in place of casamino acids, and then the temperature was shifted to 42°C by adding half the volume (250 ml) of the same medium at 65°C, and incubation was continued at 42°C. After 10 minutes, 1 mCi (1 pmol) of [³⁵S]-cysteine was added and the incubation was continued for 80 minutes. Cells were harvested by centrifugation and stored at -20°C. Using 0.5 g cells labelled with [³⁵S]-cysteine and 5 g of nonlabelled cells which were grown and harvested by the same procedure described above except for omission of [³⁵S]-cysteine, I purified 2.2 mg φ80 CI repressor as described above with purity of more than 95 %. It had a specific radioactivity of 175,000 cpm/mg.

(iii) RecA protein, λ CI repressor and LexA repressor

The wild-type RecA protein was purified to more than 98 % purity from C600(pTM2) cells as described by Kuramitsu et al. (1981) with additional gel filtration using Sephacryl S-300 superfine (Pharmacia Fine Chemicals, USA). RecA430 protein and RecA1 protein were purified to more than 95 % purity from STSrecA430(pTM2430) and STSrecA1(pTM2001) cells, respectively, using the same procedure as that used for purification of the wild-type RecA protein, and supplied by Dr. T. Ogawa.

The λ CI repressor was purified to more than 95 % purity from W3102 recA3 (pKB252) cells as described by Sauer & Anderegg (1978) and Craig & Roberts (1981) with additional DEAE-cellulose column chromatography and gel filtration using Sephacryl S-300 superfine.

The LexA repressor was purified to more than 95 % purity from W3623(pTH227) cells as described by Horii et al. (1981a).

None of the protein preparations showed any nuclease activity against single- or double-stranded DNA.

(iv) Determination of the concentrations of proteins and nucleic acids

Concentrations of proteins and nucleic acids were determined from their extinction coefficients as follows: $E_{278}^{1\%} = 5.7$ for RecA protein (Kuramitsu et al., 1981), $E_{280}^{1\%} = 13.5$ for $\phi 80$ CI repressor, $E_{280}^{1\%} = 9.92$ for λ CI repressor, $E_{260}^{1\%} = 200$ for double-stranded DNA, $E_{260}^{1\%} = 278$ for single-stranded DNA.

(e) RecA-mediated Cleavage of Repressors

Cleavage of repressors was performed at 37°C in a 50 μ l mixture containing 1.8 μ M purified RecA protein, 9.3 μ M heat-denatured calf thymus DNA, 3.3 μ M purified repressor, 12 mM Tris-HCl (pH 7.6), 5 mM MgCl_2 , 1 mM dithiothreitol, 0.05 mM EDTA, 3 mM KCl, 3 % glycerol and 1 mM ATP or dATP or 50 μ M ATP- γ -S. Reaction mixtures for the cleavage of LexA and λ CI repressors contained 4 mM NaCl in addition. Where indicated, an ATP or dATP regeneration system, consisting of 5 μ g/ml of rabbit muscle creatine kinase, 4 μ g/ml bovine serum albumin and 30 mM creatine phosphate, were included. After incubation, each reaction mixture was chilled and 12.5 μ l

lysis buffer (300 mM Tris-HCl, pH 6.8, 10 % sodium dodecyl sulfate, 25 % 2-mercaptoethanol, 25 % glycerol, 0.025 % bromophenol blue) was added. The products were analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue R-250.

(f) SDS-polyacrylamide Gel Electrophoresis

A polyacrylamide slab gel with 3 % stacking and 12.5 % resolving gels was prepared according to the method of Laemmli (1970). For the analysis of cleavage of repressors, a 12.5 % - 25 % gradient gel was used as the resolving gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

(g) Determination of the Amino Acid Composition and Sequence of Proteins

Proteins (25 µg) were hydrolyzed in 5.7 N HCl in evacuated, sealed tubes for 24 or 72 hours at 110°C. The amino acid composition of the resulting hydrolysates were determined by an automated amino acid analyzer model A3300 (Irica Instruments Inc.) essentially as described by Spackman et al. (1958). The manual Edman degradation was carried out as described (Blombäck et al., 1966) and phenylthiohydantoin derivatives were identified by high performance liquid chromatography (Zimmerman et al., 1977) and by thin layer chromatography (TLC) with the solvent V (Jeppsson & Sjöquist, 1967).

4. Results

(a) Purification of the ϕ 80 CI Repressor

In order to overproduce the ϕ 80 CI repressor, I constructed plasmid pEG70 by replacing the operator-promoter region of the ϕ 80 cI gene with the λ P_L promoter, which is regulated by the temperature-sensitive λ CI857 repressor (see Materials and Methods). The amount of heat-induced protein in the cells harboring this plasmid reached about 1 % of the total protein after incubation for 90 minutes at 42°C (Fig. II-1, lane d). From 12 g of these cells (wet weight), 4.5 mg of the protein of more than 98 % purity was obtained (Fig. II-2). Following results show this protein to be ϕ 80 CI repressor. The amino acid sequence of five residues from the amino terminus of the purified protein was NH₂-Ser-Ser-Ile-Ser-Glu- (Fig. II-3 A). This agrees with the amino terminal sequence predicted from the DNA sequence of the cI gene (Ogawa et al., 1987a), except that the predicted first residue, formylmethionine, has been removed from the initial product. In addition, the amino acid composition of the protein agrees quite well with that predicted from the nucleotide sequence of the cI gene (Table II-2). Furthermore, the purified protein bound to the operator regions of ϕ 80 DNA specifically (Ogawa et al., 1987b). The molecular weight of the protein is calculated from the nucleotide sequence of the gene to be 26,380.

(b) Cleavage of ϕ 80 CI Repressor by

Wild-type RecA Protein or RecA430 Protein

Fig. II-1

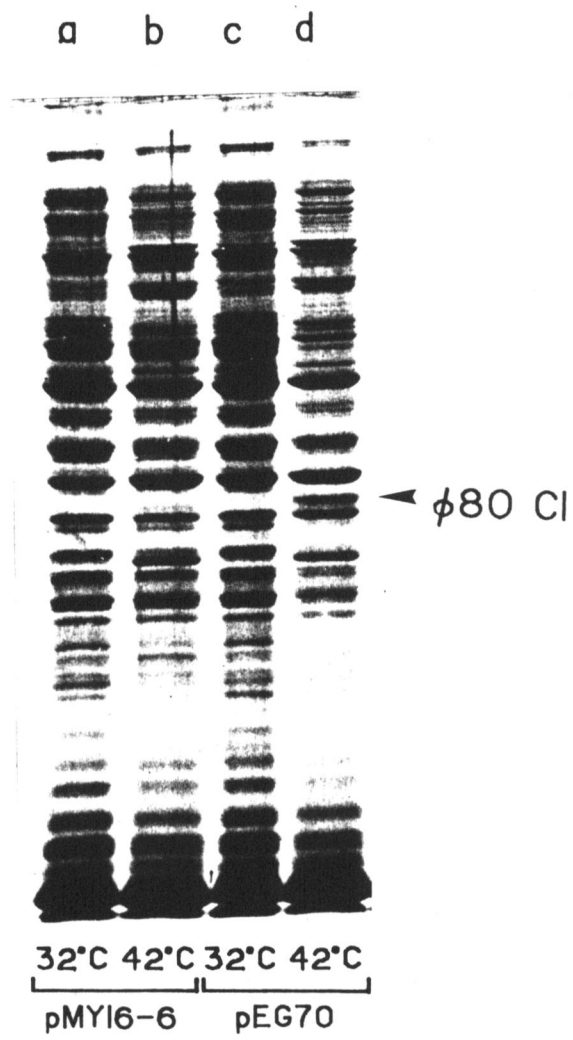


Figure II-1. Production of the $\phi 80$ CI Repressor in Heat-induced YE723(pEG70) Cells. Cells were grown in 10 ml L-broth to a density of 4×10^8 cells/ml at 32°C. Then the incubation temperature of a 5 ml portion of the culture was shifted to 42°C. After additional incubation for 1.5 hours, cells were harvested and suspended in 0.5 ml of 50 mM Tris-HCl (pH 8.0) buffer containing 10 mM EDTA. To the suspension, 0.05 ml of 10 mg/ml of lysozyme was added. After three cycles of freezing and thawing, 140 μ l of lysis buffer was added and the mixture was heated for 4 minutes in boiling water. A 20 μ l sample was analyzed by electrophoresis in 12.5 % gel of SDS-polyacrylamide. The gel was stained with Coomassie Brilliant Blue R-250. (lane a) YE723(pMY16-6) cultured at 32°C through out. (lane b) YE723(pMY16-6) followed by heat-induction at 42°C. (lane c) YE723(pEG70) cultured at 32°C through out. (lane d) YE723(pEG70) followed by heat-induction at 42°C. Arrow head indicates the position of heat-induced protein specifically in pEG70-harboring cells which was identified to be $\phi 80$ CI repressor as described in Results.

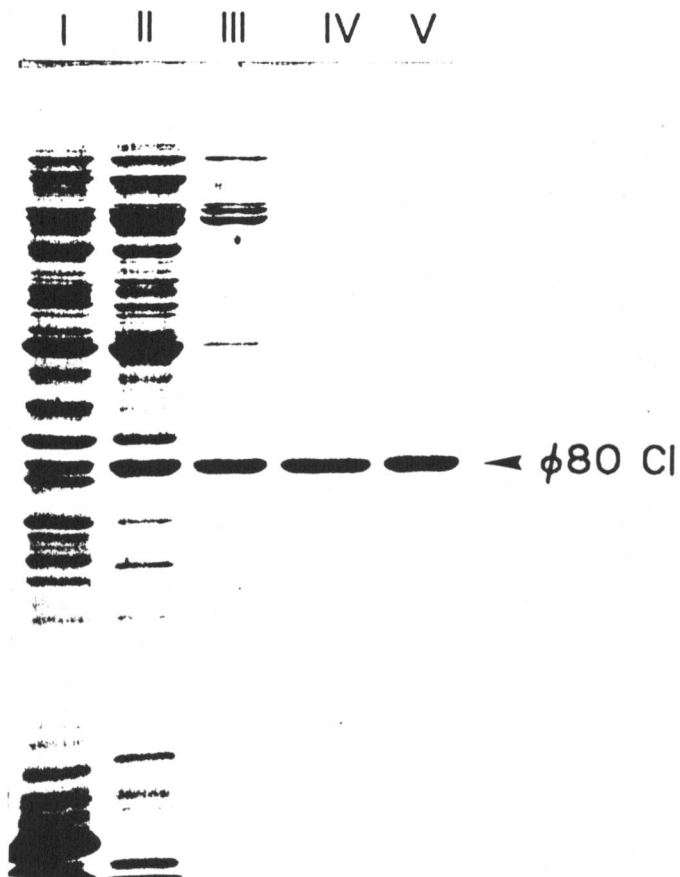
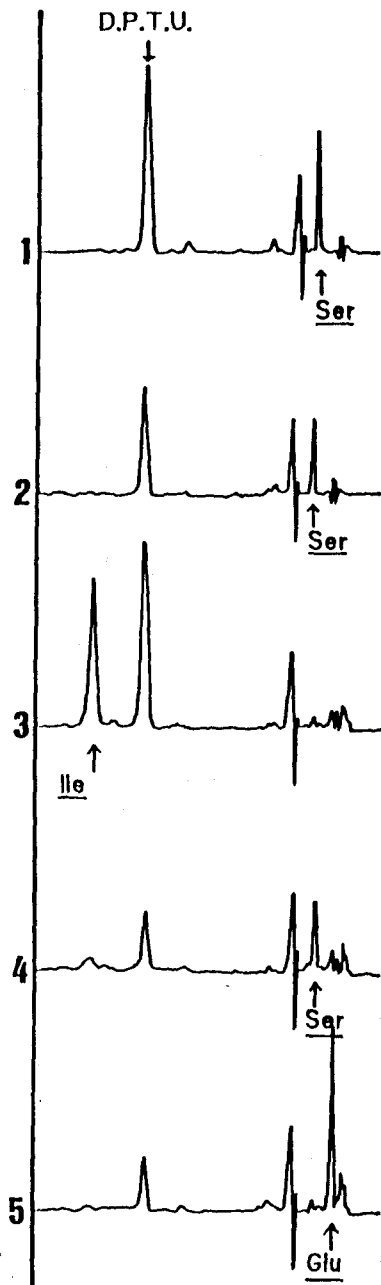


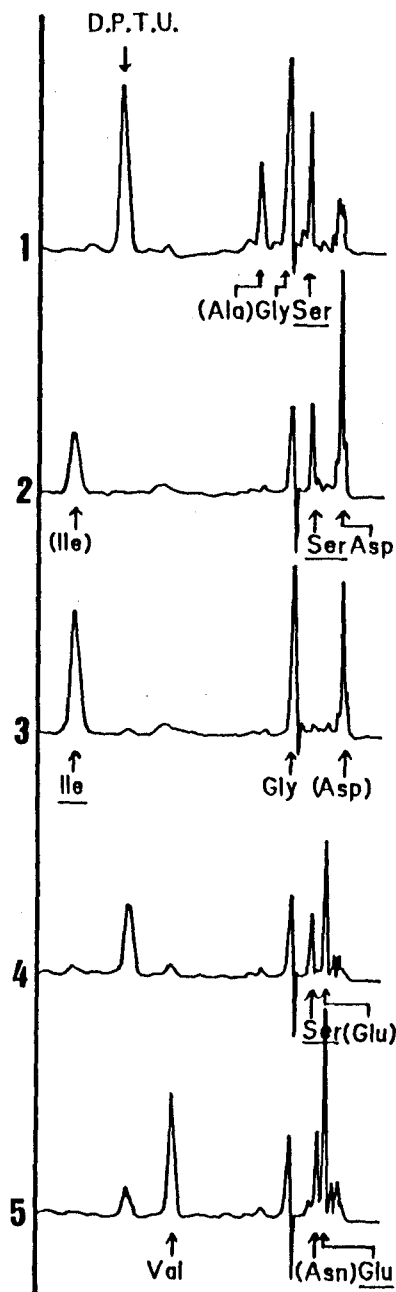
Figure II-2. Electrophoretic Analysis of Proteins during the Process of Purification of the $\phi 80$ CI Repressor. Samples from each purification step of the repressor were separated by electrophoresis in 12.5 % gel of SDS-polyacrylamide followed by staining with Coomassie Brilliant Blue R-250. Fraction I : Total cellular proteins from induced YE723(pEG70) cells. Fraction II : Dialyzate after the precipitation with 40 % - 65 % saturated ammonium sulfate. Fraction III : Eluate from DEAE-cellulose column. Fraction IV : Eluate from Affi-Gel Blue column. Fraction V : Eluate from Sephacryl S-300 superfine column. Arrow head indicates the position of $\phi 80$ CI repressor.

Fig. II-3

A. Intact $\phi 80$ cl



B. Cleaved by RecA^{wl}



C. Cleaved by RecA₄₃₀

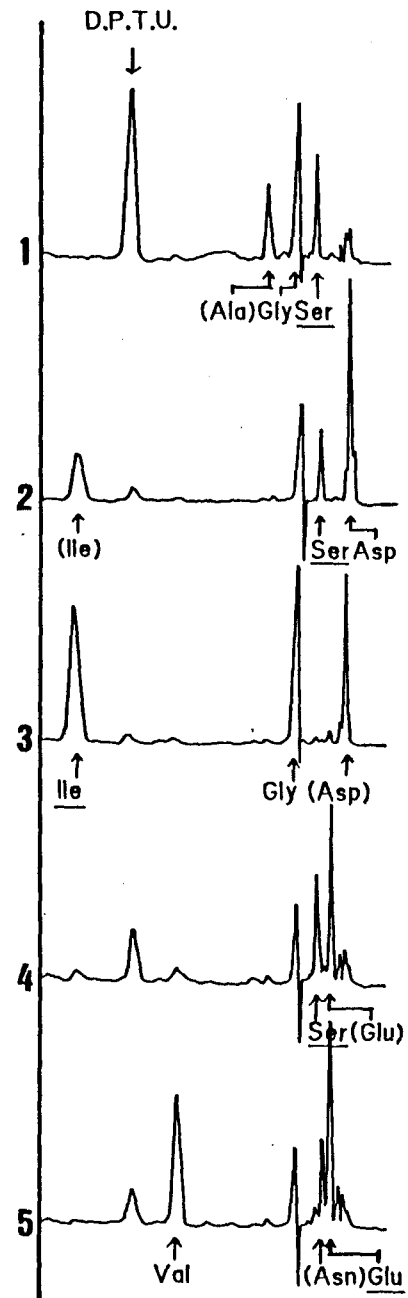


Figure II-3. Determination of the Amino Terminal Sequence of the Proteins; elution patterns of phenylthiohydantoin derivatives from high performance liquid chromatography. A : Determination of the amino terminal sequence of native repressor. 15 nmoles (400 µg) ø80 CI repressor was dialyzed against water using the Spectrapor membrane tubing (Spectrum Medical Industries Inc.) which allows passage of the substances less than 3,500 dalton. The dialyzate was lyophilized and subjected to five steps of the Edman degradation. Phenylthiohydantoin derivatives released in each step of the Edman degradations were identified using high performance liquid chromatography by absorbance at 269 nm. B and C : Determination of the sequence of the newly produced amino terminus by the cleavage of the repressor by wild-type RecA protein (B) and RecA430 protein (C), respectively. 15 nmoles ø80 CI repressor was cleaved by 5 nmoles of wild-type RecA protein or RecA430 protein in a 2 ml reaction mixture containing 10 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM ATP-γ-S, 1 mM dithiothreitol and 8.25 µg heat-denatured calf thymus DNA. After incubation for 24 hours at 37°C, the reaction mixtures were dialyzed against water using the Spectrapor membrane tubing, lyophilized and subjected to five steps of the Edman degradation. Phenylthiohydantoin derivatives were identified as described above. To confirm Ile, Val, and Asp, thin-layer chromatography was carried out. Amino acids in parentheses correspond to the amino terminal sequence of the RecA protein, NH₂-Ala-Ile-Asp-Glu-Asn-, and those underlined are amino acids released from the native amino terminus of the repressor as revealed by the results shown in A. D.P.T.U. indicates diphenylthiourea, which is a byproduct in the reaction.

Table II-2. Amino Acid Composition of the ϕ 80 CI Repressor.

Residues per Molecule		
	Analytical Value ^a	Predicted Value ^b
Asp	31.2 ^c	23
Asn		11
Thr	7.9	7
Ser	17.0	18
Glu	25.3 ^c	19
Gln		6
Pro	9.9	11
Gly	19.3	19
Ala	18.7	16
Val	16.1	13
Met	1.3	1
Ile	14.7	14
Leu	20.2	21
Tyr	4.7	6
Phe	8.6	9
Lys	14.4	15
His	2.5	2
Arg	17.6	17
Cys	ND	2
Trp	ND	5
		235

M.W. 26380

Legend to Table II-2

a : The averaged values of the number of amino acid residues obtained by acid hydrolysis for 24 hours and 72 hours are shown. Exceptions are as follows : Values for threonine and serine were obtained by extrapolation to zero time of hydrolysis. Values for valine and isoleucine were from the 72 hours hydrolysate. ND : not determined. b : Values predicted from the DNA sequence (Ogawa et al., 1987a). c : Sum of acid and amide forms.

The purified $\phi 80$ CI repressor was treated with the wild-type RecA protein, and the products were analyzed by SDS-polyacrylamide gel electrophoresis. The repressor was not cleaved by the RecA protein when ATP and ssDNA were added (Fig. II-4, lane a). However, when the ATP regeneration system, consisting of rabbit muscle creatine kinase and creatine phosphate, was included in addition to ATP and ssDNA, the repressor was cleaved by the RecA protein yielding two fragments, R_1 and R_2 of about 14,000 and 12,000 daltons, respectively (Fig. II-4, lane b). Either creatine kinase alone or creatine phosphate alone had no effect on the cleavage of the repressor (data not shown). The regeneration system is required probably for maintenance of the concentration of ATP which is hydrolyzed by the ATPase activity of the RecA protein (Ogawa *et al.*, 1978). The cleavage using dATP also requires its regeneration system (data not shown). In the presence of ATP- γ -S instead of ATP, the repressor was cleaved by the wild-type RecA protein more effectively than the case using ATP with ATP regeneration system (Fig. II-4, lane c). The cleavage reaction was not detected in the absence of either one of ssDNA, nucleoside triphosphate (Fig. II-4, lanes d and e) or $MgCl_2$ (data not shown). The optimal cleavage was observed when 5 mM $MgCl_2$ and 1 mM ATP or dATP or 50 μ M ATP- γ -S were present (data not shown). The addition of 100 mM NaCl in the complete reaction mixture reduced the efficiency of cleavage to 10 % of the efficiency observed in the absence of NaCl (data not shown). Substitution of Mn^{2+} for Mg^{2+} greatly reduced cleavage of $\phi 80$ CI repressor (data not shown) in contrast with that of λ CI repressor which was greatly enhanced by the substitution (Weinstock & McEntee, 1981). Under the condition where the wild-type RecA protein cleaves the repressor, RecA430 protein cleaves the repressor less effectively (Fig. II-4, lanes f to j). The repressor was not cleaved by RecA1 protein (Fig. II-4, lane k).

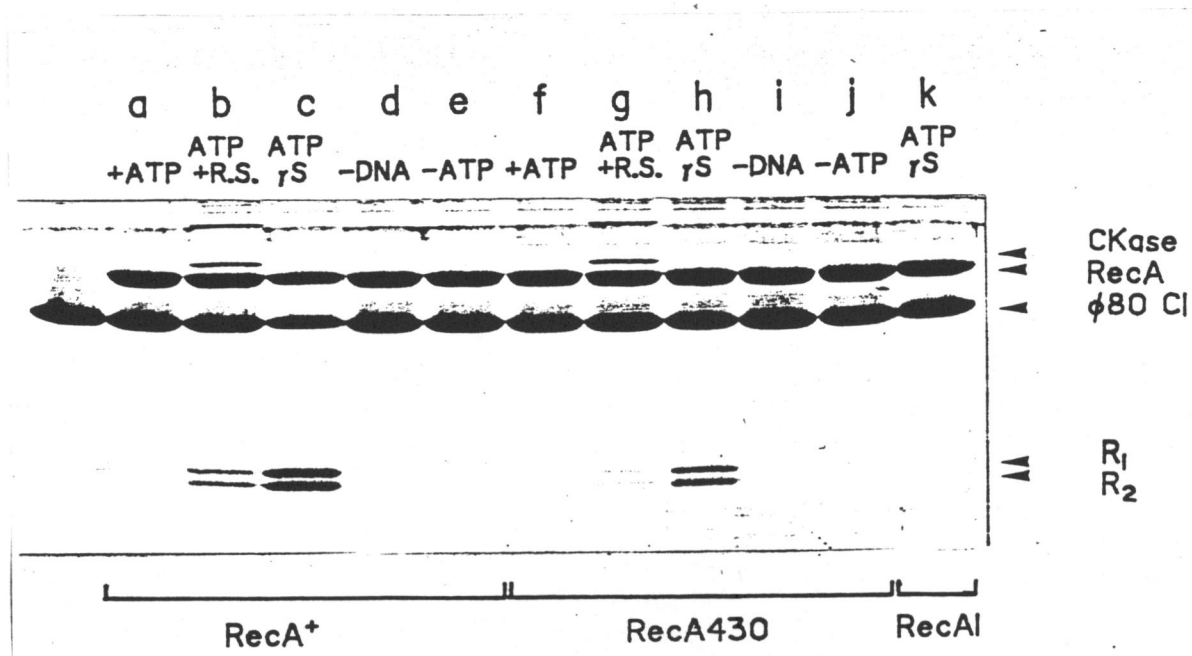


Figure 4. Cleavage of the $\phi 80$ CI Repressor by the Wild-type RecA protein, and RecA430 and RecA1 proteins. The complete reaction mixtures of 50 μ l were prepared as described in Materials and Methods except for modifications as described below. After incubation of the mixture for 6 hours at 37°C, products were analyzed by electrophoresis in 12.5 - 25 % gradient gel of SDS-polyacrylamide. The gel was stained with Coomassie Brilliant Blue R-250. Conditions used in each reaction are indicated at the top and the bottom of each lane : ATP and ATP- γ -S indicate the reactions in the presence of 1 mM ATP and 50 μ M ATP- γ -S, respectively. ATP + R.S. indicates the reaction in the presence of 1 mM ATP and ATP regeneration system. - DNA and - ATP indicate the reactions in which ssDNA or nucleoside triphosphate were omitted from the reaction mixture. R_1 and R_2 indicate the cleaved products of $\phi 80$ CI repressor, whose molecular weights were determined to be 14,000 and 12,000, respectively by separate gel electrophoresis (not shown).

I compared ability of the wild-type RecA protein and of the RecA430 protein to cleave ϕ 80 CI, λ CI and LexA repressors. The RecA430 protein cleaved both the ϕ 80 CI repressor (Fig. II-5, lane f) and the LexA repressor (Fig. II-5, lane b), but did not cleave the λ CI repressor (Fig. II-5, lane d), whereas the wild-type RecA protein cleaved all of these repressors (Fig. II-5, lanes a, c and e).

(c) Determination of the Cleavage Site in ϕ 80 CI Repressor

The ϕ 80 CI repressor was incubated with either the wild-type RecA protein or the RecA430 protein in the presence of ssDNA and ATP- γ -S, and the products were subjected to five steps of the Edman degradation. Because the samples contained the intact repressor, the cleaved products and the RecA protein, at least three kinds of amino terminal amino acids were expected to be released at each step. In both cases using the wild-type RecA protein (Fig. II-3 B) or the RecA430 protein (Fig. II-3 C), amino acid residues released in each step were found to be (Ala, Gly, Ser), (Ile, Ser, Asp), (Ile, Gly, Asp), (Arg, Ser, Glu), (Val, Asn, Glu), from the first step to fifth, respectively. The arginine residue in the fourth step was detected separately (data not shown), because, in this system, arginine and histidine residues were extracted separately to facilitate the detection of these residues. The amino terminal sequence of the intact ϕ 80 CI repressor and the RecA protein are NH₂-Ser-Ser-Ile-Ser-Glu- (Fig. II-3 A) and NH₂-Ala-Ile-Asp-Glu-Asn- (Horii *et al.*, 1980), respectively, so the sequence of the new amino terminus produced by cleavage of the ϕ 80 CI repressor was deduced as Gly-Asp-Gly-Arg-Val-. This sequence is present once, from Gly¹¹¹ to Val¹¹⁵, in the ϕ 80 CI repressor. This result

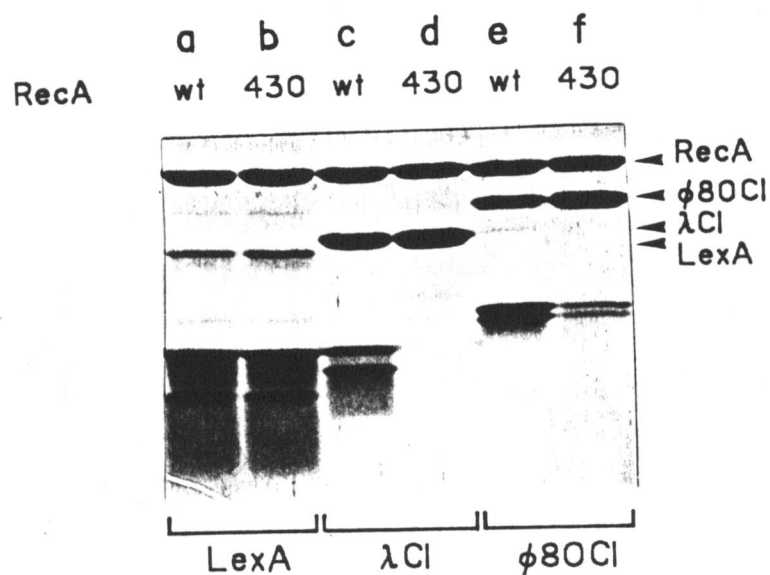
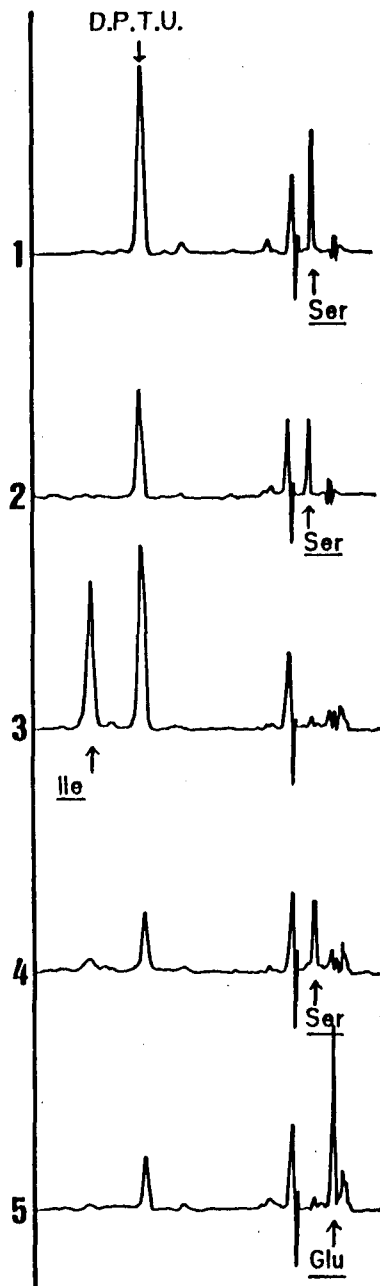


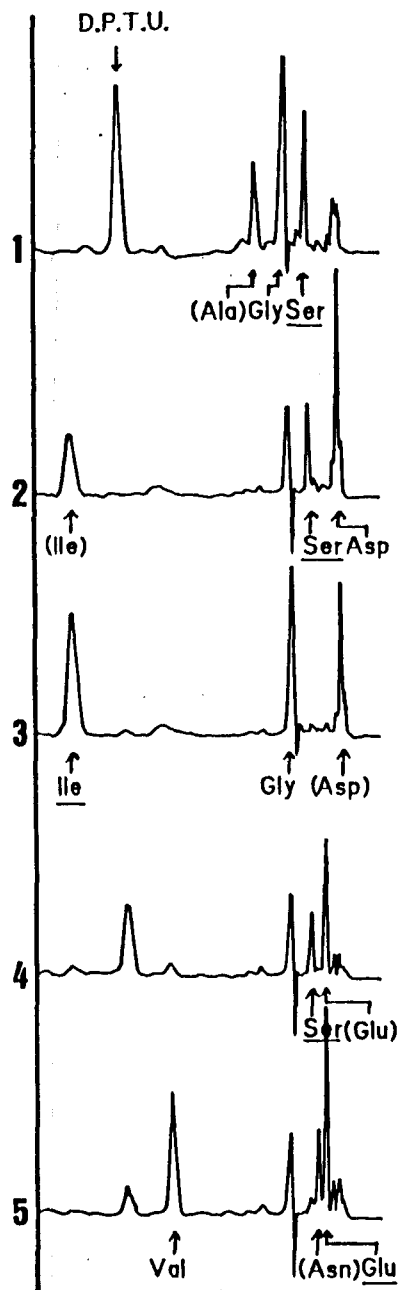
Figure II-5. Cleavage of Various Repressors by the wild-type RecA protein or RecA430 Protein. Each 50 μ l reaction mixture contained 20 μ M ATP- γ -S. After incubation for 30 minutes (for LexA repressor) or 5 hours (for λ CI and ϕ 80 CI repressors) at 37°C, reaction products were analyzed. The RecA protein and repressor used in each reaction are indicated at the top and the bottom of each lane. wt stands for wild-type.

Fig. II-3

A. Intact $\phi 80$ cl



B. Cleaved by RecA^{wt}



C. Cleaved by RecA₄₃₀

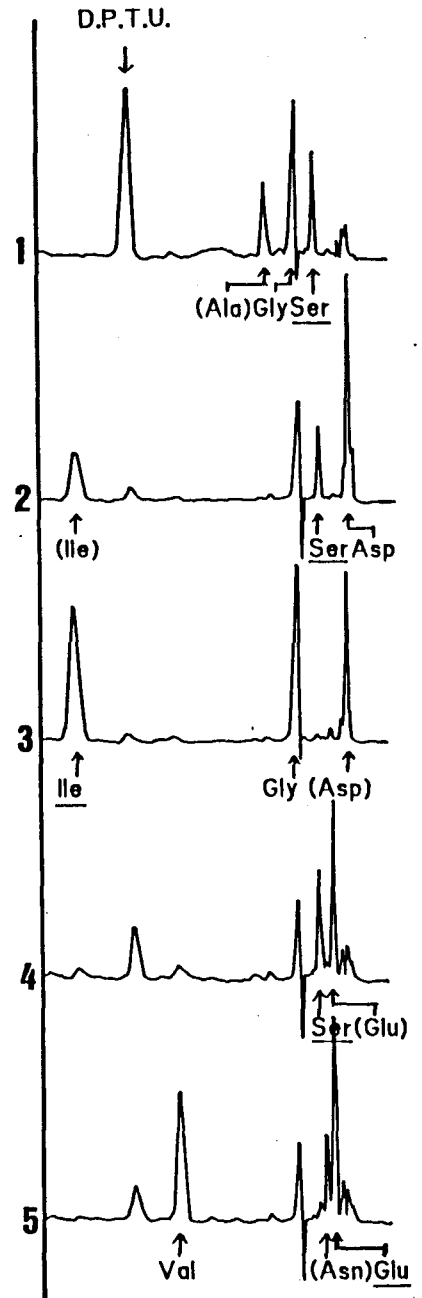


Figure II-3. Determination of the Amino Terminal Sequence of the Proteins; elution patterns of phenylthiohydantoin derivatives from high performance liquid chromatography. A : Determination of the amino terminal sequence of native repressor. 15 nmoles (400 µg) ø80 CI repressor was dialyzed against water using the Spectrapor membrane tubing (Spectrum Medical Industries Inc.) which allows passage of the substances less than 3,500 dalton. The dialyzate was lyophilized and subjected to five steps of the Edman degradation. Phenylthiohydantoin derivatives released in each step of the Edman degradations were identified using high performance liquid chromatography by absorbance at 269 nm. B and C : Determination of the sequence of the newly produced amino terminus by the cleavage of the repressor by wild-type RecA protein (B) and RecA430 protein (C), respectively. 15 nmoles ø80 CI repressor was cleaved by 5 nmoles of wild-type RecA protein or RecA430 protein in a 2 ml reaction mixture containing 10 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM ATP-γ-S, 1 mM dithiothreitol and 8.25 µg heat-denatured calf thymus DNA. After incubation for 24 hours at 37°C, the reaction mixtures were dialyzed against water using the Spectrapor membrane tubing, lyophilized and subjected to five steps of the Edman degradation. Phenylthiohydantoin derivatives were identified as described above. To confirm Ile, Val, and Asp, thin-layer chromatography was carried out. Amino acids in parentheses correspond to the amino terminal sequence of the RecA protein, NH₂-Ala-Ile-Asp-Glu-Asn-, and those underlined are amino acids released from the native amino terminus of the repressor as revealed by the results shown in A. D.P.T.U. indicates diphenylthiourea, which is a byproduct in the reaction.

indicates that $\phi 80$ CI repressor is cleaved by wild-type RecA protein or by RecA430 protein at a unique site between Cys¹¹⁰ and Gly¹¹¹.

The experiment described above does not exclude the possibility of an additional cleavage of the $\phi 80$ CI repressor by RecA protein that removes several amino acid residues from the carboxyl terminus of either fragment, because such a small peptide would be lost during the dialysis step in the sample preparation for the Edman degradation. To rule out the possibility that the cleavage might simultaneously occur at a nearby site on the amino terminal side of Cys¹¹⁰, I took advantage of the fact that the $\phi 80$ CI repressor contains two cysteine residues as the 110th and the 146th amino acids from the amino terminus. If the repressor is cleaved only between Cys¹¹⁰ and Gly¹¹¹, the Cys¹¹⁰ residue will remain in the product derived from amino terminus. On the other hand, if additional cleavage occurs at a nearby site on the amino terminal side of Cys¹¹⁰, the Cys¹¹⁰ residue will be lost from the major cleavage product. $\phi 80$ CI repressor labelled with [³⁵S]-cysteine was prepared (see Materials and Methods), treated with RecA protein, and the products were analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography of the gel. The results showed that the two cleaved products contained equal amounts of [³⁵S]-cysteine and that the total amounts of [³⁵S] were conserved in the uncleaved repressor and the cleaved fragments (Fig. II-6). This result shows that the Cys¹¹⁰ residue is part of the amino terminal product R₂ after the cleavage. Consequently, the product R₂ is composed of 110 amino acid residues and has a molecular weight of 12,422. The molecular weight of cleavage product R₁ estimated from the electrophoretic mobility is about 14,000. This value agrees with the molecular weight (13,977) predicted for a protein consisting of the 125 amino acids from position 111 to the carboxyl terminus of the CI repressor. Therefore, the carboxyl terminus of

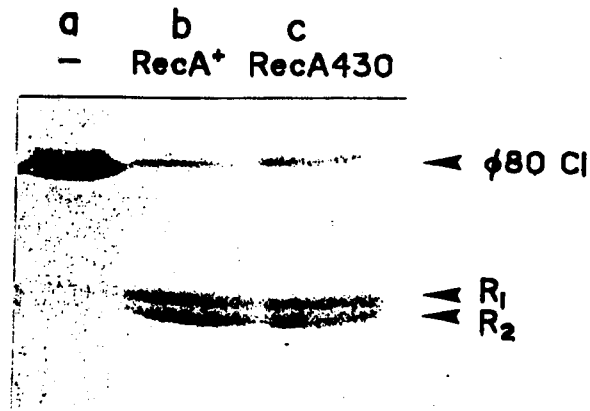


Figure II-6. Cleavage of Purified $\phi 80$ CI Repressor Labelled with [^{35}S]-cysteine with Wild-type RecA Protein or RecA430 Protein. 20 μg of the purified $\phi 80$ CI repressor labelled with [^{35}S]-cysteine was cleaved in a 1 ml reaction mixture containing 3.5 μg heat-denatured calf thymus DNA and 50 μM ATP- γ -S. After incubation for 24 hours at 37°C, the reaction mixtures were concentrated and analyzed by electrophoresis in a 12.5 - 25 % gradient gel of SDS-polyacrylamide, followed by autoradiography after treatment with ENHANCE (NEN, USA). The amount of [^{35}S]-radioactivity in the products was deduced by the results of scanning densitometry. lane a : no RecA protein, lane b : cleaved by 69 μg wild-type RecA protein, lane c : cleaved by 69 μg RecA430 protein.

the fragment R_1 is probably intact.

5. Discussion

I purified the $\phi 80$ CI repressor to more than 98 % purity from cells that overproduce the repressor. The amino acid composition and the amino-terminal sequence of the purified protein agreed with the predictions from the nucleotide sequence of the presumed $\phi 80$ cI gene except for the absence of the first residue, formylmethionine. The repressor has a molecular weight of 26,380.

The purified $\phi 80$ CI repressor was cleaved into two polypeptides by the wild-type RecA protein or RecA430 protein in the presence of ssDNA and ATP- γ -S. The RecA1 protein did not cleave the repressor under the same condition. The ability of each RecA protein to cleave the $\phi 80$ CI repressor corresponded well to the ability of each recA mutant lysogen to produce phage upon treatment with UV-light or nalidixic acid; a wild-type lysogen and a recA430 mutant lysogen can be induced but a recA1 mutant lysogen cannot.

Prophage λ is not induced by UV-irradiation in a recA430 lysogen (Blanco et al., 1975; Morand et al., 1977), while prophage $\phi 80$ is induced. Synthesis of the RecA430 protein was induced by UV-irradiation probably because of the inactivation of LexA repressor by the RecA430 protein (Devoret et al., 1983). Consistent with these in vivo results, I found that the λ CI repressor was not cleaved by the RecA430 protein (Roberts & Roberts, 1981), while the $\phi 80$ CI repressor and LexA repressor were cleaved (for LexA, Kawashima et al., 1984). The cause of the selectivity of the substrates by the RecA430 protein is not known.

Previous works showed that the Ala-Gly bond in the middle of the polypeptide was cleaved in all the repressors, such as LexA, λ CI and P22 C2 repressors, other than $\phi 80$ CI repressor (Hori et al., 1981a; Sauer et

al., 1982a). Changing the Ala-Gly sequence to Ala-Asp (in lexA3, Markham et al., 1981), Thr-Gly, Ala-Ala or Ala-Glu (in λ CI repressor, Gimble & Sauer, 1985) completely prevents the RecA-mediated cleavage (Little et al., 1980; Little, 1983; Gimble & Sauer, 1986). A variant of λ CI repressor with a Gly-Gly sequence was cleaved with much reduced rate (Gimble & Sauer, 1986). These results suggested that the RecA-mediated cleavage is highly sequence specific. The ϕ 80 CI repressor was cleaved with a significant efficiency at a unique site between Cys¹¹⁰ and Gly¹¹¹ by both the wild-type RecA protein and the RecA430 protein.

Sauer et al. (1982b) showed that the LexA, λ CI, P22 C2 and 434 repressors share extensive sequence homology in their carboxyl terminal domains. The ϕ 80 CI repressor have a sequence homologous to the other repressors in the region from Phe¹⁴⁷ to the carboxyl terminus (Fig. 7). Pabo et al. (1979) showed that the carboxyl terminal domain of λ CI repressor mediates repressor dimerization. Therefore, carboxyl terminal domain of ϕ 80 CI repressor may be responsible for its dimerization. Because dimerization would be required for efficient binding of ϕ 80 CI repressor to its operator DNA, separation of this region from the amino terminal region containing the "helix-turn-helix" sequence for binding to the operator region (Ogawa et al., 1987a) by cleavage by the RecA protein would be responsible for derepression of regulation in phage ϕ 80.

Fig.II-7

Amino Acid Sequences of Reressors

	10	20	30	40	50	60	70	homology to					
φ80	SSISERIKFLLAREGLKORDLAELSTSPOTVNNWIK-RDALSREAAQQLSEKF-GYSLDWLLNGEGSPKKDLESNIP												
LexA	MKALTARQQEVFDLIRDHISQTGMPPTRAETIAQRLGFRSP-----NAAEEHLKALARKGVIEIVSGASRGI-----												
Lambda	STKKKPLTQEQL E DARRLKAIYEKKKNELGLSQESVADKMGMGQSGVGALFNGINALNAYNAALLAKIL-KVSVEEFSPSIAREIYEMYEAVS												
P22	MNTQLMGERIRARRKKLKIRQAALGKMVGVSNVAISNWERSETEPNGENLLALS K AL-QCSPDYLLKGDL S QTNVAY----												
434	SISSRVKSKRIQLGLNQAE L AQKVGTTOQSIEQLENGKTKRPRF-LPELASAL-GVSVDWLLNGTSDSNVRF-----												
	+	*	* *	+ + *	*	+	**	+	+	++	*	LexA	
	+	+	**	*	++	*	+	**	*	+	+++	+	Lambda
	80	90	100	110	120	130	140	150					
φ80	PESEWGTVDAWDKNTPLPDDEVEVPFLKDIEFACGDGRVHDEDHNGFKLRFSKATLRRVGANSDGSGVLCF--PASGDSM-EPV----												
LexA	-----RLLNEEEGLPLVGRV--AAGEPLLAQHQHIEGHYNVDP S LFKPNAD-----FLLRVSGMSMKDIG----												
Lambda	-----MQPSLRSEYEYPVF S HV--QAGMFSELRTFTKGDAERWVSTTKASDSA-----FWLEVEGNSMTAPTGSKP												
P22	-----HSRHEPRGSYPLISWV--SAGQWMEAVEPYHKRAIENWHDTTVDCSED S -----FWLDVQGDSMTAPAGL--												
434	-----VGHVPEPKGYPLISMV--RAGSWCEACEPYDIKIDAEWYSDVNLLGNG-----FWLKEGEDSMTSPVGQ--												
	*	+++	++++	+	++++	+	*	*	**	**	+	LexA	
	*	++++	++++	+	++	*	++	*	*	**	*	Lambda	
	160	170	180	190	200	210	220	230					
φ80	-IPDGATVAVDTGNK-RNIDGELYAINQDLKRIKQLYRKPGGKILIRSINRDYDDEEAD E ADVEIIIGFVFWYSVLRYRR												
LexA	-IMDGDLLAVHKTNDVRNGQVVVARI--DDEVTVKRL-KKQGNKVELLPENSEFKPI-VVDLRQQSF T I E GLAVGVIRNGDWL												
Lambda	SFPDGM L ILVDPEQAVEPGDFCIARL-GGDEF T FFKKLIRD-SGQVFLQPLNPQYPMI-PCNESCSVVGKVIASQWPEETF G												
P22	SIPEGMIILVDPEVEPRNGKL V VAKLEGENEATFFKKLVMD-AGRKFLKPLNPQYPMI-EINGNCKIIIGVVVDAKLANLP												
434	SIPEGHMVLVD T GREPVNGSLVVAKLTDANEATFFKKLV I D-GGQKYLGKLNPSWPMT-PINGNCKIIIGVVVEARVKFV												
	*	**	+++	*	**	+	*	+	+	*	+	LexA	
	+++	+	**	+	*	+	**	+	+	+	+	Lambda	

Figure II-7. Comparisons of Amino Acid Sequences of Repressors. The amino acid sequences were aligned partially with the aid of computer using an algorithm similar to that of Toh et al. (1983), and partially by reference to the alignment reported by Sauer et al. (1982b). Numbers indicate the positions of amino acid residues from the amino terminus of the ϕ 80 CI repressor. Identical residues are indicated by underlining. * and + indicate residues of ϕ 80 CI repressor identical and with similar hydrophobicity, respectively, to those of LexA and λ CI repressors. An arrow head indicates the cleavage site by the RecA protein. "helix-turn-helix" sequence was indicated in figure. References for the amino acid sequences of repressors were Ogawa et al., (1987a) for ϕ 80 CI repressor ; Horii et al., (1981b) for LexA repressor ; Sauer & Anderegg, (1978) for λ CI repressor ; Sauer et al., (1981) for P22 C2 repressor ; Sauer et al. (1982b) for 434 repressor. Amino acid sequences of ϕ 80 CI and LexA repressors were deduced from nucleotide sequences. References for the cleavage sites were Horii et al., (1981b) for LexA repressor ; and Sauer et al., (1982a) for λ CI and P22 C2 repressors.

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CHAPTER III

Two Different Forms of RecA Protein in Cleavage of Repressor

1. Summary

Kinetics of cleavage of the $\phi 80$ CI repressor by RecA protein were analyzed. The rate of cleavage in the presence of single-stranded DNA and one of ATP, dATP or ATP- γ -S was very low in the first 1 hour and then increased sharply as the incubation was continued. The rate of cleavage was greatly increased by preincubating the RecA protein with single-stranded DNA prior to addition of ATP- γ -S, which was subsequently added to ensure cleavage of repressor. The increase of the activity of RecA protein to cleave the repressor was greatly reduced by the presence of ATP- γ -S in the preincubation mixture, which is one of essential components for the cleavage reaction. The complexes of RecA protein, single-stranded DNA and ATP- γ -S thus take two forms with different activities to cleave $\phi 80$ CI repressor.

2. Introduction

When lysogens of lambdoid phages such as λ and $\phi 80$ are treated with UV(ultraviolet)-light, growth of these prophages are induced (for review, Roberts & Deveret, 1983). The inductions of these prophages are triggered by inactivation of a repressor protein which represses the expression of all the phage genes (Roberts & Roberts, 1975). In vitro analyses showed that the repressors are inactivated by their cleavage by the RecA protein in the presence of ssDNA (single-stranded DNA) and ATP or its less hydrolyzable analogue ATP- γ -S (Craig & Roberts 1980). It was also shown that RecA protein binds tightly to ssDNA (Shibata et al., 1979) and to ATP- γ -S (Craig & Roberts, 1981) under the condition for cleavage reaction. The activation of RecA protein is thus supposed to require its binding to ssDNA and ATP or its analogues to form a ternary complex of all three components.

I showed that the phage $\phi 80$ CI repressor is cleaved by RecA protein in the presence of ssDNA and ATP or its analogue ATP- γ -S (Eguchi et al., 1987; chapter II in this thesis). In this chapter, I describe the kinetic analysis of in vitro cleavage of $\phi 80$ CI repressor by RecA protein. The results showed that cleavage of the repressor was greatly stimulated by preincubating RecA protein with ssDNA prior to addition of ATP- γ -S. The ternary complexes of these three components take two forms with different activities to cleave $\phi 80$ CI repressor.

3. Materials and Methods

(a) Chemicals

ATP and dATP were purchased from Yamasa Shoyu (Japan). ATP- γ -S and creatine phosphate were obtained from Boehringer Mannheim (West Germany). ϕ X174 viral DNA was purified according to Cunningham et al. (1980).

(b) Proteins

ϕ 80 CI repressor, λ CI repressor and RecA protein were prepared as described (Eguchi et al., 1987; chapter II in this thesis). [125 I]-labelled ϕ 80 CI repressor was prepared by iodination of the unlabelled repressor using Radioiodination System (NEN, USA) according to the instruction of supplier, followed by gel filtration and dialysis. Rabbit muscle creatine kinase and bovine serum albumin were purchased from Boehringer Mannheim and Poviet Producten N. V. (Holland), respectively.

(c) Purification of Cleaved Fragments, R_1 and R_2 , of ϕ 80 CI Repressor

ϕ 80 CI repressor (2 mg) was treated with 0.8 mg RecA protein in 8 ml of the standard reaction mixture containing 35 μ g ϕ X174 viral DNA and 50 μ M ATP- γ -S at 37°C for 12 hours. The precipitate (RecA protein - ssDNA complex) was removed by centrifugation at 3,000 rpm for 6 minutes. The obtained supernatant was diluted with the same volume of buffer P1 (10 mM potassium phosphate, pH 6.8, 7 mM 2-mercaptoethanol), and applied to 3 ml of phospho-cellulose (P-11 Whatman, USA) column previously equilibrated

with buffer P1. The flow through fractions, which contained R₂ fragment, uncleaved CI repressor and tracing amount of R₁ fragment, were pooled and used for purification of R₂ fragment as described later. The column was washed with 10 ml buffer P1 containing 0.2 M NaCl, and then the protein was eluted with 60 ml buffer P1 containing 0.5 M NaCl. The eluate was applied to 0.5 ml hydroxyapatite (Seikagaku Kogyo, Japan) column previously equilibrated with buffer P1 to concentrate the protein. The column was washed with 4 ml buffer P2 (0.1 M potassium phosphate, pH 6.8, 7 mM 2-mercaptoethanol), and protein was eluted with 1.5 ml buffer P3 (0.4 M potassium phosphate, pH 6.8, 7 mM 2-mercaptoethanol). The eluted fraction contained the R₁ fragment at greater than 95 % purity. The fraction was dialyzed against buffer G (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 2 mM dithiothreitol, 50 % glycerol) and stored at -20°C. The flow through fraction (32 ml) from phospho-cellulose column, which contained R₂ fragment, was applied to 2 ml Affi-Gel Blue (Bio Rad, USA) column previously equilibrated with buffer P1. The column was washed with 10 ml buffer P1 and protein was eluted with 40 ml buffer P1 containing 0.2 M NaCl. The protein in the eluate was concentrated with hydroxyapatite as above. The eluted fraction contained the R₂ fragment at greater than 95 % purity. The fraction was dialyzed against buffer G and stored at -20°C. Using this procedure, I obtained 0.54 mg R₁ fragment and 0.33 mg R₂ fragment. The concentration of each fragment was determined from their extinction coefficients as follows: $E_{280}^{1\%} = 8.71$ for R₁ fragment, $E_{280}^{1\%} = 18.8$ for R₂ fragment. Analysis of amino acid sequences of each fragment revealed that the R₁ fragment is derived from carboxyl-terminal half and R₂ fragment is derived from amino-terminal half (data not shown).

(d) RecA-mediated Cleavage of Repressors

Cleavage of repressors was performed essentially as described (Eguchi et al., 1987; chapter II in this thesis). The standard reaction mixtures contained 1 μ M RecA protein, 3 μ M repressor protein, 5 μ M ϕ X174 viral DNA, 12 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mM EDTA, 3 mM KCl, 3 % glycerol and 1 mM ATP or dATP or 50 μ M ATP- γ -S. When ATP or dATP was used as a cofactor, the reaction mixture always included its regeneration system, consisting of 4 μ g/ml rabbit muscle creatine kinase, 4 μ g/ml bovine serum albumin and 30 mM creatine phosphate. In the kinetic experiments, the reactions were started by final addition of ssDNA (ϕ X174 viral DNA) to the reaction mixtures, unless otherwise indicated. Incubations were performed at 37°C. At an indicated time, aliquot (40 μ l) was taken and chilled, and then 10 μ l lysis buffer (300 mM Tris-HCl, pH 6.8, 10 % sodium dodecylsulfate (SDS), 25 % 2-mercaptoethanol, 25 % glycerol and 0.025 % bromophenol blue) was added to stop the reaction. The products were analyzed by electrophoresis in a SDS-polyacrylamide gel with 3 % stacking and 17 % resolving gels essentially according to Laemmli (1970). After staining the gel with Coomassie Brilliant Blue R-250, the amounts of cleaved repressor were determined by scanning spectrophotometrically with the Toyo digital densitometer DMU-33C, using known amounts of completely cleaved repressor as a standard.

4. Results

(a) Kinetic Analysis of Cleavage Reaction of Repressors

I showed that the phage $\phi 80$ CI repressor is cleaved by RecA protein in the presence of ssDNA and ATP or dATP or ATP- γ -S (Eguchi et al., 1987; chapter II in this thesis). When ATP or dATP was used, their regeneration system consisting of creatine kinase and creatine phosphate was indispensable for cleavage. To examine the kinetics of cleavage of $\phi 80$ CI repressor by wild-type RecA protein, these proteins were incubated with $\phi X174$ viral DNA and ATP or dATP with their regeneration system or ATP- γ -S as cofactors. The reactions were started by immediate final addition of ssDNA to the mixtures, and the amounts of the cleaved products at each time were determined densitometrically after electrophoresis in a SDS-polyacrylamide gel. In the reaction mixture containing ATP or dATP with their regeneration system, cleavage of the $\phi 80$ CI repressor by the RecA protein was very slow in the first 1 hour, and the products were increased by further incubation (Fig. III-1 A). The rate of cleavage after the early slow cleavage was much larger using dATP and its regeneration system than that using ATP and its regeneration system. ATP- γ -S gave almost the same kinetics as that using dATP and its regeneration system.

To examine whether or not the slow cleavage observed in the early stage of cleavage of $\phi 80$ CI repressor was also observed in cleavage of λ CI repressor, the kinetics of cleavage of λ CI repressor by wild-type RecA protein was studied similarly (Fig. III-1 B). Cleavage of λ CI repressor by the RecA protein proceeded without a detectable lag in all cases using ATP, dATP or ATP- γ -S as one of cofactors. Cleavage was much faster when dATP with its regeneration system or ATP- γ -S was used than when ATP was

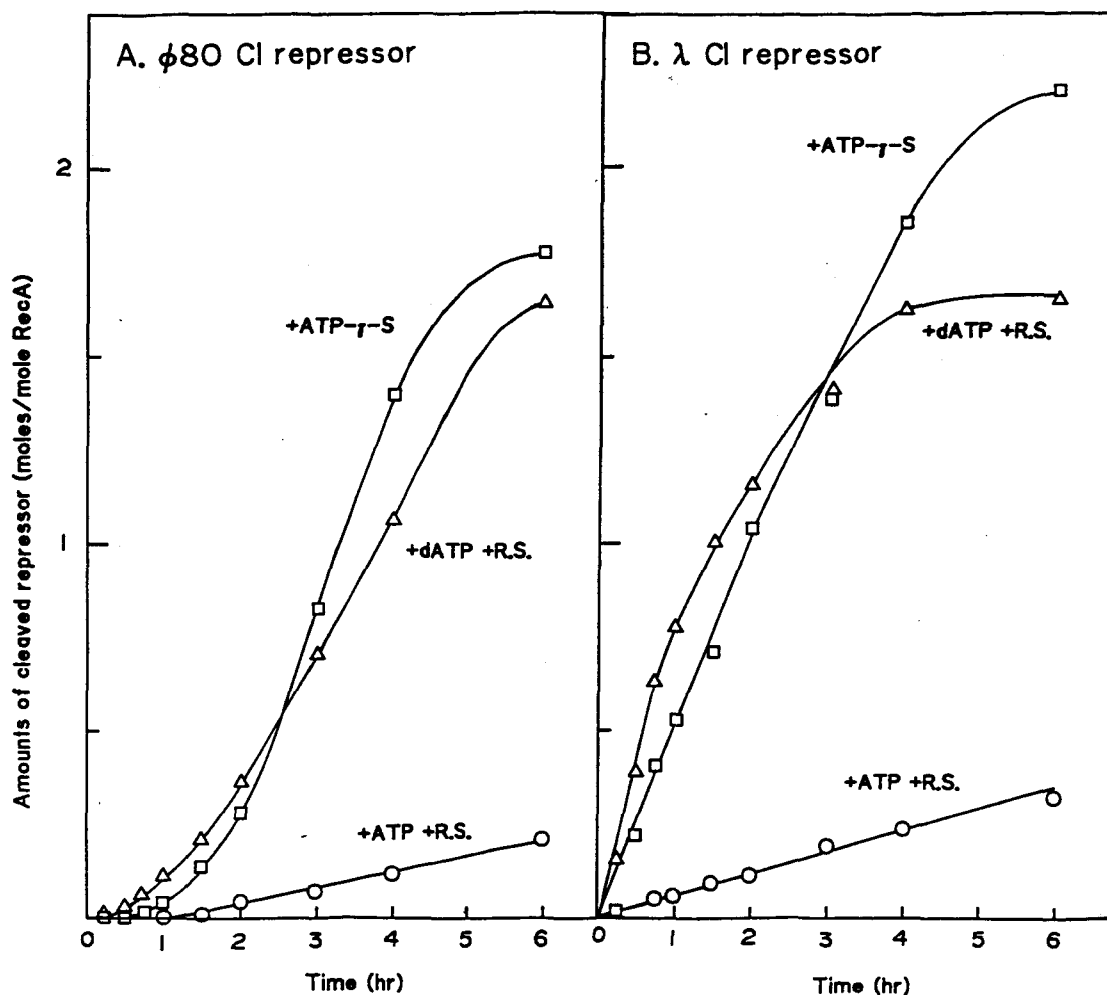


Figure III-1. Kinetics of the Cleavage Reaction of Repressors. $\phi 80$ CI repressor (3 μM) (A) and λ CI repressor (3 μM) (B) were incubated at 37°C with 1 μM wild-type RecA protein in a 500 μl reaction mixture in different conditions. Reaction was started by the immediate addition of $\phi X174$ viral DNA at the final concentration of 5 μM as the last constituent. Aliquots (40 μl) were taken at indicated times and the products were analyzed by electrophoresis in 17 % gel of SDS-polyacrylamide, followed by staining with Coomassie Brilliant Blue R-250. The amounts of the products were estimated by densitometric scanning of the band of the products in a gel. The reaction mixtures were supplemented with 1 mM ATP and ATP regeneration system (circle), with 1 mM dATP and dATP regeneration system (triangle) or with 50 μM ATP- γ -S (square).

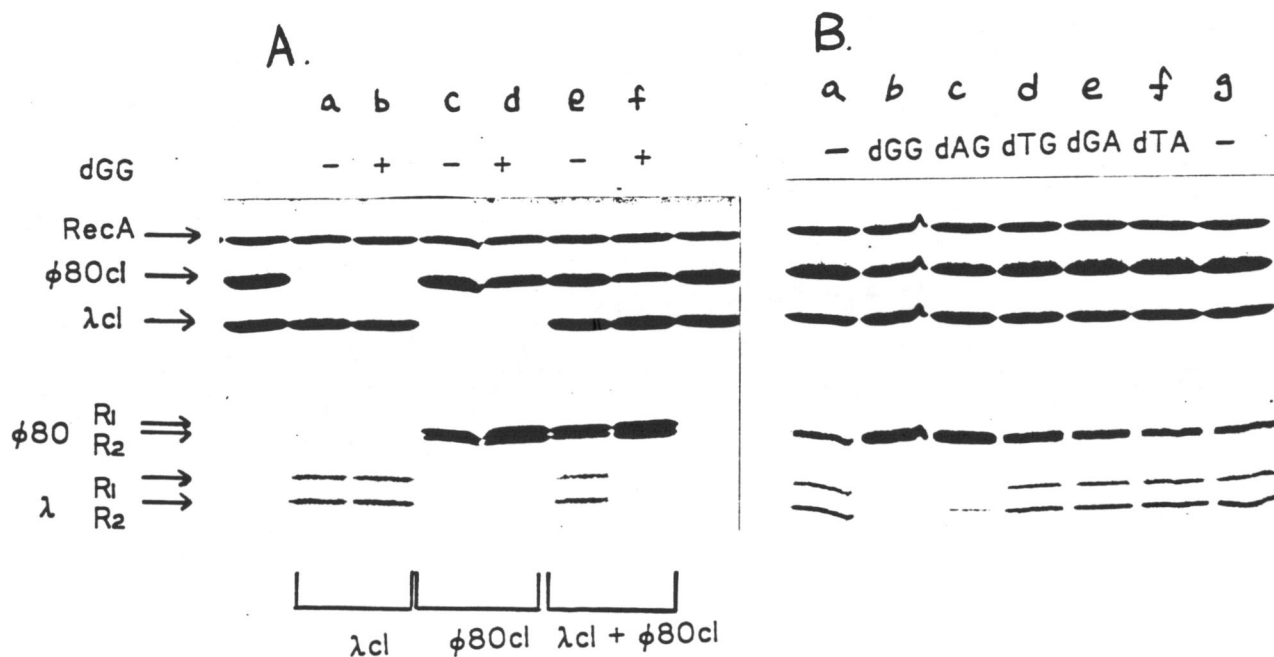


Figure IV-6. Preferential Cleavage of $\phi 80$ CI Repressor in the Presence of d(G-G) or d(A-G). $\phi 80$ CI and/or λ CI repressors ($3 \mu\text{M}$) were cleaved by RecA protein ($1 \mu\text{M}$) in $50 \mu\text{l}$ of reaction mixtures containing $50 \mu\text{M}$ ATP- γ -S for 3 hours at 37°C , and reaction products were analyzed by electrophoresis in a 17 % resolving gel of SDS-polyacrylamide. A. lanes a and b: λ CI repressor was cleaved; lanes c and d: $\phi 80$ CI repressor was cleaved; lanes e and f: λ CI and $\phi 80$ CI repressors were cleaved together in the same reaction mixture. The reaction mixtures of lanes b, d and f were contained d(G-G) at $50 \mu\text{M}$, in addition. B. λ CI and $\phi 80$ CI repressors were cleaved together in the same reaction mixture containing $50 \mu\text{M}$ of the indicated deoxydinucleoside monophosphate.

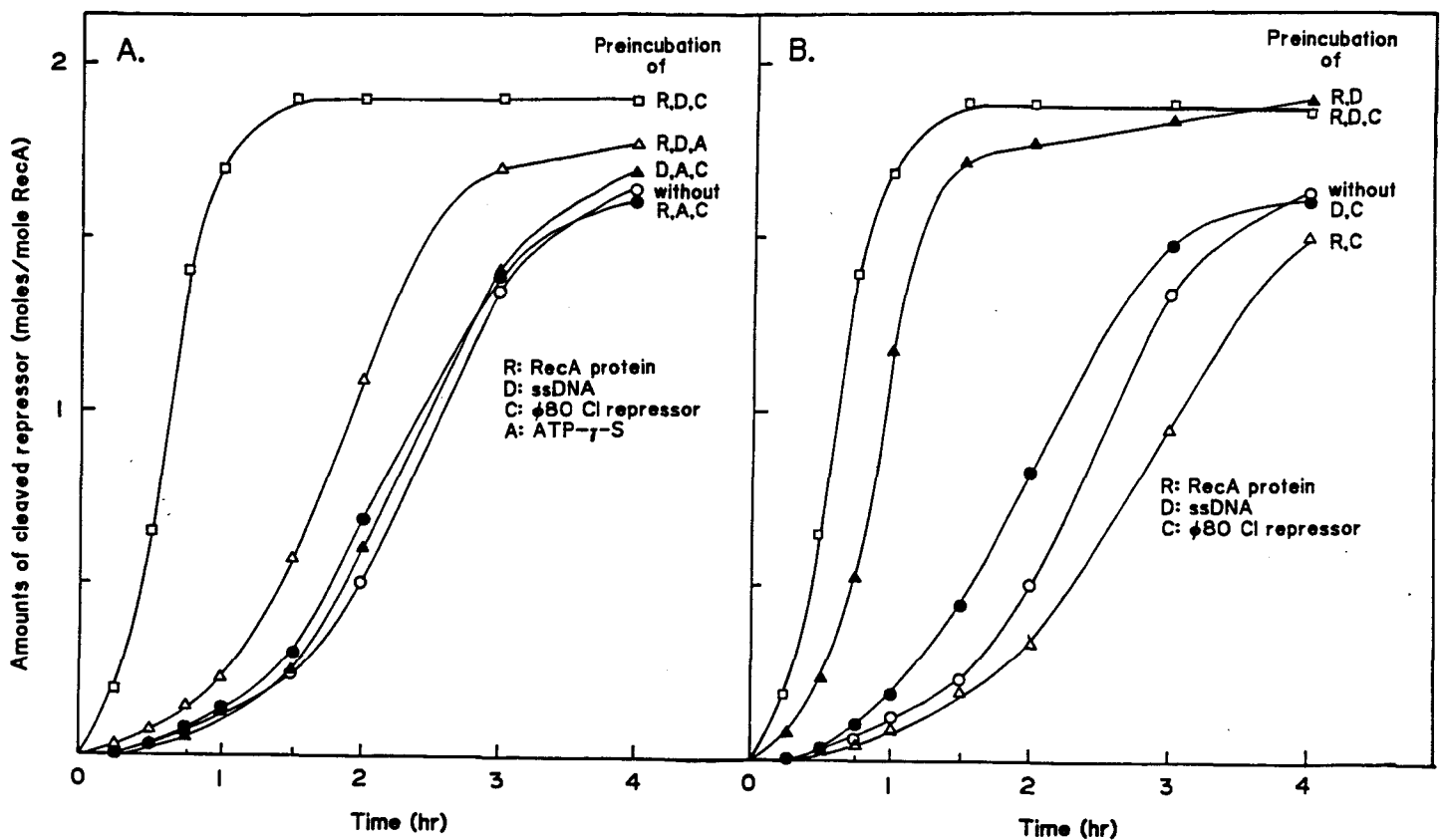


Figure III-2. Kinetics of the Cleavage Reactions of Repressors after Preincubation. The reaction mixtures were preincubated at 37°C with the omission of one component (A) or of two components (B). After 2 hours, omitted components were added to start the reaction. Aliquots (40 μ l) were taken at indicated times and the amounts of the products were analyzed as described in the legend for Figure III-1. The components presented in the initial reactions are indicated in the figure.

repressor is inhibited by the presence of ATP- γ -S, whose presence is necessary for the cleavage reaction.

(c) Incubation Time Necessary for Reduction of the Time
of Early Slow Reaction

When cleavage of ϕ 80 CI repressor by RecA protein in the presence of ATP- γ -S was started by immediate final addition of ssDNA, the repressor was cleaved very slowly; 0.03 moles/mole RecA protein in the first 30 minutes. If the RecA protein and ssDNA were preincubated in the absence of ATP- γ -S for 2 hours, the repressor was cleaved at a stimulated rate about 0.25 moles/mole RecA protein in the first 30 minutes. This difference in the rates indicates that the activity of RecA protein to cleave the repressor was changed by preincubating the protein and ssDNA. To know the time necessary for the maximum effect of preincubation, RecA protein was preincubated with ssDNA for various periods, and then the cleavage reaction was started by adding ϕ 80 CI repressor and ATP- γ -S into the mixture. After a 30-minute incubation, the amounts of the cleaved products were measured. The result showed that the amounts of products increased with increasing preincubation time and reached a plateau after about 30 minutes of preincubation (Fig. III-3). Electron microscopic study (Flory & Radding, 1982) and the filter binding study (unpublished data) showed that RecA protein bound to ssDNA rapidly. Under the condition used here, most RecA protein molecules are likely to complete binding to ssDNA in several minutes. Therefore, the requirement of 30 minutes to maximize the effect of preincubation shows that the conformation of the RecA - ssDNA binary complex must change in 30 minutes after their initial binding into an effective form for cleavage of the ϕ 80 CI repressor.

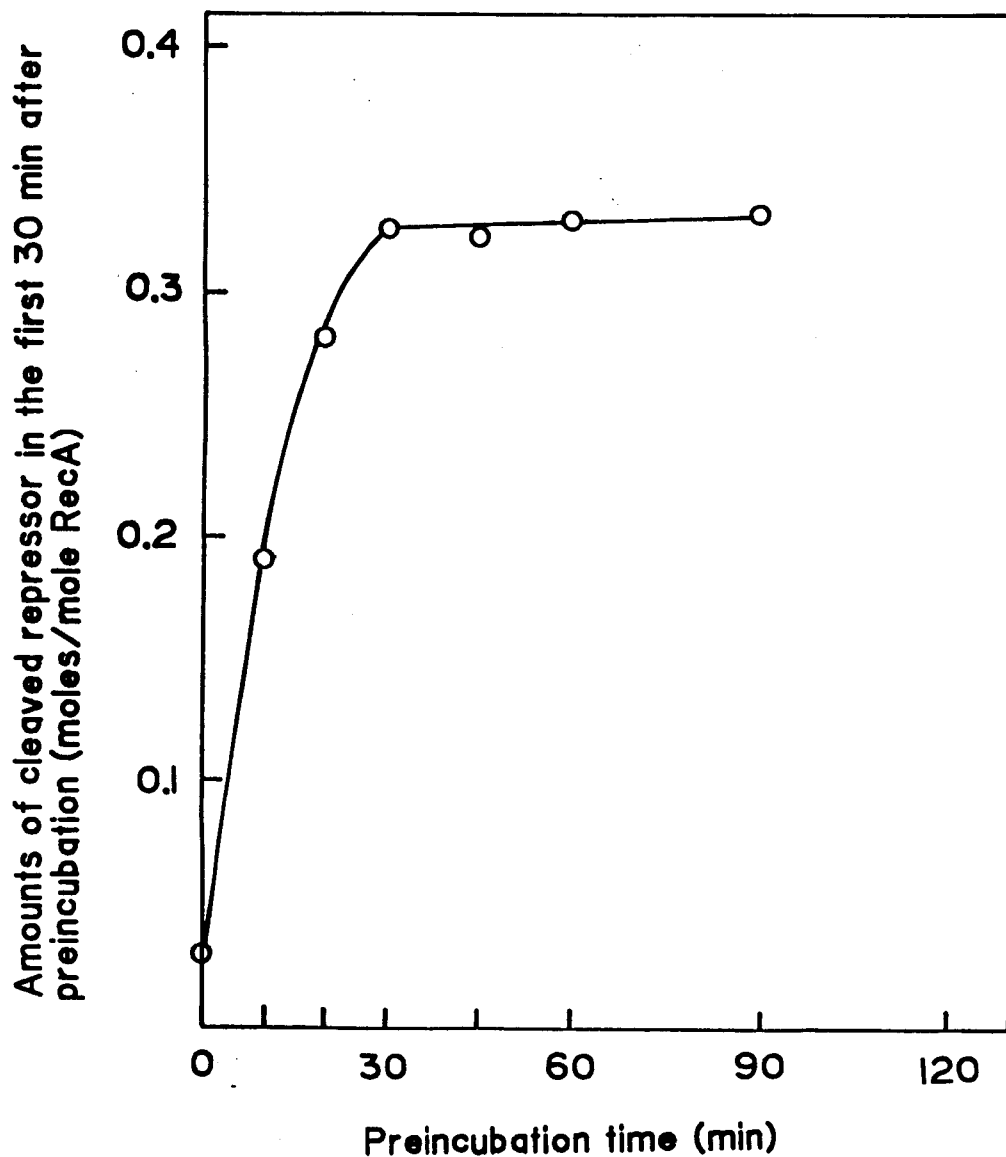


Figure III-3. Preincubation Time of RecA Protein and ssDNA Necessary for Stimulation of Cleavage of $\phi 80$ CI Repressor. RecA protein ($1 \mu\text{M}$) and ϕX174 viral DNA ($5 \mu\text{M}$) were preincubated in $40 \mu\text{l}$ reaction mixtures in the absence of $\phi 80$ CI repressor and ATP- γ -S at 37°C for indicated periods. Then $3 \mu\text{M}$ $\phi 80$ CI repressor and $50 \mu\text{M}$ ATP- γ -S was added to the mixture. After the mixtures were incubated for additional 30 minutes, products were analyzed by electrophoresis in SDS-polyacrylamide gel, and the amounts of the products were estimated densitometrically.

When RecA protein and ssDNA were preincubated in the presence of ATP- γ -S, the activity to cleave ϕ 80 CI repressor increased slightly for 4 to 5 hours preincubation, and then reached a plateau (Fig. III-4). The rate of increase of the activity by the preincubation in the presence of ATP- γ -S was about 3 % of the rate when ATP- γ -S was absent in the preincubation. This indicates that the binding of ATP- γ -S to non-preincubated RecA - ssDNA complex inhibits the formation of the RecA - ssDNA - ATP- γ -S complex formed after preincubating RecA protein and ssDNA. The activity found when the RecA protein, ssDNA and ATP- γ -S were preincubated for 5 hours was expressed by the complex that should be produced by conformational change from non-preincubated RecA - ssDNA - ATP- γ -S complex.

I also analyzed the effects of ATP- γ -S on the RecA - ssDNA - ATP- γ -S complex formed after preincubating RecA protein and ssDNA (Fig. III-5). RecA protein and ssDNA was preincubated for 2 hours to allow the formation of the effective form, and then ATP- γ -S was added and the preincubation was continued for various periods. After starting the cleavage reaction by adding ϕ 80 CI repressor, amounts of the cleaved products in the first 30 minutes were measured. It was found that the amounts of the products in the first 30 minutes were not varied. This result shows that the activity of the ternary complex formed by binding of ATP- γ -S to preincubated RecA - ssDNA complex whose formation is inhibited by ATP- γ -S is not reduced by the presence of ATP- γ -S.

(d) Stimulation Effect of Cleaved Fragment R_1
on Cleavage of ϕ 80 CI Repressor

The cleavage rate after the slow reaction in the first 1 hour

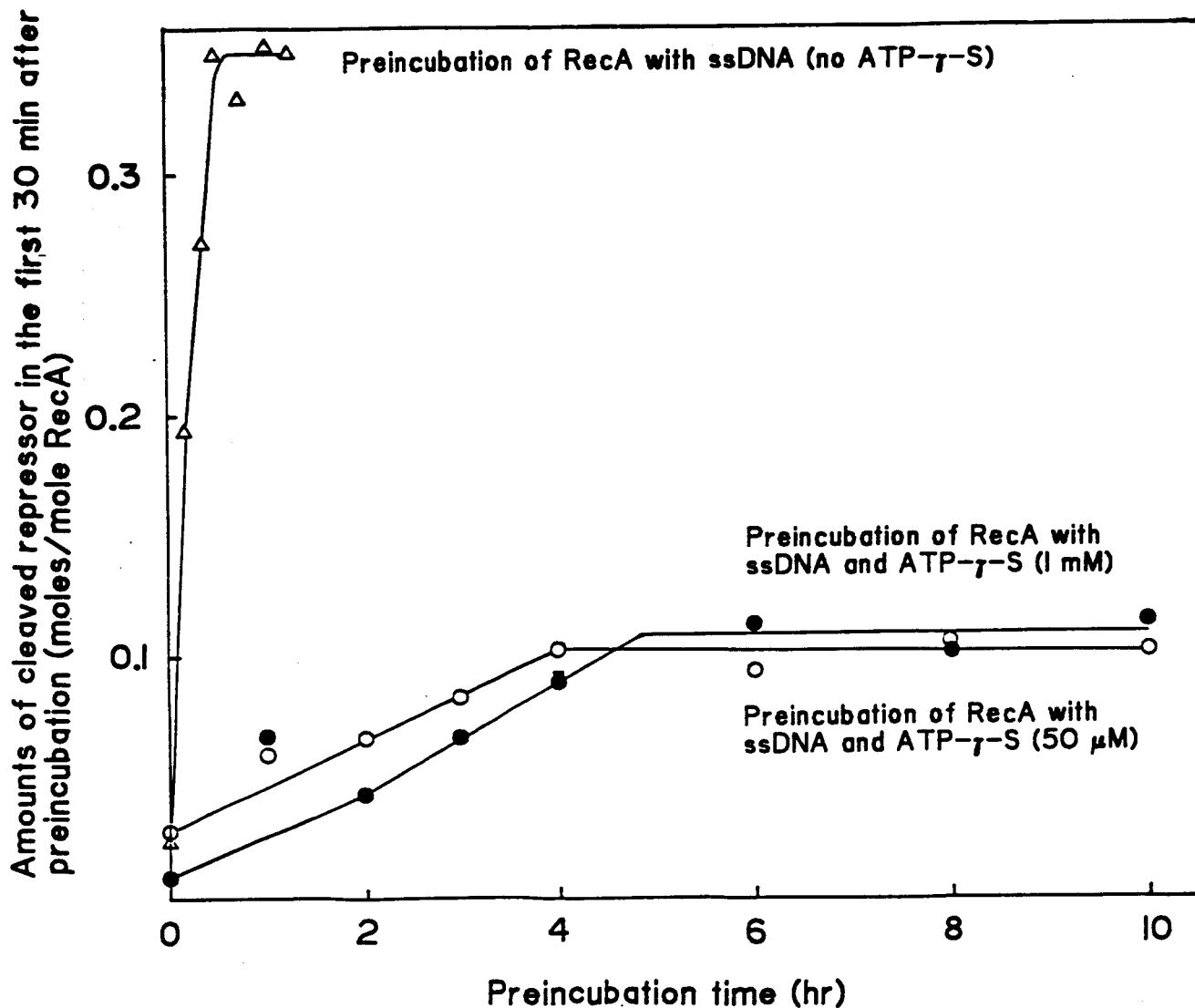


Figure III-4. The Inhibitory Effects of ATP- γ -S on the Formation of Effective RecA - ssDNA Complex. RecA protein (1 μ M) and ϕ X174 viral DNA (5 μ M) were preincubated in 40 μ l reaction mixtures containing 50 μ M ATP- γ -S (open circle) or 1 mM ATP- γ -S (closed circle) at 37°C for indicated periods. Then 3 μ M ϕ 80 CI repressor was added. After the incubation for additional 30 minutes, products were analyzed by electrophoresis in SDS-polyacrylamide gel, and the amounts of the products were estimated densitometrically.

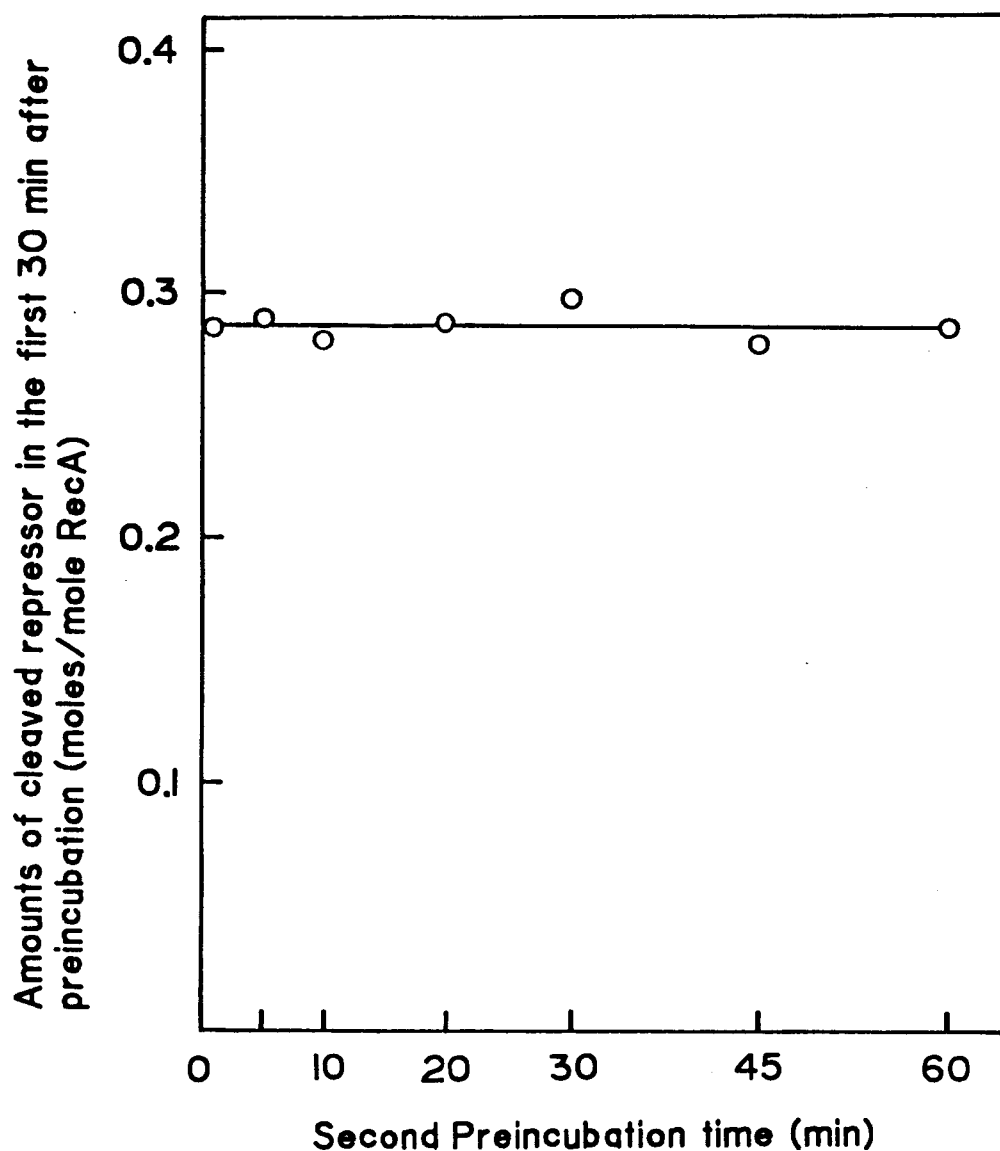


Figure III-5. No Effects of ATP- γ -S on the Preformed Effective Complex of RecA protein and ssDNA. RecA protein (3 μ M) and ϕ X174 viral DNA (5 μ M) were preincubated in 40 μ l reaction mixture for 2 hours at 37°C. Then 50 μ M ATP- γ -S was added to the mixture, followed by the second preincubation for indicated periods. ϕ 80 CI repressor (3 μ M) was then added to start the cleavage reaction, and after 30 minutes, the amounts of the products were estimated.

increased sharply as the incubation was continued. It must be partially caused by the slow accumulation of the complex formed by preincubating RecA protein and ssDNA in the presence of ATP- γ -S as shown above, but this slow accumulation of the complex was not sufficient to explain the sharp increase of the cleavage rate. For example, even if the complex is fully produced, it can cleave the repressor at the rate of about 0.1 moles per 30 minutes, while the amount of cleavage in the interval from 2 hours to 3 hours is 0.7 moles. It is possible that the cleavage products, R_1 and R_2 , which accumulate in the mixture as the reaction was continued, stimulate cleavage of the repressor. To examine this possibility, I purified these cleavage products and analyzed the effects of them on cleavage of $\phi 80$ CI repressor. [^{125}I]-labelled CI repressor was incubated with RecA protein, ssDNA and ATP- γ -S in the presence or absence of these cleavage products for 4 hours at 37°C, and the products were analyzed. The result shows that cleavage was stimulated about 3.5 fold by the presence of R_1 , while the presence of R_2 did not affect the cleavage (Fig. III-6). The presence of both R_1 and R_2 also stimulated the cleavage to a lesser extent than the presence of only R_1 .

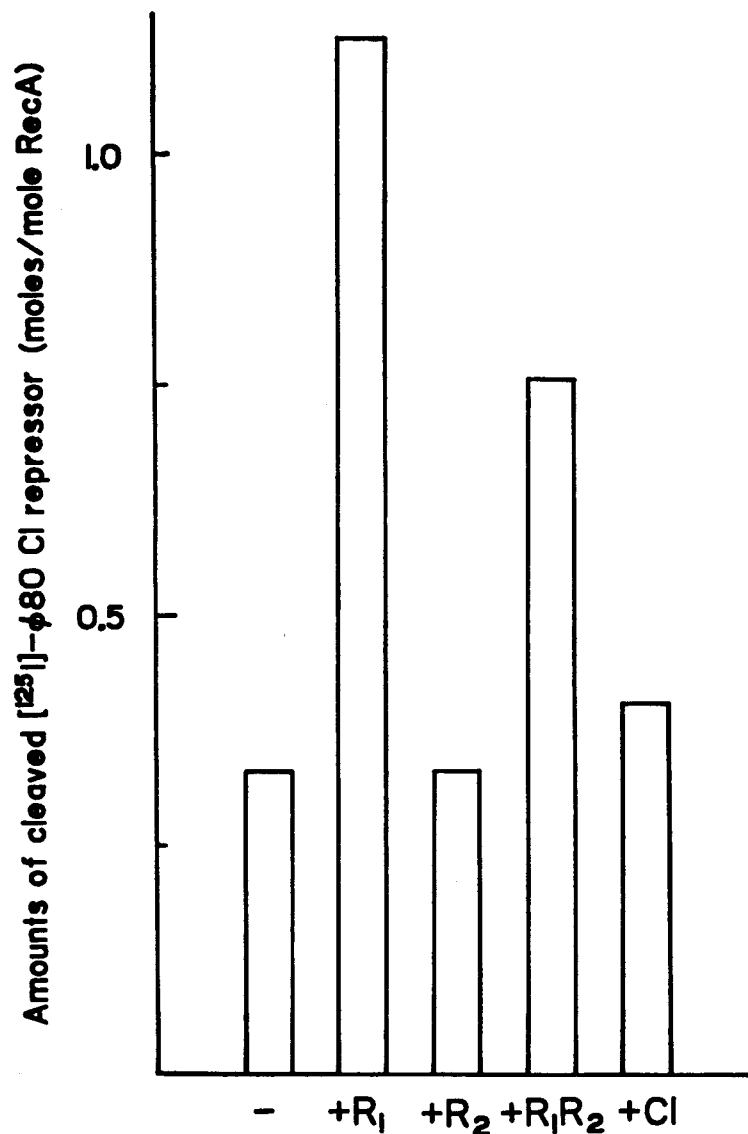


Figure III-6. Stimulation Effects of Cleaved Fragment R₁ on Cleavage of ϕ 80 CI Repressor. [¹²⁵I]-labelled ϕ 80 CI repressor (3 μ M, 2,000 cpm) was cleaved by 1 μ M RecA protein in the 50 μ l mixture which contained 3 μ M cleaved fragment, R₁ or R₂ or both, at 37°C for 4 hours. The products were analyzed by electrophoresis in a 17 % gel of SDS-polyacrylamide. The gel was stained with Coomassie Brilliant Blue R-250, dried and autoradiographed for 36 hours at -80°C using Sakura X-ray film and an intensifying screen. The density of the band corresponding to the product was analyzed by scanning spectrophotometrically, and the amounts of newly cleaved products were estimated.

5. Discussion

I found that when the reaction was started by addition of ssDNA as a final constituent, cleavage of $\phi 80$ CI repressor by RecA protein were scarcely observed during the first 1 hour and the rate of cleavage increased sharply by further incubation. While λ CI repressor was cleaved at a constant rate in the same condition. The formation of the RecA - ssDNA binary complex is the initial and slow process in the overall cleavage reaction. The interaction of RecA protein with ssDNA in the absence of ATP- γ -S was known to be very fast process. To form the complex effective for cleavage of $\phi 80$ CI repressor, some change in the binary complex between RecA protein and ssDNA should be necessary. Figure III-7 shows the schematic pathway of activation of RecA protein. When RecA protein is mixed with ssDNA, they interact each other to form complex 1 (reaction 1). In the absence of ATP- γ -S, complex 1 changes into complex 2 slowly (reaction 2). The complex 2 turns into complex 3 by binding to ATP- γ -S (reaction 3), and resulted complex 3 can cleave $\phi 80$ CI repressor efficiently. When the complex 3 was once formed, it is stable in the presence of ATP- γ -S. In the presence of ATP- γ -S, complex 1 immediately binds to ATP- γ -S to generate complex 4 (reaction 4), which is less active for cleavage of $\phi 80$ CI repressor than complex 3. When the complex 4 was once formed, it is hardly converted into complex 3. This is probably because the affinity between RecA protein and ATP- γ -S is so high that the reaction 4 proceeds to form the complex 4 and consequently the concentration of the complex 1 decreases. When the complex 4 is further incubated, it changes into complex 5 (reaction 5) which has somewhat higher activity than complex 4. The reaction 5 proceeds slower than reaction 2. It is not clear whether or not complex 5 is different from complex 4.

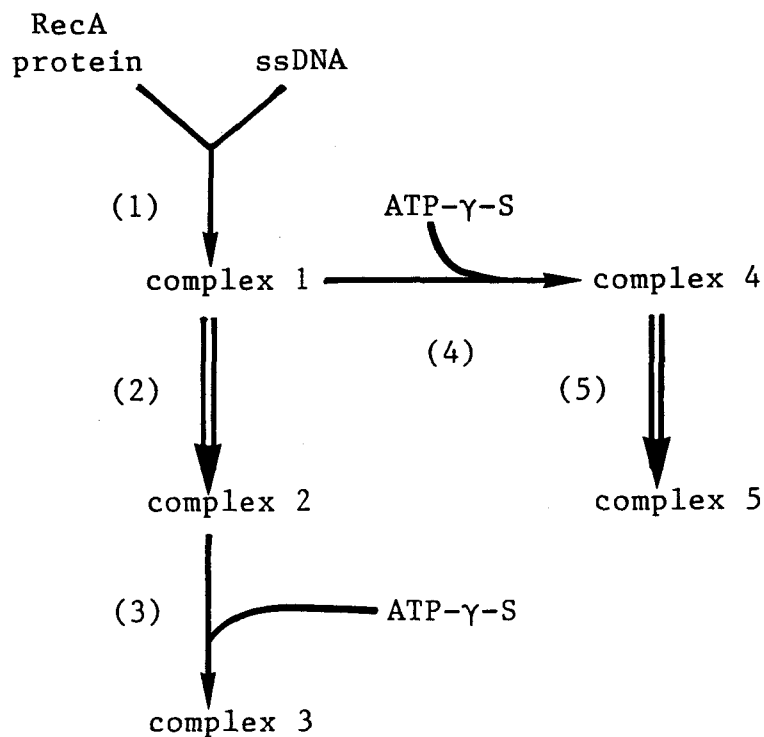


Figure III-7. Schematic Pathway of the Formation of Various RecA - ssDNA Complexes. Arrows with single line indicate the interaction of components, and arrows with double line indicate the conformational change. For details, see text.

Flory & Radding (1982) reported the structural analysis of the complex of RecA protein and ssDNA formed in the absence of ATP- γ -S by electron microscopy. Their kinetic experiments showed that collapsed circular filaments were first visualized at 2 minutes after mixing RecA protein and ssDNA (M13Goril), then about half the collapsed forms changed to extended circular filaments 60 minutes. The RecA - ssDNA complex, thus, gradually changes its form, and this transition could correspond to reaction 2.

Williams & Spengler (1986) analyzed the structure of complexes of RecA protein and ssDNA by negative-stain electron microscopy. They compared the structure of a RecA - ssDNA complex formed in the presence of ATP- γ -S and the structure of a complex made by addition of ATP- γ -S after preincubation of RecA protein and ssDNA. They found that the complex with preincubation had the same pitch of striated pattern as the complex formed without preincubation, but the latter complex was about 50 % greater in contour length than the former one. Thus the RecA - ssDNA - ATP- γ -S complex formed with preincubation of RecA protein and ssDNA had a structural difference from the nonpreincubated complex. This difference might correspond to the difference between complex 3 and complex 4.

The sharp increase of the cleavage rate after this early slow reaction cannot be sufficiently explained only by the gradual increase of complex 5 in the presence of ATP- γ -S. One of cleaved products, R_1 , stimulated the cleavage reaction of $\phi 80$ CI repressor. In contrast, cleavage product R_1 of λ CI repressor inhibits its cleavage (Sauer et al., 1982). The gradual accumulation of complex 5 and the product stimulation of cleavage would explain the gradual increase of the cleavage rate.

The major finding in this chapter is that the RecA - ssDNA - ATP- γ -S complexes take two forms; one of which is formed after preincubation of

RecA protein and ssDNA and can cleave $\phi 80$ CI repressor efficiently, and the other of which is formed without any preincubation and cannot cleave $\phi 80$ CI repressor efficiently. This difference may result from the conformational change of RecA - ssDNA complex which proceeds slowly.

6. References

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CHAPTER IV

Stimulation of RecA-mediated Cleavage of ϕ 80 CI Repressor by Deoxydinucleotides

1. Summary

The presence of either deoxyguanyryl guanosine, d(G-G), or deoxyadenyryl adenosine, d(A-G), greatly stimulates in vitro cleavage of ϕ 80 CI repressor mediated by the RecA protein. No other deoxydinucleoside monophosphates nor a riboguananyryl guanosine, r(G-G), affect the cleavage reaction. The cleavage site of ϕ 80 CI repressor is not altered by d(G-G). Requirement of single-stranded DNA and ATP for cleavage is also not altered. The cleavage reactions of the LexA and λ CI repressors by the RecA protein are not affected by d(G-G). Photoaffinity labelling experiments with [32 P]-labelled pd(G-G), which also stimulates cleavage, showed that pd(G-G) bound to the repressor under the conditions in which the repressor is cleaved by the RecA protein.

1. Introduction

Treatments that damage DNA or inhibit DNA synthesis in Escherichia coli induce the expression of a set of functions called "SOS functions" (Witkin, 1976) that are involved in DNA repair, mutagenesis, arrest of cell division, and prophage induction. Induction of SOS functions appears to be triggered by inactivation of LexA repressor or phage repressors which repress the expression of SOS functions or phage functions, respectively. In vitro analyses showed that inactivations of these repressors result from their cleavage by RecA protein in the presence of single-stranded DNA and a nucleoside triphosphate. (for reviews, see Little & Mount, 1982; Roberts & Devoret, 1983; Walker, 1984).

I showed that the purified $\phi 80$ CI repressor was cleaved by RecA protein in the presence of single-stranded DNA and ATP or its analogue (Eguchi et al., 1987a; chapter II in this thesis). The cleavage was very slow in the first 1 hour of the reaction, and after this early slow cleavage, the cleavage rate increased sharply (Eguchi et al., 1987b; chapter III in this thesis). On the other hand, LexA and λ CI repressors were cleaved much faster without a detectable lag in the initial reaction. The length of the slow reaction in early stage of cleavage of $\phi 80$ CI repressor was reduced by preincubating the RecA protein with ssDNA in the absence of ATP- γ -S to allow the formation of a certain effective complex. It is possible that some cofactor stimulates cleavage of $\phi 80$ CI repressor in vivo by stimulating the formation of the effective complex by binding to the RecA protein or by increasing affinity between the RecA protein and $\phi 80$ CI repressor by binding one or both proteins. These observations prompted me to investigate such a possible cofactor.

Irbe et al. (1981) reported that prophage $\phi 80$ in the permeabilized

lysogen was induced by addition of a deoxydinucleoside monophosphate d(G-G) or d(A-G) depending on the recA⁺ function without any other ordinary inducing treatments. Prophage λ was not induced by the same treatment. The mechanism of action of these deoxydinucleoside monophosphates was not elucidated. Assuming that they act as a cofactor in inactivation of $\phi 80$ CI repressor by a RecA-mediated reaction, I examined the effects of deoxydinucleoside monophosphates on cleavage of $\phi 80$ CI repressor by RecA protein in vitro. I found that they greatly stimulate cleavage of $\phi 80$ CI repressor by binding to the repressor to increase the affinity between the repressor and the RecA protein.

2. Materials and Methods

(a) Chemicals

ATP and dATP were purchased from Yamasa Shoyu (Japan). ATP- γ -S and creatine phosphate were obtained from Boehringer Mannheim (West Germany). Some deoxydinucleoside monophosphates, deoxydinucleotide and ribodinucleotide were supplied by Dr. M. Oishi (Tokyo University), and some others were purchased from P-L Biochemicals (USA) and Collaborative Research (USA). Deoxydinucleotide pd(G-G) labelled with [32 P] was prepared by phosphorylation of deoxydinucleoside monophosphate d(G-G) with γ -[32 P]-ATP using T4 polynucleotide kinase, followed by purification with high performance liquid chromatography. ϕ X174 viral DNA was purified according to Cunningham et al. (1980).

(b) Proteins

ϕ 80 CI repressor, LexA repressor, λ CI repressor and RecA protein were prepared as described (Eguchi et al., 1987a; chapter II in this thesis). T4 polynucleotide kinase was prepared as described by Richardson (1965). Rabbit muscle creatine kinase, trypsin and bovine serum albumin were purchased from Boehringer Mannheim, Sigma (USA) and Poviet Producten N. V. (Holland), respectively. Soy bean trypsin inhibitor and cytochrome C were gifts of Dr. H. Matsubara (Osaka University).

(c) RecA-mediated Cleavage of Repressors

Cleavage of repressors was performed essentially as described (Eguchi

et al., 1987a, 1987b; chapters II and III in this thesis). The reaction mixtures contained 1 μ M RecA protein, 3 μ M repressor protein, 5 μ M ϕ X174 viral DNA, 12 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mM EDTA, 3 mM KCl, 3 % glycerol and 2 mM ATP or dATP or 50 μ M ATP- γ -S. When ATP or dATP was used as a cofactor, the reaction mixture always included its regeneration system, consisting of 4 μ g/ml rabbit muscle creatine kinase, 4 μ g/ml bovine serum albumin and 30 mM creatine phosphate. In the kinetic experiments, the reactions were started by final addition of ssDNA (ϕ X174 viral DNA) to the reaction mixture, unless otherwise indicated, and followed by incubation at 37°C. At an indicated time, aliquot (40 μ l) was taken and chilled, and then 10 μ l lysis buffer (300 mM Tris-HCl, pH 6.8, 10 % sodium dodecylsulfate (SDS), 25 % 2-mercaptoethanol, 25 % glycerol and 0.025 % bromophenol blue) was added to stop the reaction. The products were analyzed by electrophoresis in a SDS-polyacrylamide gel with a 3 % stacking and a 17 % resolving gels essentially according to Laemmli (1970). After staining the gel with Coomassie Brilliant Blue R-250, the amounts of cleaved repressor were determined by a scanning spectrophotometer, using known amounts of completely cleaved repressor as a standard.

(d) Photoaffinity Labelling of Proteins with dinucleotide

The standard mixture in 50 μ l contained 50 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM KCl, 0.05 mM EDTA, 5 % glycerol, 10 μ M [32 P]-labelled pd(G-G) (1 μ Ci) and 10 μ M proteins. The mixture was incubated at 37°C for 10 min. Then, a droplet of the mixture was placed on a parafilm sheet resting on an aluminium block at 0°C and irradiated with

the Toshiba GL-15 (15 watt) germicidal UV lamp for 40 min at 88 erg/mm²/sec. The dose rate was measured by the Toshiba UV radiometer C-254. When the mixture contained 400 μ M ATP or its analogues, the irradiation was performed for 120 min. The effective UV dose by irradiation for 120 min in the presence of 400 μ M ATP was identical to that by irradiation for 40 min in its absence as they determined by survival of λ phage after irradiation in the reaction mixture with and without ATP. The photoaffinity-labelled proteins were analyzed by electrophoresis in SDS-polyacrylamide gel with a 13 % resolving gel. The gel was stained with Coomassie Brilliant Blue R-250, dried and autoradiographed for 48 hours at -80°C using Sakura X-ray film and an intensifying screen.

(e) A Limited Digestion of Protein with Trypsin

ϕ 80 CI repressor (10 μ g) was incubated at 37°C in a 50 μ l reaction mixture containing 12 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 2 mM dithiothreitol, 5 mM KCl, 0.05 mM EDTA, 3 % glycerol in the presence or absence of 0.5 mM deoxydinucleoside monophosphate for 5 min. Then 20 ng of trypsin was added and the incubation was continued for 45 min. The reaction was stopped by adding 80 ng of soy bean trypsin inhibitor, and the partially digested products were analyzed by electrophoresis in SDS-polyacrylamide gel with a 17 % resolving gel.

3. Results

(a) Stimulation of RecA Protein Mediated Cleavage of ϕ 80 CI Repressor by Deoxydinucleoside Monophosphate

Prophage ϕ 80 in permeabilized lysogen is induced by addition of deoxydinucleoside monophosphate d(G-G) or d(A-G) without any inducing treatments (Irbe *et al.*, 1981). The reaction depends on the recA⁺ function. I examined whether or not d(G-G) is directly involved in cleavage of ϕ 80 CI repressor using in vitro system for repressor cleavage.

d(G-G) was added to the reaction mixture containing RecA protein, ϕ 80 CI repressor, ssDNA (ϕ X174 viral DNA), ATP- γ -S and MgCl₂, and the mixtures were incubated for 2 hours at 37°C. The products were analyzed by SDS-polyacrylamide gel electrophoresis. The results showed that the amounts of the cleavage products increased about 7-fold by the presence of d(G-G) in the complete reaction mixture (compare lanes a and b in Fig. IV-1). When any one of the components, such as RecA protein, ATP- γ -S, ssDNA and magnesium ion, in the reaction mixture was removed, cleavage of the repressor in the presence of d(G-G) was not observed (Fig. IV-1, lanes c to f). These results indicate that d(G-G) stimulated cleavage of the repressor by the RecA protein in the presence of various cofactors and did not substitute for any other components. Similar results were obtained using ATP or dATP with their regeneration system instead of ATP- γ -S (data not shown).

The cleavage site of the repressor in the absence of a dinucleotide is between Cys¹¹⁰-Gly¹¹¹ (Eguchi *et al.*, 1987a; chapter II in this thesis). The cleavage site was not altered by the presence of d(G-G) (data not shown). Little (1984) reported that if LexA or λ CI repressor was kept

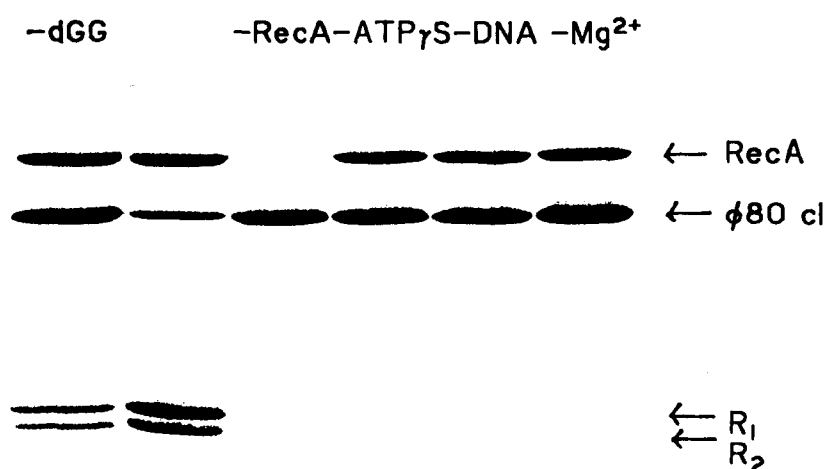


Figure IV-1. Stimulation of RecA-mediated Cleavage of $\phi 80$ CI Repressor by d(G-G) and Necessary Components for the Stimulation. $\phi 80$ CI repressor ($3 \mu\text{M}$) was incubated at 37°C for 2 hours with the wild-type RecA protein ($1 \mu\text{M}$) in a $40 \mu\text{l}$ complete reaction mixture containing $50 \mu\text{M}$ ATP- γ -S and $50 \mu\text{M}$ d(G-G) (lane b). In lane a and lanes c to f, the indicated components were omitted from the complete reaction mixture. Products were analyzed by electrophoresis in a 17 % gel of SDS-polyacrylamide. R_1 and R_2 indicate the cleaved products of $\phi 80$ CI repressor.

under a condition of a high alkaline pH, autodigestion at the usual cleavage site was observed. Few percent of $\phi 80$ CI repressor was spontaneously cleaved by incubation at pH 10 for 48 hours, but the cleavage was not stimulated by the addition of d(G-G) (data not shown).

The same stimulating effects on cleavage was observed by using various d(G-G) preparations which were obtained from different suppliers or synthesized with automated DNA synthesizer followed by purification with high performance liquid chromatography (data not shown). Therefore, stimulation was hardly due to some contaminants in these preparation of d(G-G).

(b) Effect of d(G-G) on Kinetics of Cleavage of $\phi 80$ CI Repressor

To examine the effects of d(G-G) on cleavage of $\phi 80$ CI repressor, kinetics of cleavage of the repressor in the presence or absence of 50 μ M d(G-G) were analyzed using ATP, dATP or ATP- γ -S as one of cofactors (Fig. IV-2). In the absence of d(G-G), the rate of cleavage of $\phi 80$ CI repressor varied with nucleoside triphosphate used as a cofactor; the cleavage using ATP (always together with its regeneration system) as a cofactor is very slow comparing with the cleavage using dATP (always together with its regeneration system) or ATP- γ -S (Eguchi et al., 1987b; chapter III in this thesis). When ATP or dATP was used as a cofactor, cleavage was greatly stimulated by the presence of d(G-G). The stimulation of cleavage by d(G-G) in the reaction using ATP is much larger than that using dATP, so the rate of cleavage using ATP came to be similar to that using dATP. When ATP- γ -S was used as a cofactor, the cleavage reaction was also stimulated by the presence of d(G-G), however the rate was lower than that in the

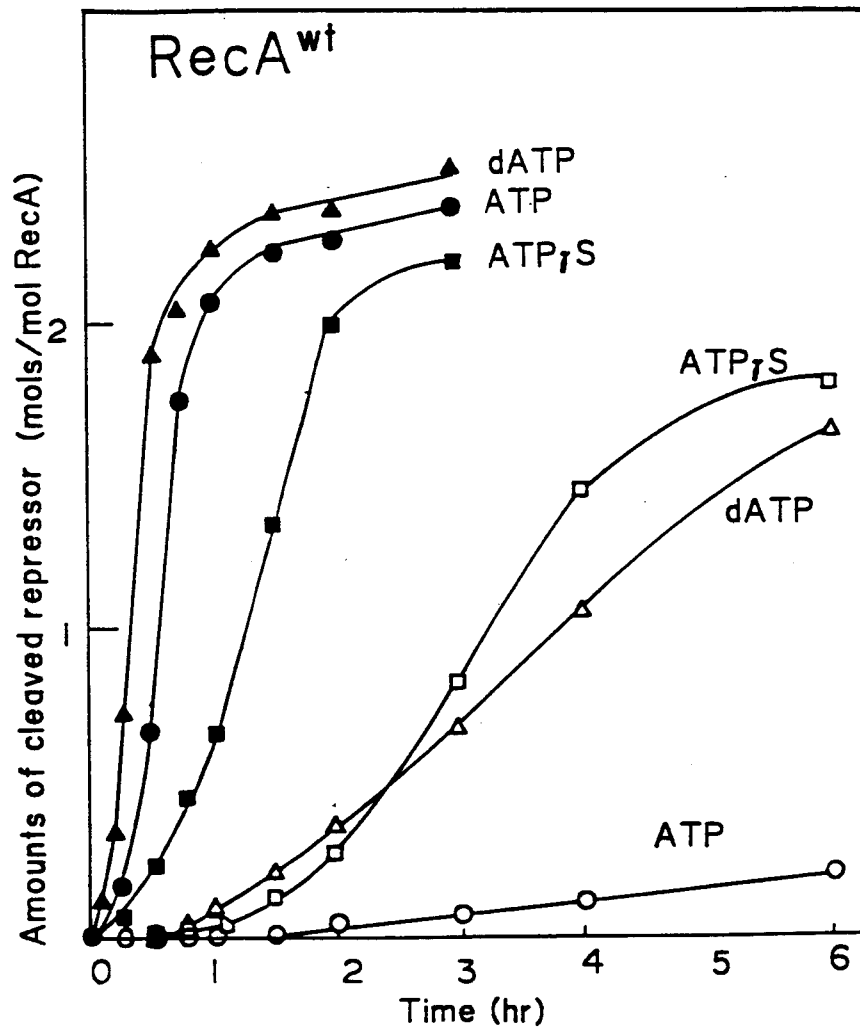


Figure IV-2. Kinetics of the Cleavage Reaction of $\phi 80$ CI Repressor in the Presence of d(G-G). $\phi 80$ CI repressor ($3 \mu\text{M}$) was incubated at 37°C with wild-type RecA protein ($1 \mu\text{M}$) in a $500 \mu\text{l}$ reaction mixture containing ATP, dATP or ATP- γ -S in the presence of $50 \mu\text{M}$ d(G-G) or in its absence. A reaction was started by adding $5 \mu\text{M}$ ϕX174 viral DNA as the last constituent to the reaction mixture. Aliquots ($40 \mu\text{l}$) were taken at indicated times and the products were analyzed by electrophoresis in a 17 % gel of SDS-polyacrylamide. The amounts of the cleaved products were estimated by densitometric scanning of the bands of the products in a gel. open symbols: the reaction in the absence of d(G-G), closed symbols: the reaction in the presence of $50 \mu\text{M}$ d(G-G). \circ and \bullet : with 1 mM ATP and ATP regeneration system, Δ and \blacktriangle : with 1 mM dATP and dATP regeneration system, \square and \blacksquare : with $50 \mu\text{M}$ ATP- γ -S.

presence of d(G-G) and either ATP or dATP.

I reported previously that the cleavage was barely detectable in the first 1 hour, and then the rate increased gradually by further incubation (Eguchi et al., 1987b; chapter III in this thesis). Here, I examined the cleavage in the first 1 hour in the presence of various concentrations of d(G-G) (Fig. IV-3). Even in the presence of 1 μ M d(G-G), the cleavage was detectable, and the rate increased as the concentration of d(G-G) was increased to 1 mM.

(c) Stimulation of Cleavage of ϕ 80 CI Repressor by Specific Deoxydinucleoside Monophosphates, d(G-G) and d(A-G)

Irbe et al. (1981) reported that among 16 deoxydinucleoside monophosphates, only d(G-G) and d(A-G) induce prophage ϕ 80 in permeabilized lysogen. I examined the stimulation of the cleavage reaction of ϕ 80 CI repressor for all possible deoxydinucleoside monophosphates. Only d(G-G) and d(A-G) significantly stimulated the cleavage reaction of ϕ 80 CI repressor when ATP was used as a cofactor (Fig. IV-4). Riboguananyryl guanosine, r(G-G), showed no effect. Irbe et al. (1981) reported that r(G-G) did not induce prophage ϕ 80 in permeabilized lysogen. 5'phosphoryl deoxyguanyryl guanosine, pd(G-G), had the effect similar to that of d(G-G) in vitro (Fig. IV-4), although it had less activity to induce prophage ϕ 80 in permeabilized lysogen (Irbe et al., 1981), probably because of poorer permeability. I obtained results similar to that shown in Figure IV-4 when dATP or ATP- γ -S instead of ATP was used as a cofactor (data not shown).

(d) Absence of Stimulation of Cleavage of LexA and λ CI Repressors by Deoxydinucleoside Monophosphates

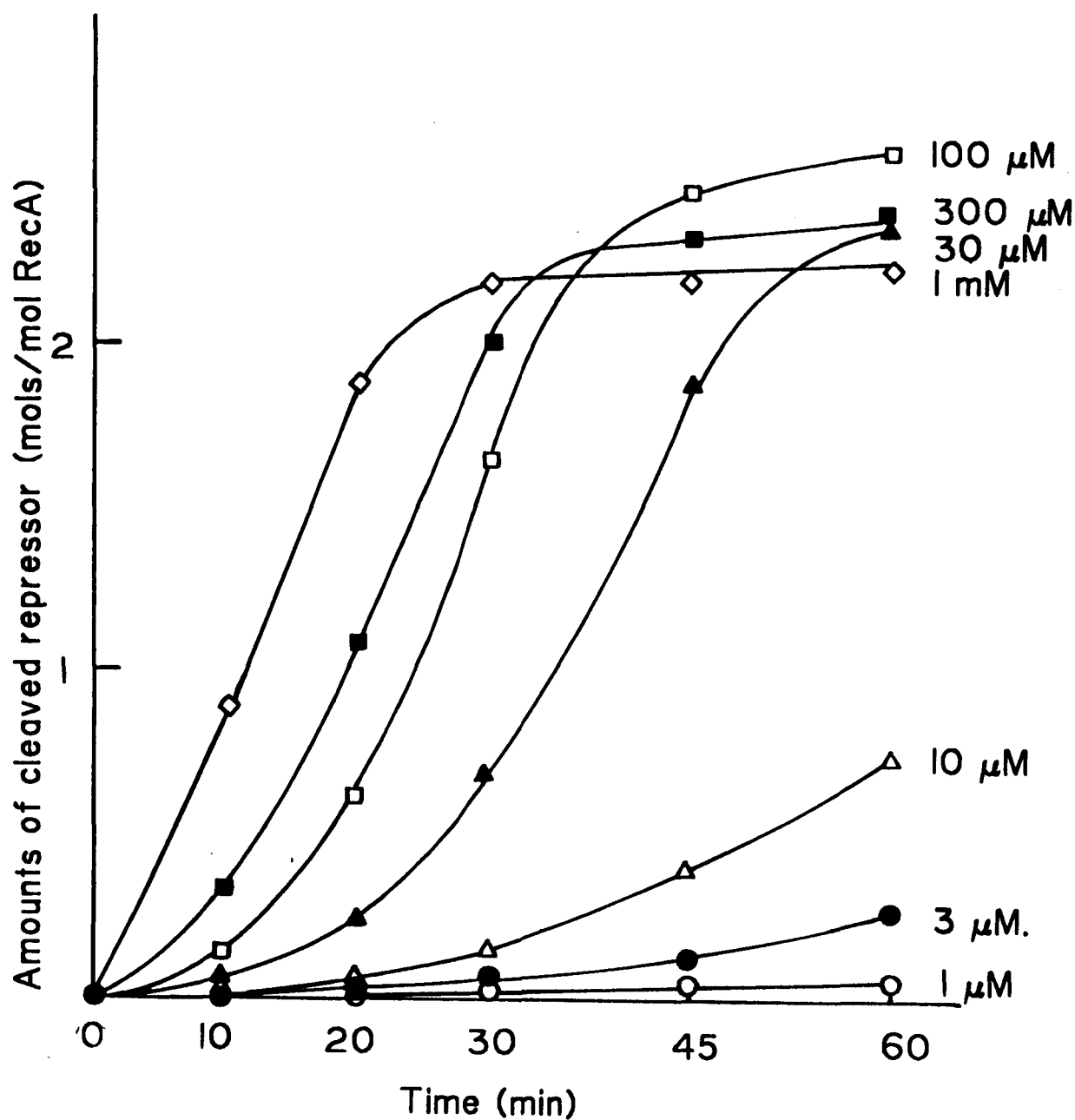


Figure IV-3. Stimulations of Cleavage of $\phi 80$ CI Repressor by d(G-G) at Various Concentrations. $\phi 80$ CI repressor (3 μM) was incubated at 37°C with the wild-type RecA protein (1 μM) in a 250 μl reaction mixture including 2 mM ATP and the ATP regeneration system and various concentration of d(G-G) as indicated. Aliquots (40 μl) were taken at indicated times and amounts of the cleaved products were measured as described in the legend to Figure IV-2.

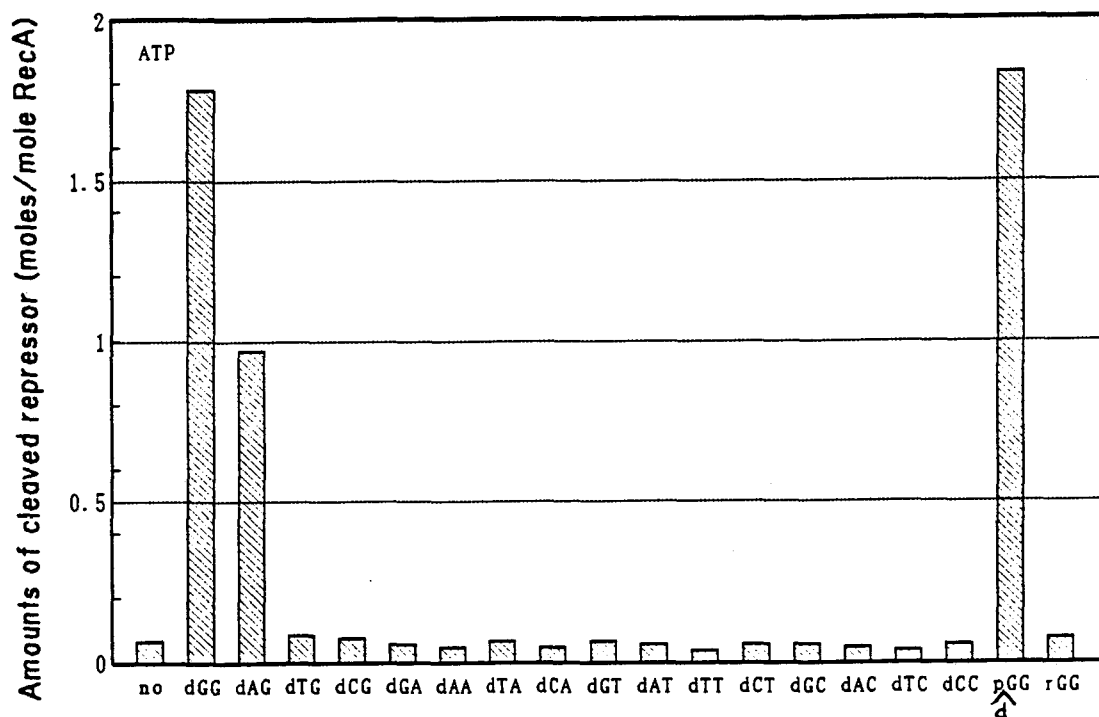


Figure IV-4. Stimulations of Cleavage of $\phi 80$ CI Repressor in the Presence of Various Deoxydinucleoside Monophosphates. $\phi 80$ CI repressor ($3 \mu\text{M}$) was cleaved by wild-type RecA protein ($1 \mu\text{M}$) in a $40 \mu\text{l}$ reaction mixture including with 2 mM ATP, and an indicated dinucleoside monophosphates at $50 \mu\text{M}$. They were incubated for 4 hours at 37°C , respectively. The products were analyzed as described in the legend to Figure IV-2.

I examined whether or not d(G-G) affects the cleavage rates of LexA and λ CI repressors. As shown in Figure IV-5, the cleavage reactions of both LexA and λ CI repressors were not affected by the addition of d(G-G) in the reaction mixture. All other deoxydinucleoside monophosphates also had no effect on the cleavage of λ CI repressor (data not shown). The stimulation effect of d(G-G) is thus specific for the cleavage of ϕ 80 CI repressor.

(e) Preferential Cleavage of ϕ 80 CI Repressor in the Presence of d(G-G)

The stimulation of cleavage of ϕ 80 CI repressor in the presence of d(G-G) can be caused by the increase of the affinity between ϕ 80 CI repressor and RecA protein or by the increase of the rate of cleavage after interaction of these two proteins. To know whether or not d(G-G) increases the affinity between ϕ 80 CI repressor and RecA protein, I analyzed possible competition between cleavage of ϕ 80 CI repressor and that of λ CI repressor by RecA protein in the presence or absence of d(G-G). When each repressor was cleaved separately, cleavage of ϕ 80 CI repressor was stimulated by d(G-G) (Fig. IV-6 A, lanes c and d), but cleavage of λ CI repressor was not affected (Fig. IV-6 A, lanes a and b). Even when ϕ 80 CI and λ CI repressors were present together at a same concentration (3 μ M each) in the same reaction mixture containing 1 μ M RecA protein but without d(G-G), each repressor was cleaved to the same extent as that when each repressor was cleaved separately (Fig. IV-6 A, lane e). Addition of d(G-G), however, stimulated the cleavage of ϕ 80 CI repressor, while cleavage of λ CI repressor was inhibited (Fig. IV-6 A, lane f). These results suggest that

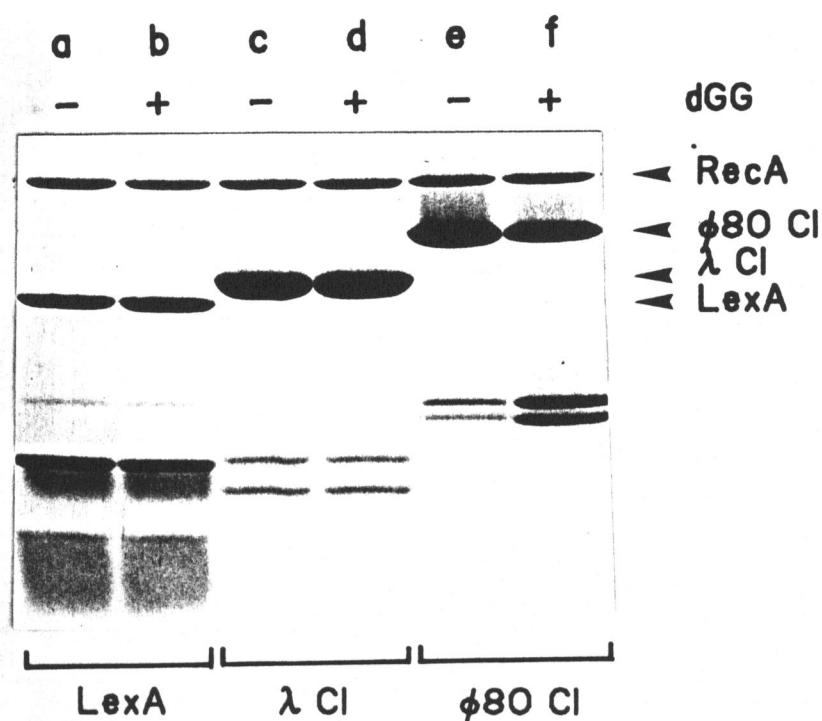


Figure IV-5. Absence of Stimulatory Effect of d(G-G) on Cleavage of LexA or λ CI Repressor. LexA, λ CI or ϕ80 CI repressors (5 μM each) was incubated with 1 μM wild-type RecA protein in a 50 μl reaction mixture including 5 μM ϕX174 viral DNA and 50 μM ATP-γ-S at 37°C for 15 minutes (for LexA repressor) or 2 hours (for λ CI and ϕ80 CI repressors). The products were analyzed by electrophoresis in a 17 % gel of SDS-polyacrylamide. lanes a and b: LexA repressor was cleaved, lanes c and d: λ CI repressor was cleaved, lanes e and f: ϕ80 CI repressor was cleaved. The reaction mixtures of lanes b, d and f contained 50 μM d(G-G), in addition.

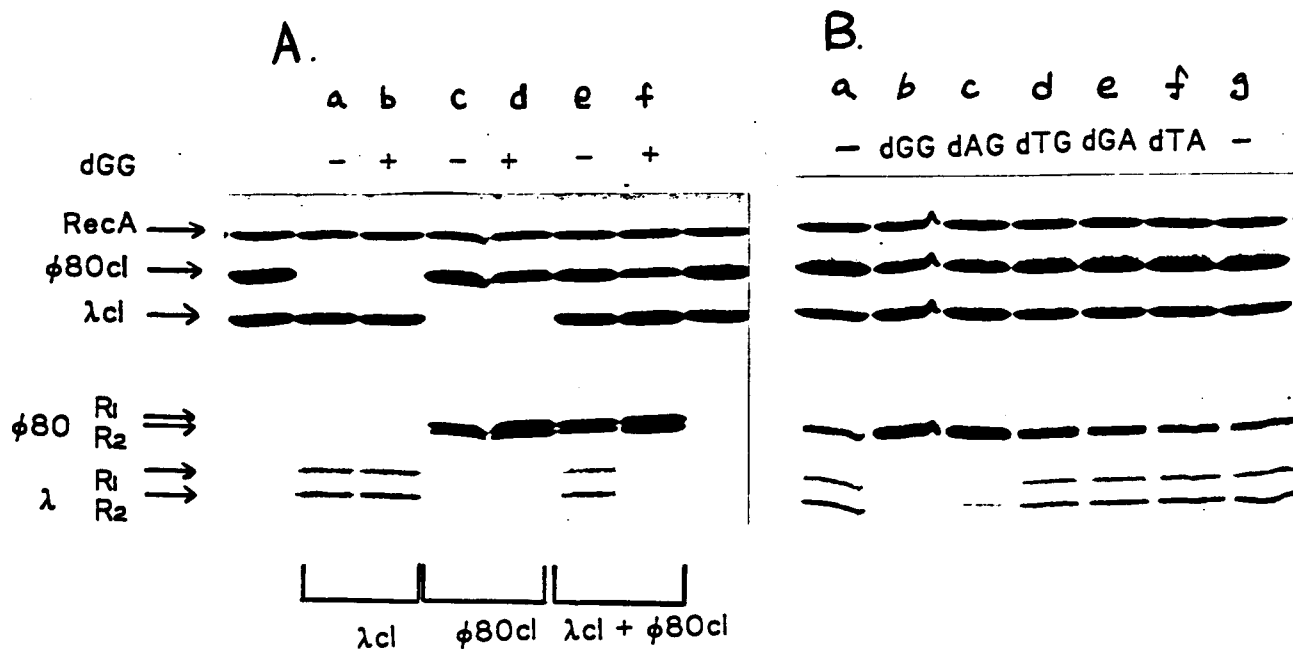


Figure IV-6. Preferential Cleavage of φ80 CI Repressor in the Presence of d(G-G) or d(A-G). φ80 CI and/or λ CI repressors (3 μM) were cleaved by RecA protein (1 μM) in 50 μl of reaction mixtures containing 50 μM ATP-γ-S for 3 hours at 37°C, and reaction products were analyzed by electrophoresis in a 17 % resolving gel of SDS-polyacrylamide. A. lanes a and b: λ CI repressor was cleaved; lanes c and d: φ80 CI repressor was cleaved; lanes e and f: λ CI and φ80 CI repressors were cleaved together in the same reaction mixture. The reaction mixtures of lanes b, d and f were contained d(G-G) at 50 μM, in addition. B. λ CI and φ80 CI repressors were cleaved together in the same reaction mixture containing 50 μM of the indicated deoxydinucleoside monophosphate.

d(G-G) enhances the cleavage reaction of ϕ 80 CI repressor by increasing the affinity between ϕ 80 CI repressor and RecA protein, and thus reduces the effective concentration of RecA protein for cleavage of λ CI repressor. d(A-G) showed similar effects as d(G-G) on the cleavage of λ CI repressor, but other deoxydinucleoside monophosphates such as d(T-G), d(G-A) and d(T-A), which did not stimulate the cleavage of ϕ 80 CI repressor, had no effects (Fig. IV-6 B).

(f) Interaction of d(G-G) with ϕ 80 CI Repressor and RecA Protein

RecA protein mediated cleavage of ϕ 80 CI repressor might be stimulated by interaction of d(G-G) with one or both of these proteins. If d(G-G) binds stably to a protein, one might be able to demonstrate the binding by photochemical fixing of the deoxydinucleoside monophosphate on the protein. To examine this possibility, I tried to fix [32 P]-pd(G-G) on a protein by a heavy UV-irradiation firstly in the absence of ssDNA and ATP or its analogues. Addition of pd(G-G) at various concentration gave the similar effect on cleavage of ϕ 80 CI repressor as d(G-G) (data not shown). [32 P]-labelled pd(G-G) was mixed with RecA protein, ϕ 80 CI repressor, bovine serum albumine and cytochrome C, and the mixture was irradiated. Irradiated proteins were separated by SDS-polyacrylamide gel electrophoresis. As shown in Figure IV-7, [32 P]-pd(G-G) cross-linked to both RecA protein and ϕ 80 CI repressor. [32 P]-pd(G-G) also cross-linked to these proteins when only one of the proteins was present in the reaction mixture. Bovine serum albumine and cytochrome C were not labelled by [32 P]-pd(G-G). The results show that pd(G-G) can bind to RecA protein and ϕ 80 CI repressor.

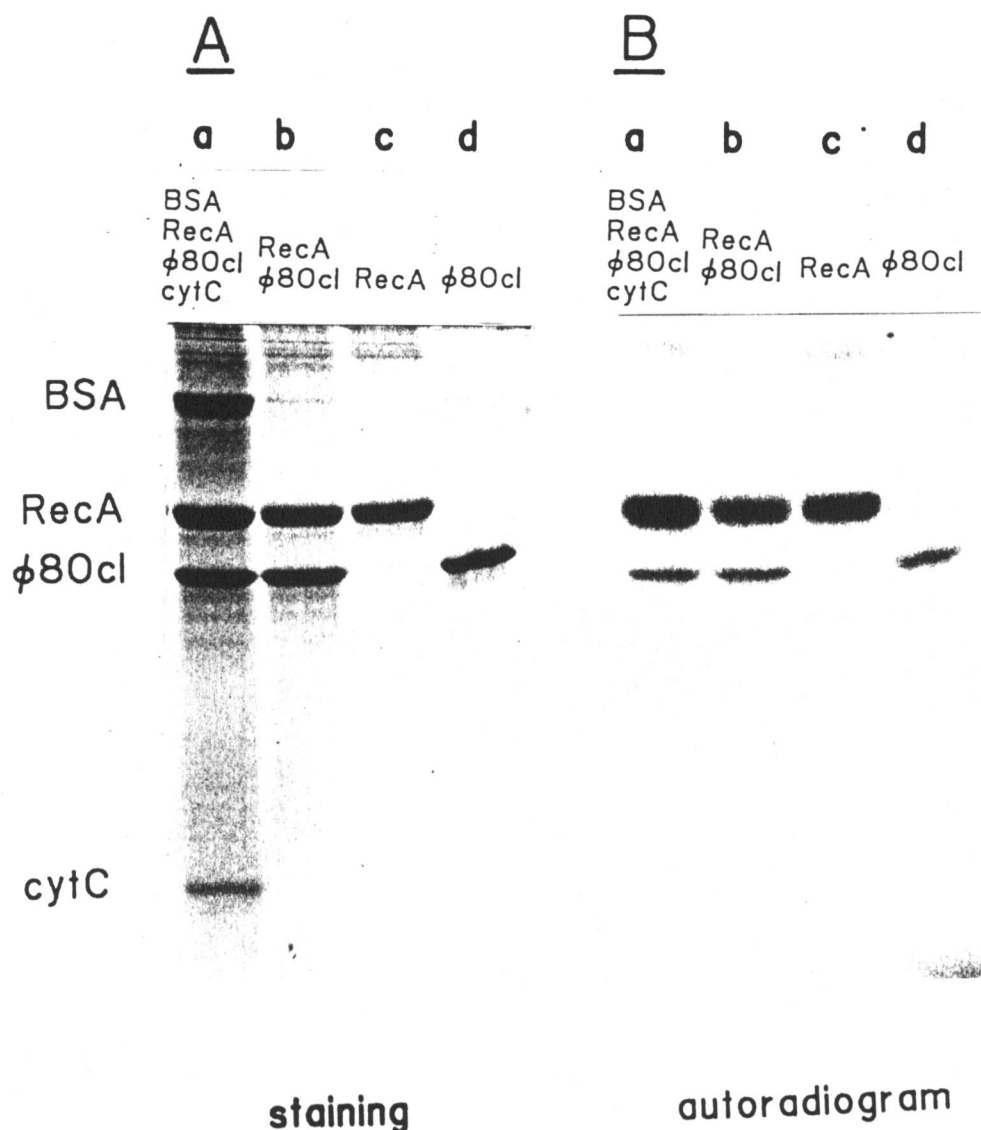


Figure IV-7. Photoaffinity Labelling of RecA protein and $\phi 80$ CI Repressor with [^{32}P]-labelled pd(G-G). Procedures of photoaffinity-labelling of proteins were described in Materials and Methods. 10 μl of each sample was analyzed by electrophoresis in a 13 % gel of SDS-polyacrylamide, followed by staining with Coomassie Brilliant Blue R-250 (A) and by autoradiography (B). The proteins presented in each reaction mixture are indicated above each lane.

Nextly, I analyzed the binding of pd(G-G) to proteins under the condition for in vitro cleavage of the repressor. In the presence of ATP- γ -S and ssDNA, the ϕ 80 CI repressor was labelled with [32 P]-pd(G-G), while labelling of RecA protein was inhibited (Fig. IV-8, lane g). Similar results were obtained using ATP, dATP or ADP instead of ATP- γ -S (data not shown). The presence of ATP- γ -S, ATP, dATP or ADP but not of ssDNA showed the similar effect as the presence of both ssDNA and ATP- γ -S, and ssDNA showed no effect. (Fig. IV-8, lanes b to f). These results indicate that under the condition for in vitro cleavage of the repressor, pd(G-G) interacts with ϕ 80 CI repressor and might not interact with RecA protein.

Figure IV-8, lane g shows that the radioactivity was also found in the cleavage products. Further analysis showed that the radioactivity was present in the R₁ fragment which was derived from carboxyl-terminal half of the repressor and was not found in the R₂ fragment (Fig. IV-9).

Interaction between ϕ 80 CI repressor and d(G-G) is further supported by an experiment of a different approach. ϕ 80 CI repressor was partially digested by trypsin in the absence or presence of 500 μ M d(G-G) and the products were analyzed by SDS-polyacrylamide gel electrophoresis. The presence of d(G-G) affected the electrophoretic pattern of the products of the limited digestion of ϕ 80 CI repressor: intensity of some bands were increased and some were decreased in the presence of d(G-G) (Fig. IV-10). The presence of d(A-G) had similar effect. These results show that the interaction of d(G-G) or d(A-G) with ϕ 80 CI repressor changed the sensitivity of the protein against trypsin digestion at a few specific sites. On the contrary, d(T-G), d(G-A) and d(T-A), any of which could not stimulate the cleavage reaction of ϕ 80 CI repressor, did not change the digestion patterns of ϕ 80 CI repressor. d(G-G) did not cause detectable change in the digestion pattern of RecA protein (data not shown).

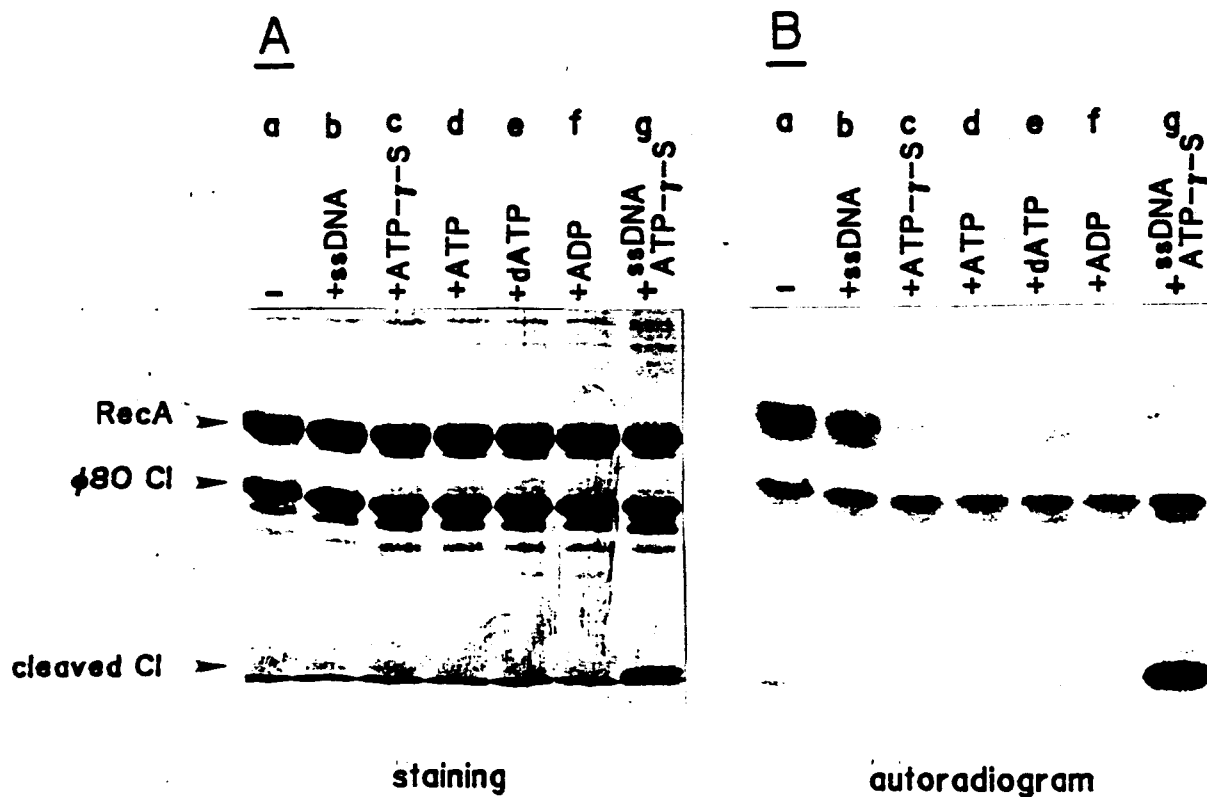


Figure IV-8. Inhibition of Photoaffinity Labelling of RecA Protein with [32 P]-labelled pd(G-G) by the Presence of Nucleoside Triphosphate. Photoaffinity labelling of RecA protein and ϕ 80 CI repressor was performed as described. Products were analyzed by electrophoresis with 13 % resolving gel, stained with Coomassie Brilliant Blue R-250 (A) and autoradiographed (B). Reaction mixture of (b) contained 50 μ M ϕ X174 viral DNA, (c) to (f) contained 0.4 mM each of ATP- γ -S, ATP, dATP, and ADP, respectively, and (g) contained both 50 μ M ϕ X174 viral DNA and 0.4 mM ATP- γ -S.

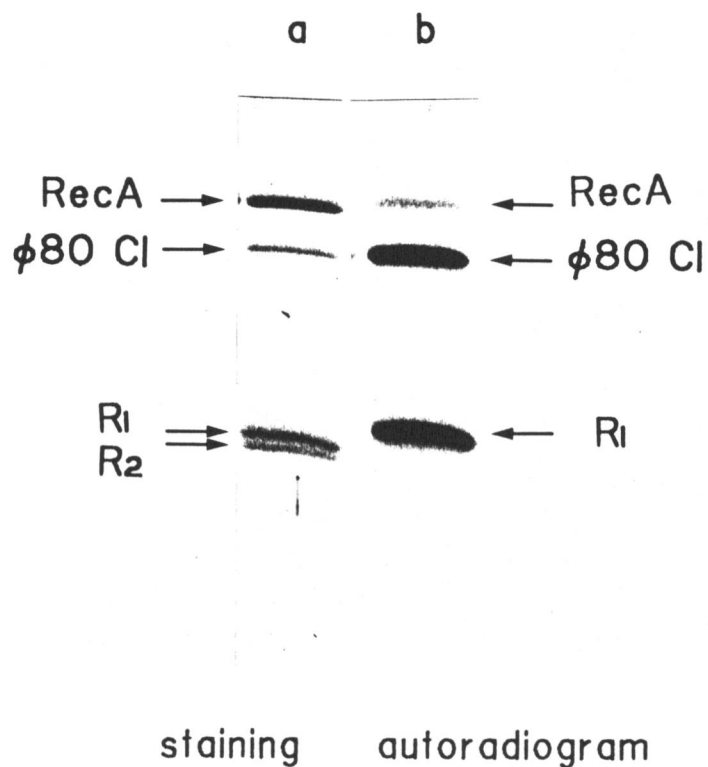


Figure IV-9. Photoaffinity Labelling of the Cleaved Products of $\phi 80$ CI Repressor by RecA Protein with [^{32}P]-labelled pd(G-G). $\phi 80$ CI repressor (10 μM) was cleaved with 10 μM RecA protein in 50 μl of the reaction mixture including 50 μM ϕX174 viral DNA and 50 μM ATP- γ -S for 3 hours. After the addition of 10 μl [^{32}P]-labelled pd(G-G), photoaffinity labelling was performed as described, and 10 μl of the sample was analyzed by electrophoresis with 17 % resolving gel, stained with Coomassie Brilliant Blue R-250 and autoradiographed. R_1 and R_2 indicate the cleavage products of $\phi 80$ CI repressor, R_1 being C-terminal half.

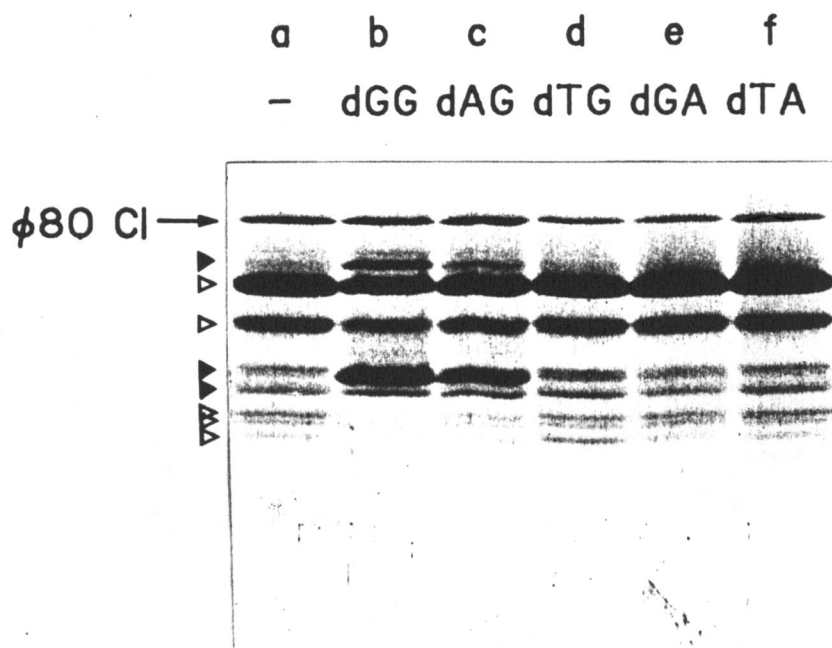


Figure IV-10. Electrophoretic Patterns of $\phi 80$ CI Repressor after Limited Digestion by Trypsin in the Presence of Deoxydinucleoside Monophosphate. $\phi 80$ CI repressor was partially digested by trypsin in the presence of 0.5 mM of an indicated deoxydinucleoside monophosphate. The products were analyzed by electrophoresis in 17 % gel. Open and filled arrow heads indicate the bands, the density of which increase or decrease by the presence of d(G-G), respectively.

(g) Effects of Preincubation of RecA Protein and ssDNA
on Cleavage of ϕ 80 CI Repressor Bound to d(G-G)

I previously reported that the preincubation of the RecA protein and ssDNA in the absence of the repressor and ATP- γ -S enhances cleavage of the repressor subsequently added (Eguchi et al., 1987b; chapter III in this thesis). I showed here that d(G-G) stimulates the cleavage. Below, I examined the effects of preincubation of RecA protein with ssDNA on cleavage of ϕ 80 CI repressor which had been bound to d(G-G) and which had not been bound to it. Preincubation of RecA protein with ssDNA stimulated cleavage of the ϕ 80 CI repressor that had not been treated with d(G-G) about 15-fold (calculated from the extent of cleavage in 60 minutes), while cleavage of the d(G-G) treated repressor was stimulated by a similar extent in the initial reaction (Fig. IV-11). These results indicate that the preincubation and binding of d(G-G) act independently in stimulation of cleavage of ϕ 80 CI repressor.

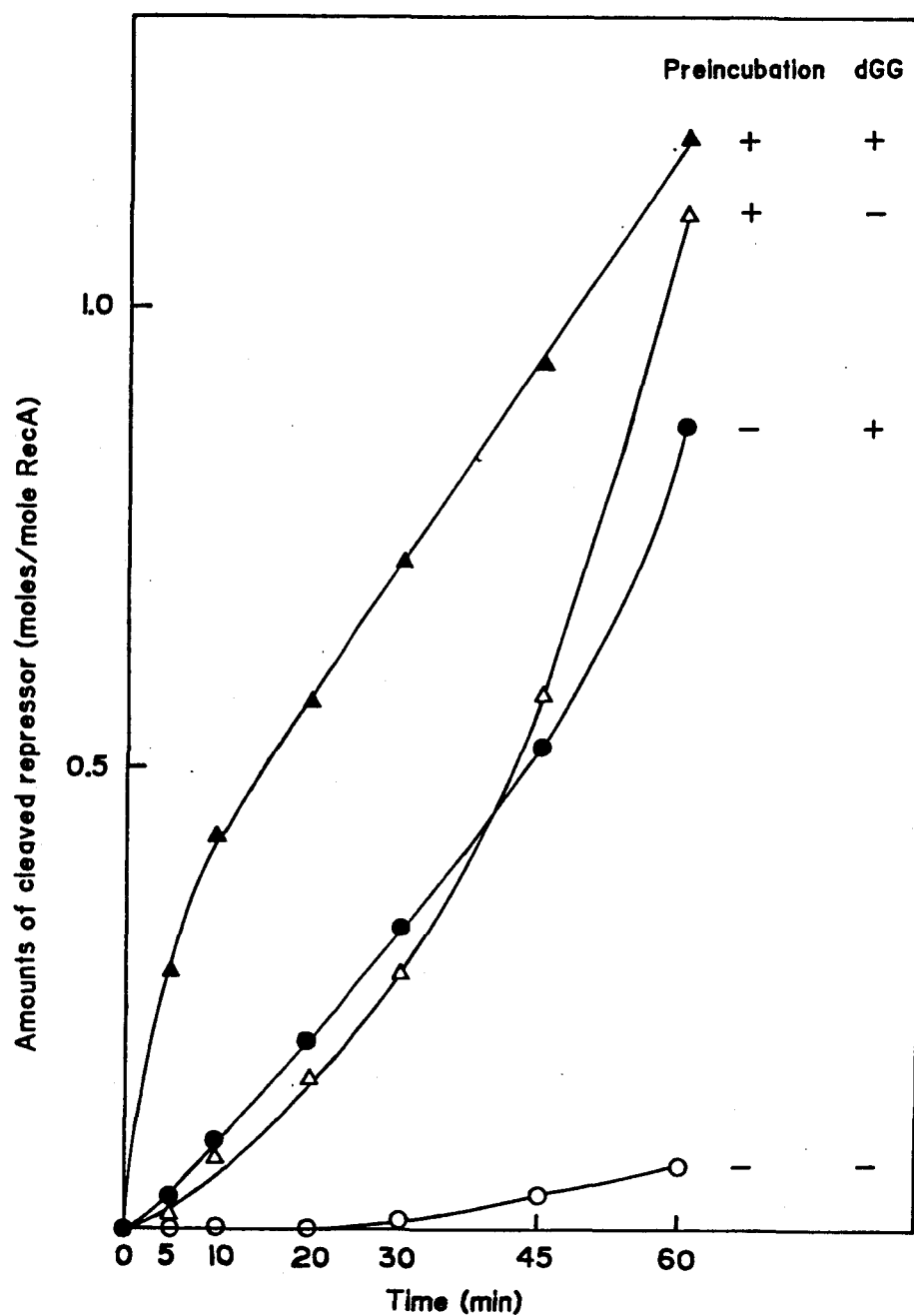


Figure IV-11. Effects of Preincubation of RecA Protein with ssDNA on Cleavage of $\phi 80$ CI Repressor bound and unbound to d(G-G). The presence or absence of preincubation or d(G-G) treatments is indicated in the figure. The conditions for preincubations of RecA protein and ssDNA were for 2 hours at 37°C. Either one of $\phi 80$ CI repressor which had been or had not been preincubated with d(G-G) for 1 hour separately was used. Aliquots (40 μ l) were taken at indicated times and the amounts of the products were analyzed.

4. Discussion

I found that d(G-G) and d(A-G) greatly stimulated the cleavage of $\phi 80$ CI repressor by RecA protein. In this stimulated cleavage reaction, d(G-G) did not substitute for either ssDNA or nucleoside triphosphate that are essential components for the cleavage. Among all deoxydinucleoside monophosphates, only d(G-G) and d(A-G) are active. This specificity is identical to the specificity of deoxydinucleoside monophosphates in the recA⁺-dependent induction of prophage $\phi 80$ in permeabilized lysogen observed by Irbe et al. (1981). The induction of prophage $\phi 80$ in permeabilized lysogen by addition of oligonucleotides probably results from the RecA-mediated cleavage of $\phi 80$ CI repressor which was stimulated by added oligonucleotides.

How does d(G-G) stimulate the cleavage reaction of $\phi 80$ CI repressor? pd(G-G) cross-linked to $\phi 80$ CI repressor but not to RecA protein by heavy UV-irradiation in the presence of ATP or its analogues which are required for the cleavage. Changes in electrophoretic patterns of the products of partial digestion of the repressor by trypsin by addition of d(G-G) or d(A-G) show that these deoxydinucleoside monophosphates bind to the repressor. The binding might change the conformation of $\phi 80$ CI repressor so that the repressor can interact with RecA protein more effectively. The kinds of deoxydinucleoside monophosphates that affect the trypsin digestion of the repressor is identical to those which stimulate the cleavage reaction of the repressor by RecA protein. It seems that the same interaction of d(G-G) and d(A-G) with the repressor that affected cleavage by trypsin may be responsible for stimulation of cleavage by RecA protein. pd(G-G) interacts with the R₁ fragment which derived from carboxyl-terminal half of the

repressor. If the carboxyl-terminal domain of the intact repressor takes a conformation similar to that of R_1 fragment, the site of interaction of d(G-G) with the intact repressor is the carboxyl-terminal domain. In the cleavage of λ CI repressor, its carboxyl-terminal domain is likely to be involved in interaction with RecA protein (Sauer *et al.*, 1982). The presence of considerable similarity between the amino acid sequence of $\phi 80$ CI repressor and that of λ CI repressor in their carboxyl-terminal regions (Eguchi *et al.*, 1987a; chapter II in this thesis), suggests that the carboxyl-terminal region of $\phi 80$ CI repressor might be responsible for interaction with RecA protein. d(G-G) increases the affinity between $\phi 80$ CI repressor and RecA protein probably by changing the conformation of the carboxyl-terminal domain of the repressor. Cohen *et al.* (1981) reported that the monomer form of λ CI repressor was preferentially cleaved by RecA protein rather than the dimer form. d(G-G) did not affect the oligomer formation of $\phi 80$ CI repressor at the concentration for the cleavage reaction, which was analyzed by glycerol gradient centrifugation (unpublished observation). d(G-G) can bind also to RecA protein at least in the absence of ATP or its analogues. Various activity of RecA protein, such as binding activity to ssDNA or ssDNA-dependent ATPase activity, were not affected by the presence of d(G-G) (unpublished observation).

I previously reported that cleavage of $\phi 80$ CI repressor in the initial stage was very slow in the absence of d(G-G) (Eguchi *et al.*, 1987b; chapter III in this thesis). The presence of d(G-G) practically eliminates the slow reaction in the initial stage. The initial slow cleavage is also eliminated by preincubating the RecA protein with ssDNA in the absence of the repressor and ATP- γ -S. Because d(G-G) increases the rate of cleavage of the repressor by RecA protein that has been preincubated with ssDNA, it seems that d(G-G) increases the affinity between the repressor and RecA

protein so that the significant amounts of the repressor could be cleaved even in the early stage, rather than it makes the repressor to interact equally with either two forms of RecA - ssDNA - ATP- γ -S complexes, which were formed with or without preincubating the RecA protein with ssDNA.

Deoxytrinucleotides containing G-G or A-G sequence are also effective to induce prophage $\phi 80$ in permeabilized lysogen (Irbe et al., 1981). It is possible that various other oligonucleotides also stimulate the cleavage reaction. It has been shown that DNA was degraded after the treatments which induce SOS response (Boyce & Howard-Flanders, 1964). Therefore, it is likely that oligonucleotides in the products of degradation of DNA stimulate the cleavage reaction of $\phi 80$ CI repressor in vivo. It should be noted that cleavage of the repressor in the presence of d(G-G) still requires ssDNA. I showed that higher concentration of d(G-G) are more effective in cleavage of the repressor. If the amount of enhancing oligonucleotides is proportional to the amount of damage of DNA, the rate of the cleavage of $\phi 80$ CI repressor might be determined by the amount of damage in DNA. When the bacterial chromosome is damaged lightly, cellular SOS functions for repair of the damages may be induced as a result of inactivation of the LexA repressor. Under such conditions, prophage $\phi 80$ would not be induced efficiently because the amount of inducing oligonucleotides would not be produced much. Therefore, most bacteria can be recover from the damage. When the bacterial chromosome is damaged heavily, inducing oligonucleotides would be accumulated and $\phi 80$ CI repressor should be cleaved efficiently resulting in induction of the prophage.

6. References

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