EFFECT OF ESCHERICHIA COLI DNA BINDING PROTEIN ON THE TRANSCRIPTION OF SINGLE-STRANDED PHAGE M13 DNA BY ESCHERICHIA COLI RNA POLYMERASE

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SUMMARY

E. coli DNA binding protein strongly inhibits the transcription of single-stranded rather than double-stranded phage M13 DNA by E. coli RNA polymerase. This inhibition cannot be significantly overcome by increasing the concentration of RNA polymerase. Nor does the order of addition of binding protein affect its inhibitory property: inhibition is evident whether binding protein is added before or after the formation of the RNA polymerase-DNA complex. Inhibition is also observed if binding protein is added at various times after initiation of RNA synthesis. Maximal inhibition occurs at a binding protein-to-DNA ratio (w/w) of about 8:1. This corresponds to one binding protein molecule covering about 30 nucleotides, in good agreement with values obtained by physical measurements.

The initiation of DNA replication on M13 ssDNA[1] requires an obligatory transcriptional reaction with E. coli RNA polymerase (1-3). The function of the DNA binding protein of E. coli [first isolated by Sigal et al. (4)] in M13 DNA replication is presumably to mask the M13 ssDNA in all but the specific region(s) where RNA polymerase can form the priming fragment (3,5). Binding protein also supports DNA polymerase III*-copolymerase III* in carrying out the

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[1]The abbreviations used are: ss, single-stranded; ds, double-stranded; RF I, replicative form I; BBOT, 2,5-bis[2-tert.-butylbenzoxazolyl]thiophene.

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DNA chain growth (3,5). It was of interest, therefore, to study the effect of DNA binding protein of E. coli on the transcription of ss phage M13 DNA by E. coli RNA polymerase.

MATERIALS AND METHODS

Substrates and Enzymes — Unlabeled ribonucleoside triphosphates were products of P-L Biochemicals, and ^{14}C-labeled ribonucleoside triphosphates were purchased from Schwarz/Mann. Bacteriophage M13 was purified through two CsCl density gradient centrifugations, and the DNA was isolated by phenol extraction following standard methods (6), then dialyzed against and stored in 0.01 M Tris (pH 7.8) buffer. The RF I was a kind gift from Dr. S. Mitra of this Division.

RNA polymerase from E. coli B was purified according to the method of Stevens (7), except that the final sucrose density gradient centrifugation step was replaced by the glycerol gradient centrifugation procedure of Burgess (8). The preparation was over 90% pure, as judged by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. It was free both of contaminating activities like polynucleotide phosphorylase and DNA polymerase and of detectable endonuclease activity, as measured by radioactively labeled RNA and DNA preparations as substrates for the release of acid-soluble radioactive material. The preparation did not introduce ss nicks in DNA, as measured by alkaline sucrose density gradient centrifugation.

DNA binding protein was purified from E. coli B according to the procedure described by Weiner et al. (5). The preparation was homogeneous, as evidenced by a single band during electrophoresis on polyacrylamide gels, and had a molecular weight of about 74,000 on native gels and about 18,500 under denaturing conditions, similar to the results of Weiner et al. (5). The protein thus appears to be a tetramer of four 18,500-dalton subunits.

Enzymatic Assays — The reaction mixture (0.2 ml) for measurement of RNA synthesis contained 20 mM Tris-HCl (pH 8.1), 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol, indicated amounts of M13 ssDNA (see figure legends), and 250 μM each of the four ribonucleoside triphosphates (UTP was labeled at 4.5 X 10^3 cpm/nmol). Indicated amounts of DNA binding protein were then added to the above reaction mixture and allowed to stand at 30° for 2 min. RNA synthesis was initiated by the addition of indicated amounts of RNA polymerase, and the isotope incorporated into acid-insoluble material was measured after a 10-min incubation period at 30°. The reaction was stopped by the addition of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate, and the solution was filtered through a Whatman glass paper (GF/C) disc. The disc was then washed extensively with the same solvent, followed by cold ethanol, then dried under an infrared heat lamp, placed in a scintillation vial, and counted with BBOT-toluene solution (4 g of BBOT in 1 liter of toluene) in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Effect of Binding Protein on Transcription of M13 ssDNA and dsDNA — Binding protein, even at very low concentrations, has a dramatic inhibitory effect on the transcription of M13 ssDNA as shown in Fig. 1. Maximal inhibition occurs at a binding protein-to-DNA ratio of about 8. In contrast,

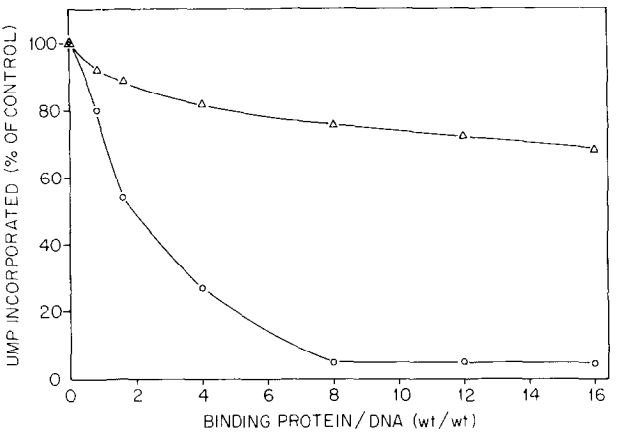

Fig. 1. Effect of increasing concentration of binding protein on the transcription of M13 ssDNA and dsDNA. Reaction conditions as described in "Materials and Methods." Isotope incorporated into acid-insoluble material was measured after a 10-min reaction at 30°. Open circles, 5 μg of M13 ssDNA with 10 μg of RNA polymerase; triangles, 2.5 μg of M13 RF 1 DNA with 5 μg of RNA polymerase.

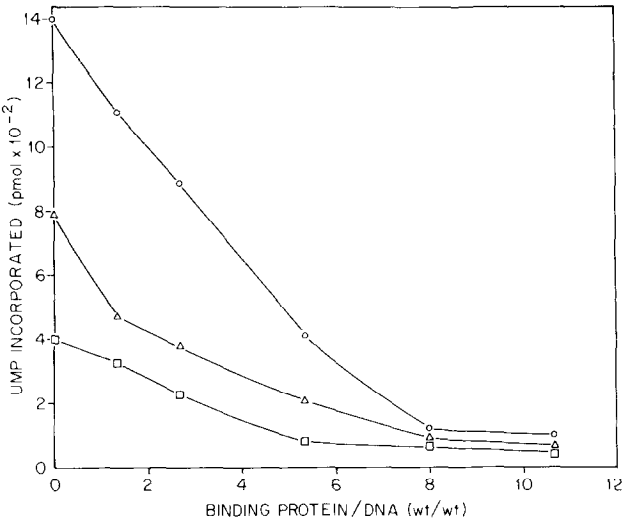

Fig. 2. Effect of increasing concentration of binding protein on transcription of M13 ssDNA with different amounts of RNA polymerase. Reaction conditions as described in "Materials and Methods." 2.5 μg of M13 DNA were used in each incubation mixture. Isotope incorporated into acid-insoluble material was measured after a 10-min reaction at 30°. Squares, 3 μg of RNA polymerase; triangles, 5 μg of RNA polymerase; circles, 10 μg of RNA polymerase.

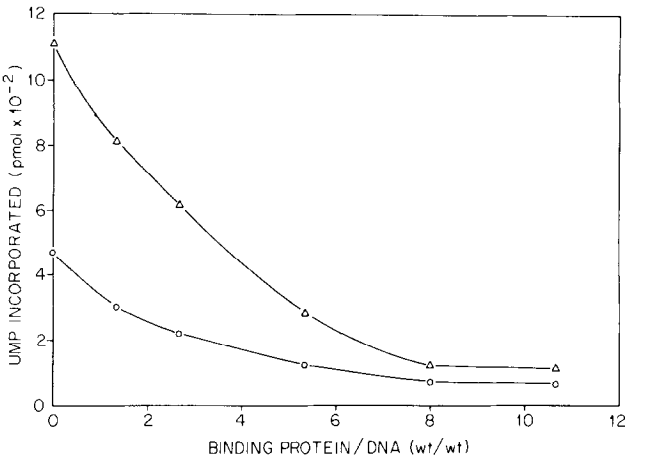

Fig. 3. Effect of increasing concentration of binding protein, added after the formation of the RNA polymerase-DNA complex, on the transcription of M13 ssDNA. RNA polymerase was added to the reaction mixture containing Tris, Mg^{2+}, 2-mercaptoethanol (as described in "Materials and Methods"), and 2.5 μg of M13 DNA. The mixture was incubated at 30° for 2 min, which is sufficient for pre-initiation complex formation (unpublished observation), followed by the addition of binding protein (as indicated in the figure) and a further incubation for 2 min at 30°. RNA synthesis was initiated by the addition of ribonucleoside triphosphates, and the isotope incorporated into acid-insoluble material was measured after a 10-min reaction at 30°. Circles, 5 μg of RNA polymerase; triangles, 10 μg of RNA polymerase.

the transcription of the M13 dsRF I DNA is inhibited to a much lesser extent by binding protein.

Effect of Enzyme Concentration — The inhibitory effect of binding protein cannot be significantly overcome by increasing the concentration of RNA polymerase (Fig. 2). Maximal inhibitions occur at a binding protein-to-DNA ratio of about 8. The same ratio was obtained when the reaction was carried out using different concentrations of M13 DNA. The effect of a great excess of RNA polymerase was studied in another experiment, in which a saturating amount of binding protein (40 μg) was bound to 5 μg of M13 DNA, and RNA synthesis was measured with different amounts of RNA polymerase. The percent inhibitions by binding protein with 10, 20, and 30 μg of RNA polymerase were 79, 77, and 76, respectively. Thus, even very large amounts of RNA polymerase were unable to substantially overcome the inhibitory effect of binding protein.

Effect of Adding Binding Protein After Formation of Enzyme-DNA Complex — It was of interest to investigate the effect of adding binding protein after

TABLE I

Inhibitory Effect of Binding Protein Added After Initiation of RNA Synthesis on M13 ssDNA

The assay mixture (0.2 ml) contained 20 mM Tris (pH 8.1); 10 mM $MgCl_2$; 20 mM 2-mercaptoethanol; ATP, CTP, GTP, and UTP (0.25 mM each); and 2.5 μg of M13 DNA. RNA synthesis was initiated (at 30°) by the addition of 5 μg of RNA polymerase. At indicated times, 20 μg of binding protein (saturing amount) were added along with [^{14}C]UTP to a final specific activity of 4.0 X 10^3 cpm/nmol. The isotope incorporated into acid-insoluble material was then measured after a 10-min incubation at 30°.

Time of addition of binding protein and [^{14}C]UTP, after initiation of RNA synthesis (min)	[^{14}C]UMP incorporated (pmol)
Control[a]	625
0	60
0.5	68
1.0	53
2.0	61
4.0	56

[a]No addition of binding protein. [^{14}C]UTP present from time zero.

RNA polymerase is bound to M13 DNA. These results, shown in Fig. 3, indicate that binding protein can inhibit the reaction even when RNA polymerase is first bound to M13 DNA. Again, maximal inhibition occurs at a binding protein-to-DNA ratio of about 8.

Effect of Adding Binding Protein After Initiation of RNA Synthesis — Table I shows the inhibitory effect of a saturing concentration of binding protein added at various times after initiation of RNA synthesis. This experiment was done in the absence of rifampicin, so that reinitiation could take place. Similar results were obtained in the presence of rifampicin. Thus, it appears that binding protein can effectively block the chain elongation step.

DISCUSSION

From the results presented above, it is clear that E. coli binding protein

has a dramatic inhibitory effect on the transcription of M13 ssDNA. The transcription of the M13 dsRF I DNA, on the other hand, is inhibited to a much smaller extent. This is expected, since E. coli binding protein preferentially binds to ssDNA (4,5).

The inhibition of transcription on M13 ssDNA cannot be substantially overcome by higher RNA polymerase concentration. The extent of inhibition depends on the ratio of binding protein to DNA, and maximal inhibition occurs at a binding protein-to-DNA (w/w) ratio of about 8. Since the molecular weights of the binding protein (5) and M13 ssDNA (9) are known, it can be calculated that, at saturation, about 200 molecules of binding protein bind per molecule of M13 ssDNA. Since the M13 genome is composed of about 6000 nucleotides (9,10), each binding protein molecule covers about 30 nucleotides. This value, obtained from enzymatic assays, agrees quite well with the value of 32 nucleotides obtained by Sigal et al. (4) using sucrose density gradient sedimentation and with the value of 30–36 nucleotides obtained by Weiner et al. (5) using a filter binding assay.

That binding protein inhibits transcription by restricting the binding of RNA polymerase to fewer sites on the M13 ssDNA has been confirmed by results presented by Schaller et al. (11) and by our own results (S. K. Niyogi and S. Mitra, to be published), which indicate that prior attachment of E. coli binding protein restricts the binding of RNA polymerase to specific site(s) on the M13 ssDNA. We are characterizing such sites in regard to length, base composition, secondary structure, and location on the M13 genome. Tabak et al. (12) and Geider and Kornberg (3) have shown that in the presence of E. coli binding protein, DNA replication is initiated from a unique site on the M13 ssDNA; in the absence of binding protein or in the presence of spermidine, initiation takes place from multiple sites.

It is interesting that the order of addition of binding protein did not affect its inhibitory property: inhibition is evident whether binding protein is added before or after the formation of the RNA polymerase–DNA complex. The extents of inhibition by binding protein in the two cases are also similar. In both cases, maximal inhibition occurs at a binding protein-to-DNA (w/w) ratio of about 8. The inhibition by binding protein after formation of the enzyme-DNA complex suggests that binding protein can also inhibit at some step subsequent to preinitiation complex formation. That this is true is confirmed by results showing inhibition by binding protein added at various times after initiation of RNA synthesis, in either the absence or presence of rifampicin. Thus, it appears that binding protein can also effectively inhibit transcription at the step of chain elongation. This is evidently achieved both by preventing free

RNA polymerase molecules from re-associating with the DNA and by preventing bound RNA polymerase molecules from further extension of RNA chains.

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