THE BINDING SITES FOR *ESCHERICHIA COLI* RNA POLYMERASE ON λ PHAGE DNA: VARIOUS TYPES OF BINDING SITES

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1. Introduction

λ DNA binds only a limited amount of RNA polymerase under appropriate conditions [1–4]. The number of binding sites on λ DNA for *E. coli* RNA polymerase which has so far been reported is between 10 and 20 [2–4]. On the other hand, the number of RNA species transcribed *in vivo* from λ DNA by a host polymerase and σ factor is estimated to be about 3 [5], which is confirmed by the *in vitro* experiments [3, 6]. These facts support the assumption that binding of RNA polymerase to a DNA molecule may not necessarily be followed by initiation of RNA synthesis [7], and suggest that various types of binding sites may exist on λ DNA.

Stead and Jones [8] suggested the presence of at least 2 types of binding by RNA polymerase to T7 DNA, one which is poorly dissociable and a more readily dissociable one. Formation of a "highly stable complex" between T7 DNA and RNA polymerase was demonstrated by Hinkle and Chamberlin [9]. In these studies, intact T7 DNA had been used to examine the interaction with RNA polymerase. Consequently, characterization of binding was rather indirect owing to the existence of many and, probably, qualitatively different binding sites on one molecule of DNA.

In order to characterize the binding sites more directly, I have studied the λ DNA–RNA polymerase interaction using the DNA fragments bearing only 1 or 2 binding site(s). The present communication shows the existence of 2 or 3 types of binding sites on λ DNA.

2. Methods

λ DNA was sheared by sonication as described previously [4]. RNA polymerase purified from *E. coli* cells [2] had a specific activity of about 8,000 units per mg protein and showed a single protein peak on gel filtration (Biogel A-1.5m in 0.2 M KCl) and on acrylamide gel electrophoresis. SDS gel electrophoresis of this preparation indicated the presence of σ factor. Complex formation between DNA and RNA polymerase was measured by the retention on Millipore filters (type HA, 0.45 μ) [10, 9]. The rate of filtration was carefully controlled by regulating the vacuum pressure between 1.8 and 2.0 cm of mercury.

3. Results

A preceding report from our laboratory [4] showed that fragmentation of λ DNA into about 30 pieces (fragments of about 1×10^6 daltons) could not change the number of binding sites per molecule of λ DNA. It was also indicated that only about half of these fragments can form a complex with RNA polymerase. Since the number of binding sites on λ DNA would be 12–14 [2–4], and since these sites would be distributed throughout a λ DNA molecule [4], it is assumed that the number of binding sites on a DNA fragment of 1×10^6 daltons would be at most only one or two. Therefore, it is likely that the binding sites on λ DNA can separately

be characterized by examining the fragment–RNA polymerase interaction.

A stable complex called an "initiation complex" which does not dissociate in the presence of high concentrations of salt is formed by the incubation of native DNA and RNA polymerase with purine nucleoside triphosphates [11–13]. Table 1 shows the formation of an "initiation complex" between the λ DNA fragment of 1×10^6 daltons and RNA polymerase. The fragment was mixed with an excess amount of RNA polymerase and ribonucleoside triphosphates as indicated. After incubation for 15 min at 37°, the mixture was brought to 0.4 M ammonium sulfate concentration and incubated for an additional 15 min. The amount of "initiation complex" formed was measured by the retention on Millipore filters. The course of complex formation was measured by varying the time of preincubation before addition of ammonium sulfate, and it was confirmed that the formation of an "initiation complex" is completed within 10 min at 37° under the conditions used. It is shown in table 1 that ATP or GTP alone, or UTP plus CTP does not cause significant retention of fragments. In contrast, about 10% of the fragments form an "initiation complex" in the presence of both ATP and GTP. The presence of UTP or CTP in addition to ATP and GTP has no effect on the amount of fragments retained. These results indicate that only one fifth of the fragments bearing the binding sites can form an "initiation complex", because it was indicated that about one half of the λ DNA fragments of 1×10^6 daltons forms a complex with RNA polymerase [4].

Table 2 shows the rate of RNA synthesis by the "initiation complex" measured in the presence of 0.5 M ammonium sulfate. The "initiation complex" formed in the presence of both ATP and GTP shows almost the same amount of RNA synthesis as in the presence of ATP, GTP, and UTP or CTP. These results are in good agreement with those presented in table 1.

Core polymerase lacking a σ factor also formed the same amount of "initiation complex" as that formed by the holoenzyme (measured by retention on Millpore filters), in which, however, core polymerase required higher concentrations of ATP and GTP than did the holoenzyme.

Hinkle and Chamberlin [9] reported that T7 DNA

Table 1
Formation of an "initiation complex" between the λ DNA fragment and RNA polymerase.

Ribonucleoside triphosphate(s) added	λ DNA retained (%)
ATP	2.2
GTP	3.4
ATP, GTP	11.2
ATP, GTP, CTP	10.3
ATP, GTP, UTP	9.4
UTP, CTP	3.0
None	1.3

The reaction mixture (0.2 ml) contained 40 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 0.1 M KCl, 0.28 μg of ^{33}P-labelled λ DNA (average molecular weight, 1×10^6 daltons), 1.2 units of RNA polymerase, and 0.25 mM ribonucleoside triphosphate(s). After 15 min at 37°, 0.05 ml of 2 M ammonium sulfate was added. The mixture was incubated for an additional 15 min and the amount of labelled λ DNA retained on a Millipore filter was determined.

Table 2
Rate of RNA synthesis in 0.5 M ammonium sulfate initiated by preincubation with ribonucleoside triphosphates in low salt.

Ribonucleoside triphosphates added during preincubation	^3H–CMP incorporated (nmoles/μg λ DNA) 5 min	15 min
None	0	0
UTP, CTP	0.04	0.10
ATP, GTP	0.15	0.27
ATP, GTP, CTP	0.14	0.30
ATP, GTP, UTP	0.14	0.32

The reaction mixture was the same as in table 1 except that 3.4 μg of intact λ DNA was used instead of the labelled fragment and 10 units of RNA polymerase was added in a final volume of 0.16 ml. After 15 min at 37°, 0.04 ml of 2.5 M ammonium sulfate was added. After an additional 15 min, ^3H-labelled CTP was added together with the lacking ribonucleoside triphosphate(s), and the amount of ^3H-CMP incorporated was measured.

can form a stable complex (a "highly stable complex") with *E. coli* RNA polymerase, depending on a σ factor and the temperature of incubation during complex formation. The results shown in fig. 1 indicate that such a "highly stable complex" can be formed between λ DNA and RNA polymerase. ^{3}H-labelled λ DNA was mixed with RNA polymerase and incubated for 30 min at 37°. Then, a 50-fold excess of unlabelled λ DNA was added. Incubation was continued at 37° and aliquots were removed at intervals and filtered. Assuming that RNA polymerase binds equally to labelled and unlabelled DNA after the addition of unlabelled DNA, then, only 1.5% or less of labelled λ DNA could be retained under the conditions used. The results obtained from the experiments with ^{3}H-labelled intact λ DNA (closed circles) indicate that a barely dissociable complex is formed between labelled DNA and RNA polymerase.

On the other hand, the results from the experiment using ^{3}H-labelled fragments of 1.6×10^6 daltons (open circles) clearly indicate that 2 types of binding exist. About 60% of the fragment is retained after incubation with an excess amount of polymerase (see [4]) and about two thirds of these complexes are dissociated within 30 min after the addition of unlabelled DNA. The remaining one third is shown to be less dissociable. These results suggest that out of 20 pieces of fragments (1.6×10^6 daltons) derived from one molecule of λ DNA (32×10^6 daltons), 8 pieces of fragments can form weak binding with RNA polymerase and 4 pieces can form a "highly stable complex".

It is of considerable interest to see whether the sites which can form a "highly stable complex" can also form an "initiation complex". To investigate this, ^{3}H-labelled fragments (1.6×10^6 daltons) were mixed with polymerase and incubated for 30 min at 37°. Then, a 150-fold excess of unlabelled λ DNA was added to remove weakly bound polymerase from fragments. After incubation for 30 min at 37°, ATP and GTP were added and the amount of "initiation complex" between fragments and polymerase was measured as described under table 1. Thus, the amount of fragments which can form not only a "highly stable complex" but also an "initiation complex" can be measured. The results presented in table 3 indicate that a part of the fragments which can form a "highly stable complex" can also form an "initiation complex". It is suggested that out of 20 fragments derived from one molecule of λ DNA, 4 fragments (20%) can form a ' highly stable complex", of which 2 (10%) would be able to form an "initiation complex". However, the amount of "initiation complex" measured without addition of unlabelled DNA differs between exp. 1 and exp. 3. This may be caused by the difference in experimental conditions that the complex was incubated for 1 hr at 37° before additions of ATP and GTP in exp. 1, while in exp. 3, purine nucleotides were added immediately after the mixing of fragments with polymerase. It seems possible from this that one other fragment forming an "initiation complex" may be present.

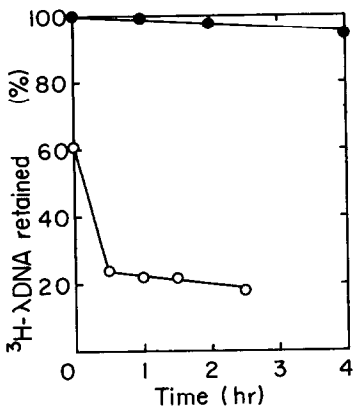

Fig. 1. Dissociation rate of the λ DNA–RNA polymerase complex. The reaction mixture (0.15 ml) contained 40 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 0.1 M KCl, 0.1 mM dithiothreitol, 30 μg of bovine serum albumin, 1.6 units of RNA polymerase, and 0.6 μg of ^{3}H-labelled intact λ DNA (●–●–●) or 0.24 μg of ^{3}H-labelled λ DNA of 1.6×10^6 daltons (o–o–o). After preincubation (30 min at 37°), an aliquot (25 μl) was removed at zero time. The remaining solution (125 μl) was immediately mixed with an equal volume of unlabelled λ DNA (30 μg) solution containing 40 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 0.1 M KCl, and 0.1 mM dithiothreitol, and incubated at 37°. Aliquots (50 μl) were removed at intervals and filtered.

Table 3
Relationship between a "highly stable complex" and an "initiation complex".

	Additions during incubation			^{3}H-λ DNA retained
	Unlabelled λ DNA	ATP and GTP	$(NH_4)_2SO_4$	(%)
Exp. 1	+	+	+	8.8
	–	+	+	9.8
Exp. 2	+	+	+	11.4
	+	+	–	19.3
Exp. 3	–	+	+	14.1

Formation of a "highly stable complex" between ^{3}H-labelled λ DNA of 1.6×10^6 daltons and RNA polymerase was carried out as in fig. 1. The mixture was incubated for 30 min at 37° after addition of excess unlabelled λ DNA, and then ATP and GTP were added to a final concentration of 0.25 mM. In exp. 3, however, purine nucleotides were added immediately after mixing the fragment with polymerase. The amount of "initiation complex" formed was measured as in table 1.

4. Discussion

The results presented in this paper reveal that: 1) of the λ DNA fragment forming a complex with RNA polymerase, one third can form a "highly stable complex", while two thirds form a less stable one, 2) only one fifth of the fragments bearing the binding sites can form an "initiation complex", and 3) one half or more of the fragments forming a "highly stable complex" would also form an "initiation complex". These results indicate that there are at least 3 types of binding between the fragments and RNA polymerase. The previous report from our laboratory [4] showed that fragmentation of λ DNA to about 30 pieces or less had no essential effect on the specificity of binding between λ DNA and RNA polymerase. Cohen et al. [14] reported that the regions of λ DNA transcribed *in vitro* were not significantly changed by shearing or sonication of the template. Therefore, the results presented in this paper are considered to indicate that various types of binding sites exist on λ DNA. Since a "highly stable complex" [9] as well as an "initiation complex" [15] would be related to the initiation of RNA synthesis, the presence of the fragment which can form another type of complex (a less stable one) than the above 2 suggests that the binding sites which can not make initiation of RNA synthesis would exist on λ DNA. Seifert [16] reported that T4 polymerase can initiate RNA synthesis on T4 DNA at one third of the initiation sites of *E. coli* polymerase, whereas it binds not only to the same number of sites on T4 DNA as *E. coli* polymerase but really the same sites. These facts seem to be favourable to a thinking that a different type of polymerase, probably, having the λ-specific σ factor [3] may be able to initiate RNA synthesis at the inactive binding sites on λ DNA where *E. coli* RNA polymerase can not initiate.

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