Kinetic Analysis of λ DNA Digestion Catalyzed by Restriction Endonuclease, *Eco*RI
−Effect of Anti-DNA Antibody on the Kinetic Constants−

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Synopsis

The rate constant values of the reaction catalyzed by restriction endonuclease, *Eco*RI, at individual recognition sites in λ DNA were estimated in the presence or absence of monoclonal anti-DNA antibody. The time-course of the product formation was experimentally obtained by agarose-gel electrophoresis, and the data were analyzed by numerical analysis of a differential equation derived from the kinetic model for the λ DNA degradation to each fragment. From the data-fitting procedure, the rate constant value of the cleavage at the recognition site located near the terminal of λ DNA was found to be considerably larger than those at the sites far from the terminal. The rates of cleavage were enhanced by preincubating the DNA with monoclonal anti-DNA antibodies obtained from systemic lupus erythematosus patients. The enhancement was more intensive at the recognition site located near the terminal than those at other sites. These findings suggest that the thermal motion of the λ DNA molecule might be involved in the recognition and binding mechanisms of the enzyme and the antibodies toward the DNA molecule.

Introduction

It has been recognized that DNA-protein interaction plays an important role in regulation of gene expression. Although a lot of works on the DNA-protein interaction have been done, the recognition mechanism of DNA molecule by the proteins have not been understood yet. A restriction endonuclease reaction which recognizes a specific nucleotide sequence of DNA molecule can be considered as one of the most appropriate system for the elucidation of the recognition mechanism. Most of the works on the reaction mechanism of endonucleases were performed with oligonucleotides as the substrate[1,2]. However, polynucleotides should have a conformational factor which does not occur in oligonucleotides, and the reaction of endonucleases toward polynucleotides might exhibit some aspect different from that in the enzymatic reaction toward oligonucleotides.

The analysis of the λ DNA-cleavage reaction by restriction endonuclease, *Eco*RI, was first made by Thomas and Davis[3] with an electron microscope observation, and demonstrated that the cleavage frequencies of the individual recognition sites were different from each other. This result indicated that the restriction enzyme, *Eco*RI, recognizes not only the sequence composed of six nucleotides (GAATTC) but one of the other structural factors in the DNA molecule. For the elucidation of the recognition mechanism, therefore, it is essential to determine the kinetic constant of the cleavage reaction at each

recognition site under various conditions. On the other hand, Halford et al.[4,5] reported the kinetic analysis of the DNA-cleavage reaction by *Eco*RI. Since they used a derivative of the λ DNA molecule, which carry only one recognition site for *Eco*RI, as the substrate, the result obtained seems to contain some uncertainty. In fact, the results obtained in the report are inconsistent with the results obtained by Thomas and Davis[3]. Here, it is highly desirable to establish the method which can afford the kinetic constant of the cleavage at each restriction site using the intact λ DNA.

In the studies on the monoclonal anti-DNA antibodies obtained in the sera of patients with systemic lupus erythematosus, the antibodies were found to exhibit an broad specificity toward various types of DNA[6]. An electron microscopic observations by means of a ferritin-antibody conjugate suggested that the antibody sparsely binds to the surface of the DNA molecule (Goto et al., unpublished), indicating that there might be some specificity in the recognition of DNA molecule by the antibodies as in the case of the endonuclease recognition. Therefore, it is quite interesting to investigate the effect of the anti-DNA antibodies on the kinetic parameters for the reaction catalyzed by the endonuclease, *Eco*RI.

This paper deals with a method to obtain the rate constant of the λ DNA cleavage by *Eco*RI at each recognition site, and reports that the rate constants of the individual cleavage sites are considerably different from each other and the rates of the cleavages are enhanced by preincubation of the λ DNA with DNA antibody.

Materials and Methods

Materials —— λ DNA (from cI857 Sam 7 Lysogen of *E. coli* M65) and restriction endonuclease *Eco*RI (from *E. coli* RY13) were purchased from Nippon Gene Co. Ltd. Acridineorange used for staining of DNA fragments in agarose-gel was from Wako Pure Chemical Co. Monoclonal anti-DNA antibodies, 1A2 and 2C10, were obtained by the method previously reported[7]. All other reagents were of analytical grade commercially available.

Enzyme and substrate concentrations —— The concentration of the enzyme was determined from units of enzyme activity according to the method of Halford[4], and that of λ DNA was from the absorbance at 260 nm using $E_{260\ nm} = 20$. The molar concentrations of DNA in solutions are determined in terms of the entire λ DNA molecule taking 30.8×10^6 for the molecular weight of the λ DNA.

Enzymatic reaction —— λ DNA (3.7 nM) and *Eco*RI (0.74 nM) were mixed in 80 mM Tris-HCl buffer (pH 7.5) containing 60 mM NaCl, 5 mM $MgCl_2$, and 6 mM 2-mercaptoethanol at an appropriate temperature. After incubation of a given period, an aliquot withdrawn from the reaction mixture was mixed with 0.5 M EDTA solution in order to terminate the reaction. 0.5 % agarose gel electrophoresis was performed to separate the reaction products, and the gel was stained with 0.4 % acridineorange in 1 M acetic acid for 60 min. After destaining with 0.2 N acetic acid for 15 hr, gel-scanning was carried out with a dual wavelength TLC scanner, Shimadzu CS-910. In the measurement, the wavelengths for the sample and the reference were fixed at 492 nm and 410 nm, respectively. From the peak areas obtained by the gel-scanning, the molar concentrations of the DNA fragments were calculated and plotted against the reaction time.

For investigation of the effect of DNA antibody, λ DNA and the antibody, 1A2 or 2C10, were mixed and preincubated for 30 min under the same conditions as those for the enzymatic reaction. The enzyme was then added to the mixture to obtain the reaction time-course with the method described above.

Kinetic model —— In the degradation process of λ DNA to each *Eco*RI-fragment, 14 species of the intermediate fragments can be considered as

shown in Fig. 1. Here, 21 reaction species which include one initial substrate and six final reaction products were taken into consideration for the construction of the mathematical model.

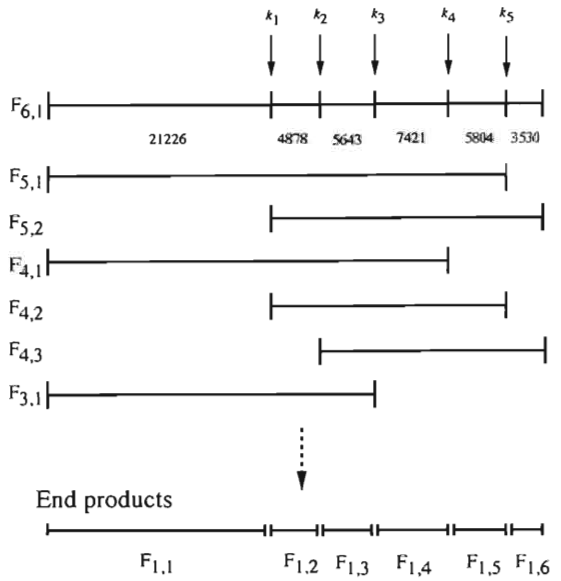

Fig. 1. Reaction model for λ DNA cleavage by restriction endonuclease, *Eco*RI. Each cleavage process is assumed to be first order reaction.

Furthermore, one cleavage reaction process by *Eco*RI was assumed to be one-step first order reaction with the rate constant, k_i (i = 1, 2, 3, 4, and 5). Thus, the simultaneous differential equation for the reaction species can be described as follows;

$$\frac{d[F_{m,n}]}{dt} = k_{n+m-1} \cdot \sum_{i=m+1}^{7-n} [F_{i,n}] + k_{n-1} \cdot \sum_{i=2}^{n-1} [F_{n+m-i,i}] - [F_{m,n}] \cdot \sum_{i=0}^{m-2} k_{n+i} \quad (m=1\sim6,\ n=1\sim7-m) \quad (1)$$

where $F_{m,n}$ indicates the DNA fragments including the initial substrate and all possible intermediate fragments, and k_i the rate constant of the cleavage at each restriction site, as shown in Fig. 1. $F_{6,1}$ is the initial substrate, the entire molecule of λ DNA. $F_{m,n}$ (m = 2 ~ 5) are the intermediates consisting of m pieces of *Eco*RI fragments of λ DNA. $F_{1,n}$ are the end products of the *Eco*RI digestion of λ DNA. The differential equation was numerically solved by Gear's method[8], and the reaction time-course of the λ DNA cleavage was theoretically obtained.

Estimation of rate constants —— The values of the rate constants (k_i = 1, 2, 3, 4, and 5) were estimated by sensitivity method. First, roughly guessed values were allocated to five rate constants and plausible values of the rate constants which gave theoretical time-courses more fitted to the experimental ones were found by repeating the calculation with revised values. Next, further calculations were conducted with the plausible values varied within a narrow range. The deviation between theoretical and experimental profiles was evaluated by the equation;

$$D = \sum_{t} \sum_{m} \sum_{n} ([F_{m,n}]t^c - [F_{m,n}]t^e)^2 \quad (2)$$

where e and c are the experimental and the calculated concentrations at each reaction time, and t the reaction time. The values of D were plotted against the rate constant values used for the calculations. Finally, a set of the rate constant values which gave the smallest D value (deviation) was chosen as the most reliable values of the rate constants.

Results

Determination of DNA fragments produced by enzymatic digestion —— At first, λ DNA and *Eco*RI were mixed and incubated for 30 min at pH 7.5, and at 37°C. The reaction conditions allow a complete digestion of the DNA. After the separation of the reaction mixture with agarose gel electrophoresis, the gel was stained with 0.4% acridineorange and scanned by means of the 492 nm light absorption derived from the acridineorange molecules bound to DNA fragments. The typical profile of the electrophoresis is shown in Fig. 2a. Although the peaks for the fragments, 5804 bp and 5643 bp, were overlapped, the other fragments were successfully separated from each other. The peak

areas would reflect the amounts of acridineorange bound to equimolar DNA fragments. The relationship between the peak area and the length of λ DNA fragment is shown in Fig. 2b.

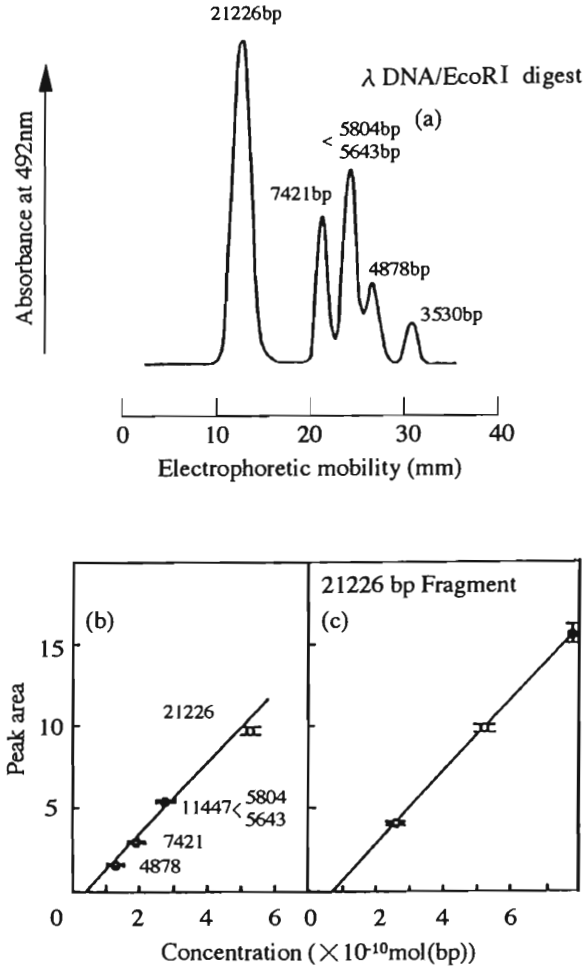

Fig. 2. (a) Electrophoretic profile of λ DNA/*Eco*RI digest. 0.5% agarose gel was used for the separation, and the gel-staining was done with 0.4% acridineorange in 1M acetic acid for 60 min. The profile was obtained by a dual wavelength TLC scanner, Shimadzu CS-910. The individual peaks were assigned to 21226 bp, 7421 bp, (5804+5643) bp, 4878 bp, and 3530 bp, respectively, from left to right. (b) Relationship between the peak area obtained and the length of the DNA fragments. The DNA length was converted into the molar concentration in terms of the base pair unit. (c) Relationship between the peak area and the concentration of 21226 bp fragment obtained by the complete digestion with *Eco*RI.

The numbers on the abscissa indicate the molar concentration in terms of base pairs calculated from the length of the DNA fragment. Since the fragments, 5804 bp and 5643 bp, were overlapped, the peak was regarded as the total of the two fragments in this figure. A linear relationship was found between the peak area and the length of the DNA fragment. The complete digestion experiments were further conducted with different concentrations of initial substrate, λ DNA, and the relationship between the peak area and the concentration of each DNA fragment was investigated. As shown in Fig. 2c, a linear relationship was found between the peak areas and the DNA concentrations as well as in Fig. 2b, and the slopes of the lines were almost identical between these figures (Figs. 2b and 2c). This indicates that the peak area detected by acridineorange bound to DNA fragment reflects the molar concentration of each fragment in terms of base pair.

Time-course of λ DNA digestion by EcoRI — λ DNA and *Eco*RI were incubated at pH 7.5, and at 15℃. The electrophoretic profile of each reaction time are shown in Fig. 3a. Since the peaks for the fragments, whose sizes are more than 20 kbp, were overlapped, 21226 bp end product could not be determined. From the profiles, however, the rate of the production of the 3530 bp fragment was found to be higher than those of the other fragments. The production rate of the 4878 bp fragment was lower than those of the other fragments. The peak areas were converted into molar concentrations of the fragments using the linear relation obtained in Fig. 2, and plotted against reaction time. The result is shown in Fig. 3b. The overlapped peak for 5804 bp and 5643 bp fragments was regarded as the total amounts of the fragments.

Effect of anti-DNA antibody on the λ DNA digestion by EcoRI — λ DNA was preincubated with 3-fold or 9-fold molar excess of the monoclonal antibody, 1A2, and was applied to the

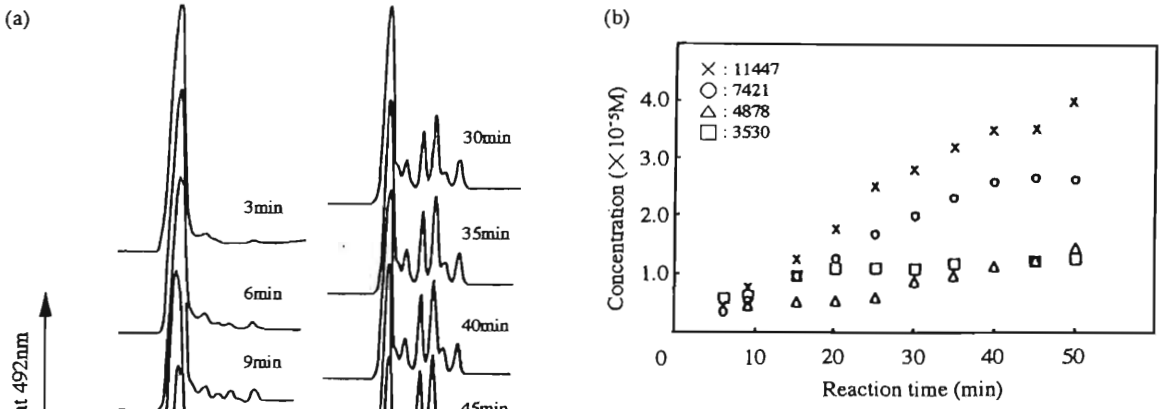

Fig. 3. (a) Time-dependent profile of the electrophoretic separation of the DNA fragment obtained by λ DNA digestion with *Eco*RI. The reaction conditions were described in **Materials and Methods**. (b) Time-course of λ DNA digestion with *Eco*RI. The concentrations were calculated from the peak area obtained in the electrophoretic profile.

*Eco*RI digestion. As shown in Fig. 4a and 4b, the preincubation of λ DNA with the antibody clearly enhanced the rate of the digestion by the endonuclease, *Eco*RI, and the enhancement appears to be dependent upon the amount of the antibody added. λ DNA was preincubated with 500-fold molar excess of monoclonal antibody, 2C10, and then applied to the *Eco*RI digestion. The product formations were dramatically enhanced by the preincubation, as shown in Fig. 5a.

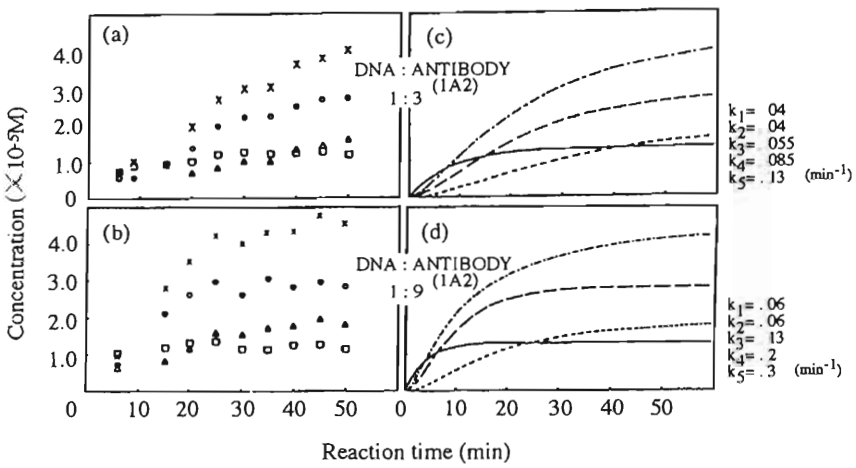

Fig. 4. (a) Experimental time-course of λ DNA/*Eco*RI reaction in the presence of the anti-DNA antibody, 1A2. The molar ratio of the antibody to λ DNA is 3. (b) Experimental time-course of λ DNA/*Eco*RI reaction in the presence of the anti-DNA antibody, 1A2. The molar ratio of the antibody to λ DNA is 9. (c) Theoretical time-course best fitted to the time-course in (a). The rate constant values used for the calculation were $k_1 = 0.04$, $k_2 = 0.04$, $k_3 = 0.055$, $k_4 = 0.085$, and $k_5 = 0.13$ min^{-1}. (d) Theoretical time-course best fitted to the time-course in (b). The rate constant values used for the calculation were $k_1 = 0.06$, $k_2 = 0.06$, $k_3 = 0.13$, $k_4 = 0.20$, and $k_5 = 0.30$ min^{-1}.

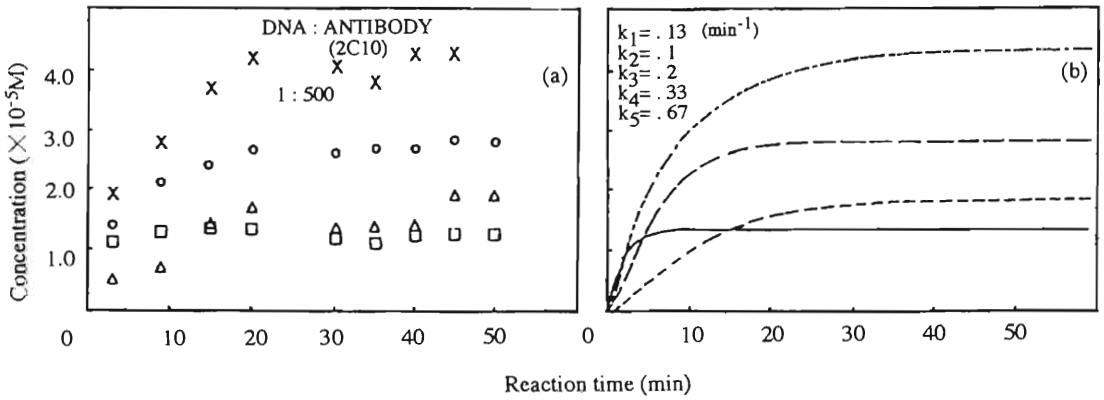

Fig. 5. (a) Experimental time-course of λ DNA/*Eco*RI reaction in the presence of the anti-DNA antibody, 2C10. The molar ratio of the antibody to λ DNA is 500. (b) Theoretical time-course best fitted to the time-course in (a). The rate constant values used for the calculation were k_1=0.13, k_2=0.10, k_3=0.20, k_4=0.33, and k_5=0.67 min^{-1}.

Analysis of experimental time-courses ——First, the calculation was performed with the assumption that the rates of the cleavages are the same at any restriction sites. In practice, all of the values of the rate constants, k_1, k_2, ..., k_5, were set at 0.06 min^{-1}. The time-courses thus calculated were considerably different from the experimental time-courses (Fig. 6a). This clearly indicated that the rate constants of the cleavage at the individual restriction sites were different from each other.

From the fact that the rate of production of 4878 bp fragment was extremely lower than those of the others, it is speculated that the k_1 and k_2 were considerably smaller than the other rate constants. Thus, the calculation was performed with 0.04 min^{-1} for k_1 and k_2, and with 0.1 min^{-1} for k_3, k_4, and k_5. However, the calculated result could not be fitted to the experimental data. In the time-dependent profiles of electrophoretic separation of the DNA fragments (Fig. 3a), the

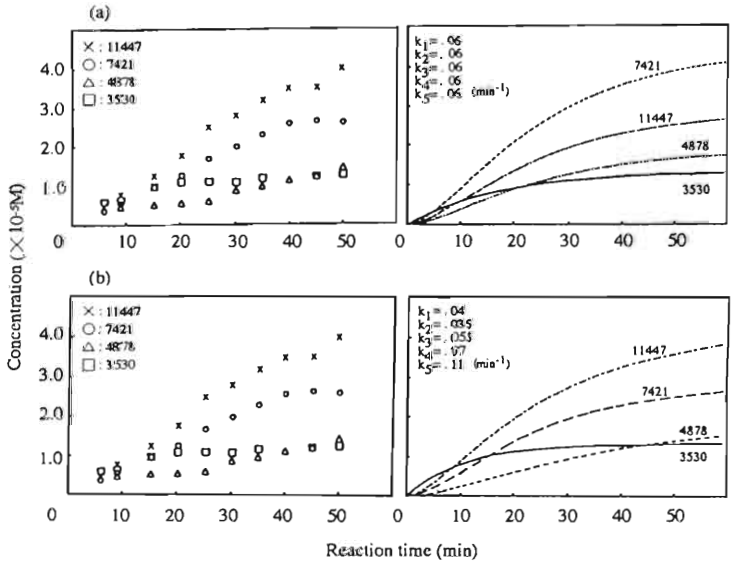

Fig. 6. (a) Experimental (left panel) and theoretical time-courses (right panel) of end product formation of λ DNA/*Eco*RI reaction. The theoretical time-course was obtained assuming that the rate of the cleavage at each recognition site is identical to each other k_1=k_2=k_3=k_4=k_5=0.06 min^{-1}. (b) Experimental (left panel) and theoretical time-courses best fitted to the experimental ones (right panel). The theoretical time-course was obtained with k_1=0.04, k_2=0.035, k_3=0.055, k_4=0.07, and k_5=0.11 min^{-1}.

amount of the 3530 bp fragment was larger than those of the others at 3 min of the reaction time. This result led us to calculate with the higher k_5 value. In practice, the calculation was done with $k_1=0.04$, $k_2=0.04$, $k_3=0.07$, $k_4=0.07$, and $k_5=0.1$ min^{-1}, and this gave a better fitness of the theoretical time-courses to the experimental ones. Starting from these values roughly allocated to the five rate constants, the time-courses were repeatedly calculated with finely revised values to obtain the best fitting to the experimental data. Finally, the values of the rate constants which gave a minimum of D value (equation (2)) were determined to be $k_1=0.04$, $k_2=0.035$, $k_3=0.055$, $k_4=0.07$, and $k_5=0.11$ min^{-1}. The theoretical time-courses calculated with these values are shown in Fig. 6b.

The reliability of the values of the rate constants estimated was examined by a sensitivity analysis; that is, calculations were conducted with changing the values of the rate constants, and effects of the rate constant values on the profile of the time-courses were investigated. The changes in the values of k_2, k_3, k_4, and k_5 by 0.01 min^{-1} considerably affected the profile of the time-courses, indicating that the deviations of the values estimated are within about 0.005 min^{-1} For the value of k_1, however, 3-fold increase in the value could not cause a significant change in the profile of the time-course, indicating an uncertainty of the estimated value.

Effect of the anti-DNA antibody on the rate constant of EcoRI cleavage —— The rate constant values of the *Eco*RI cleavages of λ DNA preincubated with the antibodies were estimated by the same procedure as described in the previous section on the basis of the experimental time-courses shown in Figs. 4a, 4b, and 5a. First, the time-courses were calculated with the assumption that the rate of the cleavage at each recognition site is accelerated with the same proportion. However, the calculated time-courses could not be fitted to the experimental data, indicating that the antibody binding accelerated the rate of the cleavage at individual recognition sites with different proportions. Finally, the rate constant values for each experimental time-course were obtained as listed in Table 1. The calculated time-courses with the estimated values are shown in Figs. 4c, 4d, and 5b, respectively.

Table. 1. Rate constant values of λ DNA cleavage catalyzed by restriction endonuclease, *Eco*RI, at individual recognition sites in the presence or absence of anti-DNA antibodies.

	λDNA:antibody (molar ratio)	k_1	k_2	k_3	k_4	k_5
		(min^{-1})				
control	1:0	0.04	0.035	0.055	0.07	0.11
monoclonal anti-DNA antibody 1A2	1:3	0.04	0.04	0.055	0.085	0.13
	1:9	0.06	0.06	0.13	0.20	0.30
monoclonal anti-DNA antibody 2C10	1:500	0.12	0.10	0.20	0.33	0.67

Discussion

A number of restriction endonucleases have been found and routinely used in gene manipulation. Although the sequences of DNA cleaved by the restriction endonucleases have

been identified, it has recently been considered that more complicated processes than expected so far might be involved in the recognition of DNA molecule by the restriction enzymes. Halford et al.[4] demonstrated the significance of a interaction between the enzyme and DNA sequences surrounding each recognition sequence. Furthermore, a DNA conformation depending upon its nucleotide sequence is considered to play an important role in the recognition and the cleavage of DNA molecule by the restriction enzymes[9]. Thus, the recognition mechanism of DNA sequence by the restriction enzymes is getting more distinct, but not understood definitively. The values of the rate constants of the cleavages at recognition sites in various conditions can give us an useful information on the recognition mechanism.

Although the detection of DNA fragments in agarose gel has usually been performed by taking a photograph of the gel stained with ethydium bromide, the method is considered to be inappropriate for a quantitative analysis. Thus, the scanning of the gel stained with acridineorange was employed for the quantitative analysis of the DNA fragments in the present study. The method could give experimental time-courses of λ DNA digestion by *Eco*RI, which have a sufficient accuracy for kinetic determination. From the analysis of the experimental data shown in Figs. 4, 5, and 6, the values of the rate constants were finally obtained as listed in Table 1. On the other hand, Thomas et al.[3] found that the frequency of the cleavage at the recognition site corresponding to the rate constant k_5 was considerably larger than the others. Halford et al.[4] also found that the values of the rate constants, k_4 and k_5, were estimated to be about twice larger than that of k_2. These findings are consistent with the result obtained in this study, indicating the reliability of the values estimated by the kinetic method employed in this study.

As seen from the rate constant values obtained in the absence of the antibody, the values of the rate constants are closely related to the distance of the recognition site from the terminal of the λ DNA; the values at the recognition site near the terminal of the λ DNA are larger than those at the site far from the terminal. Halford et al.[4] suggested that the different reactivities of *Eco*RI at the individual recognition sites were caused by the interactions between the enzyme and nucleotide sequence surrounding each recognition site. From the nucleotide sequence of λ DNA reported by Sanger et al.[10], however, any relationship could not be found between the rate constants and the nucleotide sequences surrounding the recognition sites. In addition, at present, it is impossible to correlate the nucleotide sequence at each recognition site with a specific conformation of a DNA molecule which is considered to participate in the *Eco*RI recognition[1]. Thus, the data in Table 1 cannot be rationalized from the nucleotide sequences at or near the recognition sites of λ DNA. Nevertheless, one possible explanation of the data in Table 1 might be as follows. A thermal motion at the terminal region of the λ DNA molecule is likely more intensive than that at the inside region. This can rationalize the result that the rate constant values at the terminal region of the λ DNA are larger than those at the inside region. From these consideration, the recognition of the DNA sequence by *Eco*RI may be closely related to the intensity of the thermal motion of DNA molecule. In this situation, however, the rate constant value at the inside region of the λ DNA molecule should increase with progress of the enzymatic reaction, because the recognition site at the inside region should gradually become closer to the terminal by the enzymatic cleavage. The reaction model, in which the rate constant value at each recognition site is assumed to be dependent upon the length of the substrate, might result in the better fitness between the experimental and theoretical data.

From Table 1, it can be concluded that the binding of the anti-DNA antibodies to λ DNA molecule affects the steric structure of the DNA and changes its conformation into that favourable for *Eco*RI recognition. The preincubation of

λ DNA with 9-fold molar excess of the antibody, 1A2, caused 3-fold increase in the k_5-value and 1.5-fold increase in the k_2-value, while the preincubation with the antibody, 2C10, caused 6-fold and 3-fold increase, respectively. In both cases, the antibodies enhance the rate of the *Eco*RI cleavage more efficiently at the terminal region of λ DNA than at the inside region. These results can be explained from the situation similar to the *Eco*RI recognition; that is, the thermal motion at the terminal region of λ DNA is more intensive than those at the inside region, enhancing the antibody binding ability at the terminal region. Although the molecular mechanism of the recognition of DNA molecule by anti-DNA antibody has been unknown yet, there should be a common factor controlling the DNA recognition by endonucleases and antibodies, such as the thermal motion of the DNA molecule.

References

1) C.A. FREDERIC, J. GRABLE, M. MELIA, C. SAMUDZI, L. JEN-JACOBSON, B.C. WANG, P. GREENE, H.W. BOYER, and J.M. ROSENBERG : *Nature*, 309, 327-331 (1984)
2) L.W. MCLAUGHLIN, F. BENSELER, E. GRAESER, N. PIEL, and S. SCHOLTISSEK : *Biochemistry*, 26, 7238-7245 (1987)
3) M. THOMAS and R.W. DAVIS : *J. Mol. Biol.*, 91, 315-328 (1975)
4) S.E. HALFOLD, N.P. JOHNSON, and J. GRINSTED : *Biochem. J.* 191, 581-592 (1980)
5) S.E. HALFORD, and N.P. JOHNSON : *Biochem. J.* 191, 593-604 (1980)
6) Y. KANAI, T. AKATSUKA, T. KUBOTA, S. GOTO, and B.D. STOLLAR : *Clin. Exp. Immunol.* 59, 139-145 (1985)
7) T. KUBOTA, T. AKATSUKA, and Y. KANAI : *Clin. Exp. Immunol.* 62, 321- 328 (1985)
8) C.W. GEAR : *in Numerical Initial Value Problems in Ordinary Differential Equations*, Prentica-Hall, Englewood Cliffs. (1971)
9) Y. NISHIMURA, : *Adv. Biophys.* 20, 59-74 (1985)
10) F. SANGER, A.R. COULSON, G.F. HONG, D.F. HILL, and G.B. PETERSEN, *J. Mol. Biol.* 162, 729-773 (1982)

(Received : September 30, 1999)

制限酵素*Eco*RIによるλDNA分解反応の速度論的解析
—抗DNA抗体の反応速度定数に対する影響—

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要　約

制限酵素 *Eco*RIによるλDNA分解反応の速度定数を各認識部位それぞれについて推定した。まず、アガローズ電気泳動法によって分解反応の経度変化を実験的に得ることができた。さらに、推定された分解反応モデルから導かれる連立微分方程式を数値的に解くことによって、理論的な経度変化を得、データフィッティング法によって、信頼できる速度定数を推定することができた。その結果、λDNAの末端付近の認識部位においては、λDNA内部領域にある認識部位よりも効率よく分解が行われていることがわかった。また、SLE患者から得られた抗DNA抗体を作用させたλDNAを用いて同様の実験を行うと、分解反応の速度は促進されており、その促進の程度は末端に近い認識部位ほど大きかった。これらの結果より、制限酵素*Eco*RIと抗DNA抗体のDNA認識の分子機構には、DNA分子の熱運動といった共通の要因が存在することが明らかになった。