CHARACTERIZATION OF E.COLI RNA POLYMERASE-BINDING SITES ON f1 RFI DNA

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SUMMARY

E. coli RNA polymerase-binding sites on f1 RFI DNA were isolated as DNA fraction(1.9% of total DNA)protected by RNA polymerase against the nucleolytic digestion. These polymerase-protected DNAs had the properties as follows:(1) They were not obtained in the absence of sigma factor. (2) They were short double-stranded DNA fragments(35-40 base pairs long) and enriched in A-T base pairs(65%). (3) After heat-denaturation, they exhibited the resistance to the single strand-specific nuclease to some extent, but the nuclease-resistance was very temperature dependent. They behave not like GC-rich regions on f1 DNA, which have very stable hairpin-like structure.

INTRODUCTION

Previously, we isolated two sorts of hairpin-like structure, AT-rich fragments(61.3%AT,(1)) and GC-rich fragments(41.3%AT,(2)) from coliphage f1 DNA. The binding of E. coli RNA polymerase to the f1 RFI DNA was inhibited by both phleomycin and the AT-rich fragments, and the binding of RNA polymerase to the AT-rich fragments was inhibited by phleomycin(3). Phleomycin is an antibiotic which has been reported to bind specifically to the A-T pairs in DNA(4,5). These evidences indicate that E. coli RNA polymerase bind preferentially to AT-rich regions on DNA template.

One direct approach to study the structure of promoter regions is to isolate RNA polymerase-binding sites. Taking advantage that the binding sites on DNA were protected by polymerase against nucleolytic digestion, several groups have looked for peculiarities in base composition of these sites, as compared with that of total DNA. These results show many common features. Protection requires in addition to holoenzyme, elevated temperatures. The protected fractions in fd RF(6,7), λ(8-10), T_5(11) and T_7(11) DNAs are AT-rich (65-67%) and consist of about 35-50 nucleotide residues. Although the nucleotide distribution of the protected sequences obtained

from various DNAs all show a fairly good A=T and G=C correlation, there are few proof of double-strandedness(7). Moreover it is very interesting whether the protected sequences have self-complementary hairpin-like structure or not(1,2, 12-14). In order to solve these problems, the authors investigated the sensitivity of the protected DNA fragments to S1 nuclease, a single strand-specific endonuclease(15,16) with or without previous heat-denaturation.

MATERIALS AND METHODS

RNA polymerase holoenzyme and core polymerase were purified from *E. coli* as described by Berg et al(17). Enzymes of Fractions 6 and 7B in their method were used as holoenzyme and core polymerase, respectively. The Fraction 6 appears to contain approximately 0.8 equivalent of sigma.

Uniformly labelled(^{32}P)RFI DNA was obtained from f1-infected *E. coli* K12 W2252 growing in a Tris-glucose medium containing 9 x 10^{-4} M phosphate and 15μCi/ml $^{32}PO_4H_3$. Tritiated thymidine-labelled RFI DNA was obtained from f1-infected *E. coli* K12 W2252 thy$^-$ mutant as described previously(3). Cold and tritiated thymidine-labelled AT- rich and GC-rich fragments were prepared from f1 DNA by the method reported previously(3). These two sorts of fragments correspond to size of 45-50 and 35-40 nucleotide residues, respectively. Salmon sperm DNA and poly U were purchased from Calbiochem. and Miles Laboratories, respectively. Tritium labelled poly U(Miles Lab.) was a generous gift from Dr. K. Sakaguchi of Mitsubishi Kasei Institute of Life Sciences.

Isolation of RNA polymerase-binding sites was performed as described in legends of figures and table.

S1-digestion of nucleic acids and analysis of digestion products, fragmentation of DNA, and measurement of hyperchromicity were carried out as described previously(16).

RESULTS AND DISCUSSION

It is known that the complex of DNA-RNA polymerase can be retained on the filter even after nucleolytic digestion. As shown in Fig.1, the complex of f1 RFI DNA and *E. coli* RNA polymerase was hydrolyzed with pancreatic DNase in the presence of an excess unlabelled heat-denatured salmon sperm DNA. In the experiment using RNA polymerase holoenzyme, about 1.9% of total DNA was retained on the filter by binding of 1.5 μg of the enzyme. This per cent did not change

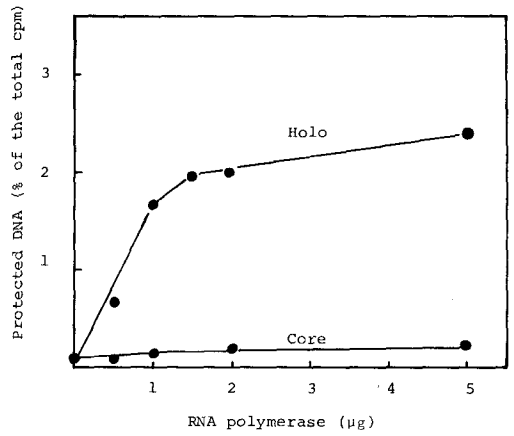

Fig.1. Hydrolysis of the RFI DNA-RNA polymerase complex with pancreatic DNase.

Each mixture contained 2 μg of(^{32}P)RFI DNA (about 1 x 10^5cpm) and indicated amounts of RNA polymerase holoenzyme and core polymerase in 0.5 ml of binding buffer(10 mM Tris,pH 7.9, 10 mM $MgCl_2$, 50 mM KCl, and 0.1 mM dithiothreitol). After incubation at 37°C for 7 min, 20 μg of unlabelled heat-denatured salmon sperm DNA and 50 μg of pancreatic DNase I (Worthington Biochem. Co.) were added and the incubation was continued for 25 min. The mixtures were chilled, diluted with cold binding buffer and then passed through membrane filters(Millipore,HA, 0.45μ). After washing with cold binding buffer, radioactivity retained was counted in a toluene-based scintillator.

by the omitting the unlabelled DNA. On increasing the amount of RNA polymerase, the per cent of protected fraction did not exceed about 2.5%. These results suggest that RNA polymerase holoenzyme bind specific sites on f1 RFI DNA and the DNA regions initially associated with the polymerase could be isolated as a protected fraction. On the other hand, in the experiment using core polymerase, the protected fraction was not obtained. Therefore, sigma is required for the isolation of the protected fraction.

The protected DNA fraction(1.9%) was eluted from the filter with 0.2% SDS, and gel-filtrated on a Sephadex G-50 column. The average chain length of isolated DNA moieties was estimated to be 35-40 nucleotide residues, from the ratio of total ^{32}P to terminal ^{32}P released by E. coli alkaline phosphatase. The observed result suggests that the protected fraction (105 out of 5,500 nucleotide residues (18)) may be mixture of three DNA fragments. Previously, Sugiura et al(19) reported that RFI DNA of fd, closely related f1, provide at

NUCLEOTIDE COMPOSITION OF PROTECTED DNA FRAGMENTS

	A	T	G	C	A+T%	Pu/Py
PROTECTED DNA	32.3	32.8	17.6	17.3	65.1	1.00
TOTAL RF I DNA	29.2	29.8	20.5	20.5	59.0	0.99

Average of three independent preparations.

Table 1. Nucleotide composition of protected DNA fragments.

The complex of 20 μg of (^{32}P)RFI DNA(about 1 x 10^6cpm) and 15μg of RNA polymerase holoenzyme was digested with 50μg of DNase I as described in Fig. 1. The 1.9%-protected DNA fraction was obtained as the materials retained on the filter. Materials retained were eluted with 0.2% SDS-150 mM NaCl-10 mM Tris,pH 7.8. The eluate was treated with phenol saturated in advance with 150 mM NaCl-10 mM Tris, pH 7.8, concentrated after removing phenol in the aqueous layer with ether, and gel-filtrated on a Sephadex G-50 column equilibrated with the above buffer. The eluate was concentrated and precipitated by two volumes of ethanol in the presence of 100μg of salmon sperm DNA as a carrier. The precipitates were dissolved and hydrolyzed to mononucleotides by the treatments with DNase I and subsequently with venom phosphodiesterase(Worthington Biochem. Co.). The mononucleotides wereseparated by paper chromatography. The each nucleotide spot was cut out and counted in a toluene-based scintillator. As a control, total(^{32}P)RFI DNA was treated as described above.

least three initiation sites for RNA synthesis.

Table 1 shows the nucleotide composition of the protected DNA fragments determined from the distribution of ^{32}P. The binding sites are rich in AT (65%), as compared with total RFI DNA (59%). The A=T and G=C correlation was observed, suggesting the double-strandedness of the binding sites. Then we investigated the resistance of the binding sites to the single strand-specific S1 endonuclease. As a control, fragmented f1 RFI DNA and fragmented f1 DNA were used. DNA samples were digested with 750 units of S1 at 30°C for the indicated period in the reaction mixture and the digests were passed through a Sephadex column after treatment with phenol and ether, as described in Materials and Methods. Fig.2-(a) shows the results of the gel filtration. The first fraction(peak I) contained materials relatively resistant to S1. Fig.2-(b) shows the yields of the S1-resistant fraction as a function of time of digestion. The figure

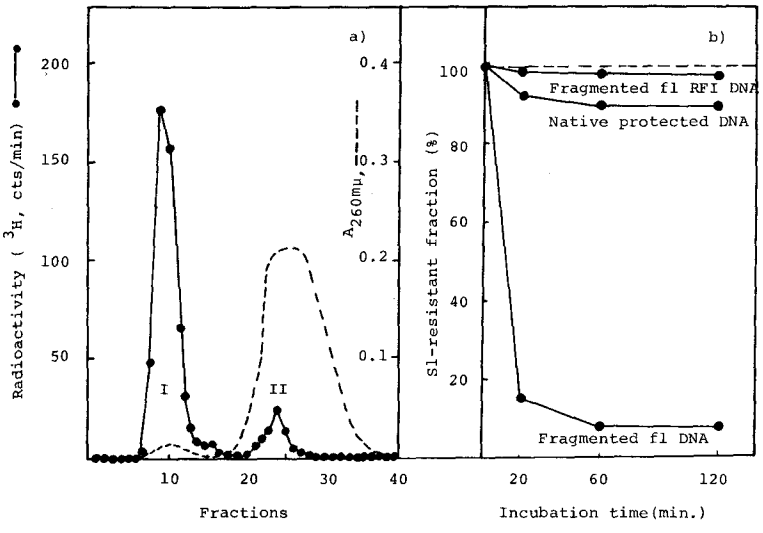

Fig. 2. Nuclease S1-resistance of protected DNA fragments

The complex of 20 μg of (^{3}H)RFI (about 1.5 x 10^5cpm) and 15 μg of RNA polymerase holoenzyme was digested with 50 μg of DNase I and then the protected DNA fragments were obtained as described in Figs. 1 and 2.

(a). Elution profile of S1-treated protected DNA fragments from Sephadex column.

0.1 μg(about 750cpm) of (^{3}H)protected DNA fragments were treated with 750 units of S1 nuclease at 30°C for 120 min in the buffer(100mM NaCl-10mM acetate buffer,pH5.0) containing 100 μg of unlabelled heat-denatured salmon sperm DNA and then passed through Sephadex column as described in Materials and Methods. After measurement of $A_{260\,m\mu}$, each fraction was concentrated and dropped on glass fiber filter paper (The Toyo GB-100). Radioactivity on the paper was counted in a toluene-based scintillator.

(b). Percentage of fraction I in (a) as a function of time of digestion

As a control, same amounts of (^{3}H)-fragmented RFI DNA and fragmented f1 DNA were treated with the enzyme for indicated periods(20, 60, and 120 min) and the percentages of fraction I were obtained.

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indicates that the binding sites were hardly hydrolyzed (10% $>$). But appearance of a very limited hydrolysis might reflect the presence of a short single-stranded terminal region in double-stranded structure of the binding sites. Heyden et al reported that the two strands of the binding sites of fd RF are of unequal length(7).

Next, in order to study the presence of self-complementary sequence in the binding sites, the S1-sensitivity after heat-denaturation was investigated(Fig.3). As a control, AT-rich and GC-rich hairpin-like fragments obtained from f1 DNA, fragmented f1 DNA of

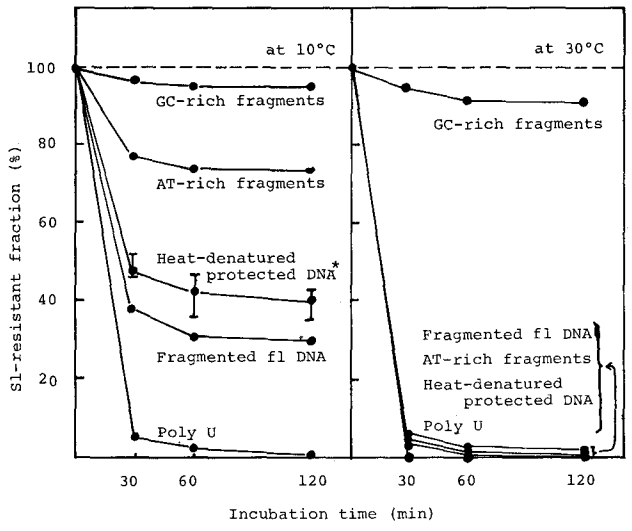

Fig. 3. Nuclease S1-sensitivity of protected DNA fragments after heat-denaturation.

The mixture of 0.1 µg(about 750cpm) of heat-denatured (^{3}H)protected DNA fragments and 100 µg of unlabelled heat-denatured salmon sperm DNA was treated with 750 units of S1. Digestion was carried out at 10°C and/or 30°C for indicated periods(30,60 and 120 min). The percentages of resistant fraction were obtained as described in Fig.2. As a control, same amounts of (^{3}H)-GC-rich fragments, AT-rich fragments, fragmented f1 DNA and poly U were treated with the enzyme.

*The percentages obtained from three independent preparations of the protected DNA fragments all fall within a narrow range.

similar size to the binding sites, and poly U were used. At 10°C of hydrolysis temperature, the heat-denatured binding sites were less resistant to S1 than AT-rich and GC-rich fragments and were slightly more resistant than fragmented f1 DNA. Poly U was degraded rapidly. At 30°C, the binding sites, AT-rich fragments and fragmented DNA were almost hydrolyzed. But the resistance of GC-rich fragments to the enzyme remains much the same.

Fig.4 indicates that the hyperchromicity of various DNA samples and poly U. From the results of Figs.3 and 4, we have reached the conclusion that the heat-denatured binding sites might contain the base-paired structure to some extent. However, this structure is very temperature-sensitive. Recently, the similar result was obtained by Schaller et al in the system of fd RF DNA and by Talaer et al in that of T_5 DNA(personal communications).

DNA conformation of the promoter regions under the specific

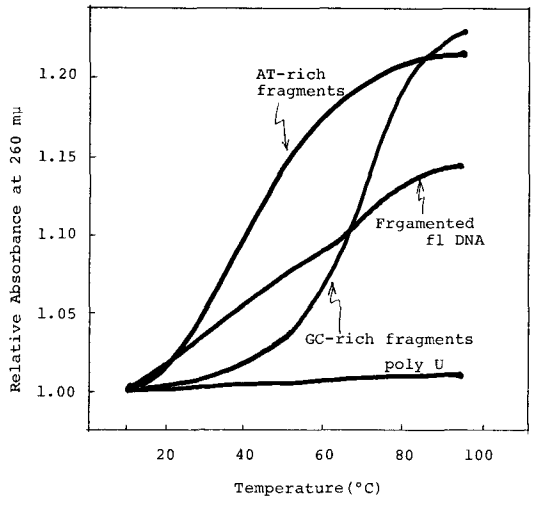

Fig. 4. Hyperchromicity of GC-rich fragments, AT-rich fragments, fragmented f1 DNA and poly U.
Measurement of hyperchromicity was performed in the reaction mixture by the method described in Materials and Methods.

interaction with RNA polymerase is still uncertain. The twisted covalently closed duplex RFI form of the DNA is the transcription form of the phage circular DNA; it is formed immediately after infection and transcribed rapidly to product mRNA. Termination of new transcription appears to be correlated with the conversion of DNA to the relaxed circular duplex RF. If the binding of RNA polymerase unwinds DNA, it is predicted that the twisted form DNA with negative superhelical turns has a higher affinity for the polymerase since superhelicity can supply enough free energy to denature several turns of DNA duplex. Hayashi and Hayashi(20) showed that ØX RFI is much more rapidly transcribed than ØX RFII. The lower template activity of the RFII might be due to failure of RNA polymerase to form appreciable amounts of the productive binary complex(probably base pair opening state), and the structure of ØX 174 promoters appears to be more stable to denaturation in the untwisted RFII form

SV 40 twisted form DNA could be cleaved by S1 nuclease at two unique sites(21,22) and one of these sites was suggested to be at or near the origine of transcription *in vitro* by *E. coli* RNA polymerase(21). Also in our preliminary data, this S1-specific cleavage of twisted form DNA was observed in f1 RFI DNA. The direct approach

to the correlation between the S1-cleavage sites and RNA polymerase binding sites is in progress.

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REFERENCES

(1) Shishido,K. and Ikeda,Y.(1971), J. Mol. Biol. 55,287-291.

(2) Shishido,K. and Ikeda,Y.(1971), Biochem. Biophys. Res. Comm. 42, 482-489.

(3) Shishido,K. and Ikeda,Y.(1971), Biochem. Biophys. Res. Comm. 44, 1420-1428.

(4) Falashi,A. and Kornberg,A.(1964), Federation Proceedings, 23, 940-945.

(5) Shishido,K. and Ikeda,Y.(1972), FEBS LETTERS, 25, 353-356.

(6) Okamoto,T., Sugiura,M. and Takanami,M.(1972), Nature, New Biol. 237, 108-109.

(7) Heyden,B., Nüsslein,C., and Schaller,H.(1972), Nature, New Biol. 240,9-12.

(8) Le Talaer,J-Y, and Jeanteur,Ph.(1971). FEBS LETTERS, 12, 253-256.

(9) Le Talaer,J-Y. and Jeanteur,Ph.(1971). Proc. Natl. Acad. Sci. U.S.A. 68, 3211-3215.

(10) Le Talaer,J-Y. and Jeanteur,Ph.(1972). FEBS LETTERS, 28,305-308.

(11) Le Talaer,J-Y., Kermici,M. and Jeanteur,Ph.(1973), Proc. Natl.Acad Sci. U.S.A. in press.

(12) Schaller,H.,Voss,H. and Gucker,S.(1969). J. Mol. Biol. 44,445-458.

(13) Gierer,A. (1966), 212,1480-1481.

(14) Sobell,H.M.(1972), Proc. Natl. Acad. Sci. U.S.A.69, 2483-2487.

(15) Ando,T.(1966), Biophys. Biochim. Acta, 114,158-168.

(16) Shishido,K. and Ando,T.(1972), Biophys. Biochim. Acta,287,477-484.

(17) Berg,D., Barrett,K. and Chamberlin,M. (1971), Methods in Enzymology, Vol.21, pp506-519,Academic Press, New York.

(18) Hall,J.B. and Sinsheimer,R.L. (1963), 6, 115-127.

(19) Sugiura,M., Okamoto,T. and Takanami,M.(1969), 43, 299-315.

(20) Hayashi,Y. and Hayashi,M.(1971), Biochemistry,10, 4212-4218.

(21) Beard,P., Morrow,J. and Berg,P.(1973), J. Virology, in press.

(22) Morrow,J. and Berg,P.(1973), J. Virology, in press.