

Inhibition of the Type I Restriction-modification Enzymes *EcoB* and *EcoK* by the Gene *0-3* Protein of Bacteriophage T7

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The gene *0-3* protein of bacteriophage T7 is a potent inhibitor of the restriction-modification enzymes *EcoB* and *EcoK*, both *in vivo* and *in vitro*. We have analyzed the ability of purified *0-3* protein to inhibit different steps in the reactions of *EcoB* and *EcoK* with DNA. Most of our experiments were done with *EcoK*, but selected tests with *EcoB* indicate that the two enzymes are affected by *0-3* protein in the same way. Purified *0-3* protein binds tightly to free enzyme, apparently to one of the small subunits, and prevents it from binding to DNA. If *EcoK* is allowed to form specific recognition complexes with unmodified DNA before *0-3* protein is added, relatively low levels of *0-3* protein prevent the nuclease activity that would otherwise appear upon addition of ATP, but considerably higher levels are needed to prevent formation of filter-binding complexes or ATPase activity. This, together with other results, suggests that the binding site for *0-3* protein is protected in recognition complexes and in the early stages of the ATP-stimulated reactions, but that it becomes accessible again before cleavage of the DNA, perhaps after the translocation step. If added after the nuclease reaction is substantially over, *0-3* protein has little effect on ATPase activity, and indeed, the subunit having the binding site for *0-3* protein apparently dissociates from the enzyme-DNA complex. The methylase activity of *EcoK* on hemi-methylated recognition sites is strongly inhibited by *0-3* protein added at any stage of the reaction.

1. Introduction

EcoB and *EcoK*, in a complex set of reactions, modify the DNA that is resident in their own cell but degrade unmodified foreign DNA (see review by Yuan, 1980; Endlich & Linn, 1981). The DNA of bacteriophage T7 contains six recognition sites for *EcoB* and four for *EcoK* (Dunn & Studier, 1983). However, when T7 infects *Escherichia coli* B or K-12, its DNA is neither degraded nor methylated by *EcoB* or *EcoK*, because gene *0-3*, the first T7 gene, directs the synthesis of a protein that prevents both

the restriction and modification activities (Studier, 1975). The *0-3* protein is a small protein of 116 amino acids, and it has been purified and shown to inactivate the nuclease and ATPase activities of *EcoB in vitro* (Mark & Studier, 1981; Dunn *et al.*, 1981). The inhibition requires stoichiometric rather than catalytic amounts of *0-3* protein, suggesting that inactivation occurs by binding. Indeed, the *0-3* protein is very acidic, which raises the possibility that it could compete for the DNA-binding site of the restriction enzymes. We have explored further the interaction between purified *0-3* protein and *EcoB* and *EcoK*, and the effects of this interaction on different stages of the complex interaction between these restriction enzymes and DNA.

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2. Materials and Methods

(a) DNAs and proteins

(i) DNA

Wild-type T7 DNA and lambda *cI857 Sam7* DNA were prepared by extraction of purified phage particles with phenol, and pBR322 DNA was prepared essentially as described by Clewell & Helinski (1969). Random heteroduplexes between *EcoK*-modified and unmodified lambda DNA were prepared by denaturing an equal mixture of the DNAs in 0.5 M-NaOH and renaturing at 10 µg/ml in 100 mM-Tris·HCl (pH 7.4), 100 mM-NaCl, 1 mM-EDTA and 50% formamide for 12 h at 30°C. The renatured DNA was precipitated with ethanol and dissolved in 10 mM-Tris·HCl (pH 8.0), 10 mM-NaCl, 0.5 mM-EDTA at a final concentration of 50 to 100 µg/ml. Using molecular weights calculated for the sodium salts of T7 DNA (26.4×10^6 ; Dunn & Studier, 1983), lambda DNA (32.1×10^6 ; Sanger *et al.*, 1982) and pBR322 DNA (2.9×10^6 ; Sutcliffe, 1979), 100 ng of these DNAs would contain 2.3×10^9 , 1.9×10^9 , or 2.1×10^{10} molecules, respectively.

(ii) *EcoB* and *EcoK*

The preparation of *EcoB* used in these experiments has been purified through the DEAE concentration step (Eskin & Linn, 1972a), and was a kind gift from S. Linn. This is the same as the preparation used by Mark & Studier (1981). It had a protein concentration of about 2 mg/ml, 1 to 2% of which was estimated to be *EcoB*. *EcoK* was purified as described by Yuan *et al.* (1980). Three different preparations of *EcoK* were used, which we designate *EcoK*-1, *EcoK*-2 and *EcoK*-3. *EcoK*-1 is the same as the preparation described by Yuan *et al.* (1980): it had a protein concentration of 64 µg/ml and was essentially pure *EcoK*. Using a molecular weight of 450,000 for *EcoK* (Meselson *et al.*, 1972), the preparation is calculated to contain 8.5×10^{10} molecules of *EcoK* per µl. *EcoK*-2 had a protein concentration of about 30 µg/ml, 30 to 40% of which was estimated by gel electrophoresis to be *EcoK*, and *EcoK*-3 had a protein concentration of about 300 µg/ml, 10 to 20% of which was estimated to be *EcoK*. Based on apparent concentrations of *EcoB* or *EcoK* subunits observed by gel electrophoresis, and the relative activities in cutting DNA, we estimate that the preparation of *EcoB* contained about 2×10^{10} molecules of active enzyme per µl, *EcoK*-1 about 8.5×10^{10} /µl, *EcoK*-2 about 10^{10} /µl, and *EcoK*-3 about 5×10^{10} /µl. These are the numbers we used to estimate stoichiometries between *EcoB* or *EcoK* and *0-3* protein or DNA, and we think they are likely to be accurate to within a factor of 2.

(iii) *0-3* protein

0-3 protein was prepared from extracts of *E. coli* B834 that had been infected with the T7 triple mutants H3, *am193*, *LG3*, essentially as described by Mark & Studier (1981). Crude extracts were loaded onto a column of DEAE-cellulose in buffer containing 0.3 M-NH₄Cl, conditions where virtually all of the protein retained by the column is *0-3* protein, and the *0-3* protein was eluted by a gradient of NH₄Cl from 0.3 M to 1 M. [³⁵S]methionine-labeled *0-3* protein (1 µCi/µg *0-3* protein) was radiochemically >99% pure at this stage, and the peak column fractions were pooled, dialyzed against 50 mM-Tris·HCl (pH 8), and used directly to test for binding to *EcoB* and *EcoK*. Peak fractions of unlabeled *0-3* protein were further purified, essentially to homo-

geneity, by two or more cycles of precipitation with trichloroacetic acid and solubilization with ethanol (Mark & Studier, 1981). The ratio of absorbance at 280 to 260 nm in such preparations is near 2, and the concentration of *0-3* protein in a solution having an absorbance of 1.0 at 280 nm is about 0.75 mg/ml, as estimated from amino acid analyses of samples of known absorbance. Using a calculated molecular weight of 13,678 for *0-3* protein (Dunn *et al.*, 1981), 1 ng would contain 2.2×10^{10} molecules of dimer *0-3* protein.

(b) Assays for *EcoB* and *EcoK* activities

(i) Assay buffer

Buffers used to assay activities of *EcoB* and *EcoK* typically contained 50 to 100 mM-Tris·HCl or HEPES buffer (pH 8.0), 6 mM-MgCl₂, 0.5 mM-EDTA, 12 mM-mercaptoethanol, and sometimes 50 µg bovine serum albumin per ml. When present, the concentration of AdoMet† was typically 20 µM, and that of ATP was 400 µM.

(ii) Binding of DNA to filters

The ability of *EcoB* or *EcoK* to bind labeled DNA to nitrocellulose filters was assayed essentially as described by Meselson & Yuan (1971).

(iii) Gel filtration to measure binding of *EcoK* to DNA.

Reaction mixtures of 50 µl contained 1.5 µg T7 DNA (3.5×10^{10} molecules) and 20 µl of *EcoK*-3 (estimated to be 10^{12} molecules), a ratio of approximately 30 molecules of *EcoK* per molecule of T7 DNA. When added to the reaction, 3 µg of *0-3* protein contained 6.6×10^{13} dimer molecules, or an estimated 66 per molecule of *EcoK*. Reactions without *0-3* protein were for 10 min; reactions to which *0-3* protein was added were 10 min before and 5 min after addition of the *0-3* protein. All binding reactions contained AdoMet. Reactions without ATP were incubated at room temperature; reactions in the presence of ATP were incubated at 37°C. The entire reaction mixture was applied to a 1 ml column of Bio-Gel A-5m (BioRad) that had been equilibrated with 100 mM-Tris·HCl (pH 8), 6 mM-MgCl₂, 0.26 mM-EDTA, 12 mM-mercaptoethanol. The column was eluted with the same buffer, collecting 2-drop fractions (45 µl). To each fraction was added 12 µl of a solution of 5% sodium dodecyl sulfate, 40% glycerol, 5% mercaptoethanol, and the resulting mixture was heated for 5 min at 65°C and analyzed by electrophoresis in a Tris·HCl-glycine stacking buffer system in the presence of 0.1% sodium dodecyl sulfate on a 10% to 20% gradient polyacrylamide slab gel with a 5% stacking gel (Studier, 1973). The gel was stained with ethidium bromide to locate the DNA and then stained with Coomassie blue to visualize the protein bands.

(iv) Endonuclease assays

Degradation of DNA was analyzed by electrophoresis on agarose gels. DNA was visualized by staining the gel with ethidium bromide solution and photographing the fluorescence induced by ultraviolet light.

(v) Electron microscopy

Enzyme-DNA complexes were fixed by the addition of an equal volume of 0.2% glutaraldehyde. Samples were

† Abbreviation used: AdoMet, *S*-adenosyl-methionine.

prepared procedure

(vi) ATPa

Hydrolytic conversion chromatography release of ability to

(vii) Methy

Transfer heteroduplex modified Burekhard

(a) Bi

Inactive protein a than cate Studier, inactivating restriction directly, allowed to a variety of *0-3* protein by gel filtration denaturing, both assays, informative

As shown in itself (lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100). Antibody the label confirming preparatio with T7 I labeled bands does not as when the *EcoB* or *E* to position proteins 0 (lanes 3, 1 two major identified, bound to c the pattern (lane 17), bands may

According dimer *0-3* I shown in I the reactive than one restriction protein, but molecule of *EcoK* (sam)

prepared for electron microscopy by the protein-free procedure described previously (Yuan *et al.*, 1980).

(vi) ATPase assays

Hydrolysis of ATP was measured either by following the conversion of [³H]ATP to ADP, using thin-layer chromatography (Yuan *et al.*, 1972), or by following the release of ³²PO₄ from [³²P]ATP, as measured by loss of ability to adsorb to Norit (Eskin & Linn, 1972a).

(vii) Methylase assays

Transfer of ³H from [methyl-³H]AdoMet into random heteroduplex DNA (between EcoK-modified and unmodified lambda DNA) was measured as described by Burckhardt *et al.* (1981).

3. Results

(a) Binding of 0·3 protein to EcoB and EcoK

Inactivation of EcoB and EcoK by purified 0·3 protein appears to require stoichiometric rather than catalytic amounts of 0·3 protein (Mark & Studier, 1981; this work), indicating that inactivation is due to binding of 0·3 protein to the restriction enzyme. In order to test for such binding directly, purified ³⁵S-labeled 0·3 protein was allowed to react with purified EcoB or EcoK under a variety of conditions, and association between the 0·3 protein and the restriction enzymes was assayed by gel filtration or by gel electrophoresis under non-denaturing conditions. Binding was detected in both assays, but gel electrophoresis was the more informative.

As shown in Figure 1, the labeled 0·3 protein by itself (lanes 1, 11 and 19) migrated largely as a single band near the bottom of the gel, with perhaps minor amounts of higher aggregates. Antibody against purified 0·3 protein caused all of the label to be found at the origin (lane 16), confirming the radiochemical purity of the preparation. Incubation of the labeled 0·3 protein with T7 DNA did not affect the mobility of the labeled bands (lane 10), indicating that 0·3 protein does not associate strongly with T7 DNA. However, when the labeled 0·3 protein was incubated with EcoB or EcoK before electrophoresis, label moved to positions on the gel that would correspond to proteins of considerably higher molecular weight (lanes 3, 12, 13, 17 and 18). In the case of EcoK, two major and two minor bands of label can be identified, which apparently represent 0·3 protein bound to different forms of the restriction enzyme; the pattern of binding to EcoB was similar (lane 17), although the mobilities of the labeled bands may have been slightly different.

According to our estimates, the molar ratio of dimer 0·3 protein to EcoB or EcoK in the reactions shown in Figure 1 was 0·34, 1·7, 4·3 or 8·5. All of the reactions that were estimated to have more than one molecule of dimer 0·3 protein per restriction enzyme had residual unbound 0·3 protein, but the one reaction estimated to have one molecule of dimer 0·3 protein per three molecules of EcoK (sample 18) had no free 0·3 protein. Although

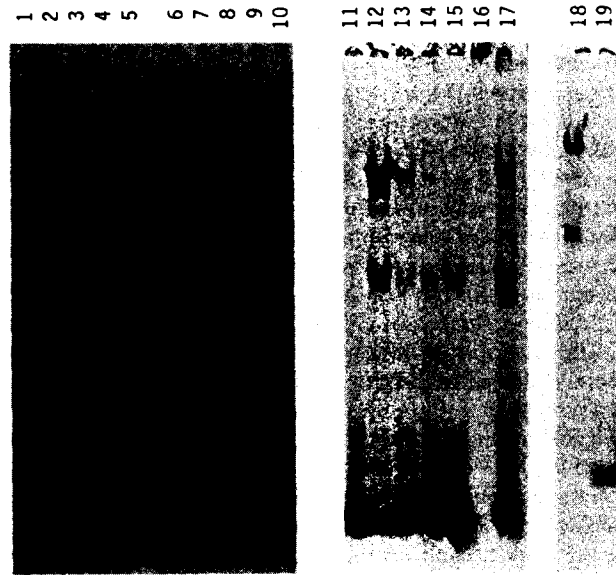


Figure 1. Binding of labeled 0·3 protein to EcoK and EcoB. [³⁵S]methionine-labeled 0·3 protein was incubated in nuclease assay buffer plus 10% glycerol, with or without EcoK or EcoB, AdoMet, ATP or DNA in various combinations. Reaction mixtures (30 µl) were incubated at room temperature, and for 10 min after the last addition. At the end of the incubation, the reaction mixtures, without any denaturing treatment, were applied directly to a 3% to 10% gradient polyacrylamide slab gel, and subjected to electrophoresis in 40 mM-Tris acetate (pH 8). The gel was dried, and the label was visualized by autoradiography. Electrophoresis of samples 1 to 10 was for 15 h at 35 V, and of the remainder for 2 h at 150 V, which accounts for the difference in sharpness of the labeled bands. (No sample was analyzed in the lane between samples 5 and 6.) Each reaction mixture contained an estimated 7·5 ng of labeled 0·3 protein ($1·7 \times 10^{11}$ dimer molecules). Reactions 5 to 8 and 10 contained 500 ng of T7 DNA ($1·2 \times 10^{10}$ molecules) and 14 to 15 contained 50 ng; reaction 9 contained 775 ng of K-modified lambda DNA ($1·5 \times 10^{10}$ molecules). EcoK was used at three different levels: the samples in lanes 2 to 9 and 12 contained 10 µl of EcoK-2 (10^{11} molecules); those in lanes 13 to 15 contained 2 µl of EcoK-2 (2×10^{10} molecules); and that in lane 18 contained 10 µl of EcoK-3 (5×10^{11} molecules). The sample in lane 17 contained 2 µl of EcoB (4×10^{10} molecules). The estimated molar ratios of 0·3 protein dimer to restriction enzyme in the different reactions are given in parentheses. The reactions contained the following components: 1, 0·3; 2, 0·3 + EcoK (1·7 : 1) + AdoMet; 3, 0·3 + EcoK (1·7 : 1); 4, 0·3 + 100 ng unlabeled 0·3 + EcoK (24 : 1) + AdoMet; 5, 0·3 + EcoK (1·7 : 1) + AdoMet (5 min); + T7 DNA; 6, 0·3 + EcoK (1·7 : 1) (5 min); + T7 DNA; 7, EcoK + T7 DNA + AdoMet + ATP (5 min); + 0·3 (1·7 : 1); 8, EcoK + T7 DNA + AdoMet (5 min); + 0·3 (1·7 : 1); 9, EcoK + K-modified lambda DNA + AdoMet (5 min); + 0·3 (1·7 : 1); 10, 0·3 + T7 DNA + AdoMet; 11, 0·3; 12, 0·3 + EcoK (1·7 : 1); 13, 0·3 + EcoK (8·5 : 1); 14, EcoK + T7 DNA + AdoMet (15 min); + 0·3 (8·5 : 1); 15, EcoK + T7 DNA + AdoMet + ATP (15 min); + 0·3 (8·5 : 1); 16, 0·3 + antibody to 0·3; 17, 0·3 + EcoB (4·3 : 1); 18, 0·3 + EcoK (0·34 : 1); 19, 0·3 + 3 µg unlabeled 0·3.

there is some uncertainty in the estimates of the concentration of *EcoB* and *EcoK*, these results suggest that binding of *O*-3 protein to *EcoB* and *EcoK* is relatively strong, and that perhaps only one or two molecules of dimer *O*-3 protein bind to each molecule of *EcoB* or *EcoK*.

EcoB has been isolated in several forms, containing subunits of molecular weight 135,000, 60,000 and 55,000 in various proportions (Eskin & Linn, 1972a), and *EcoK* has been isolated in a single form containing subunits of 135,000, 62,000 and 52,000 in a ratio of 2 : 2 : 1 (Meselson *et al.*, 1972). Mobilities of the labeled bands in Figure 1 relative to oligomers of bovine serum albumin analyzed in the same gel (not shown) are consistent with the largest, most intensely labeled band in both *EcoB* and *EcoK* being a holoenzyme of molecular weight close to 450,000. The smallest band, which is also well labeled, migrates slightly behind monomer bovine serum albumin, consistent with it being one of the two small subunits of the enzyme plus bound *O*-3 protein. The two minor bands just ahead of the largest band may represent small amounts of other forms of the enzyme, perhaps partially dissociated.

Apparently, the binding site for *O*-3 protein in both *EcoB* and *EcoK* is in one of the small subunits of the enzyme. It seems likely that *O*-3 protein binds whether the small subunit is free or is complexed with other subunits, although our experiments did not determine whether the dissociated small subunit and the apparently partially dissociated forms of the enzymes pre-existed in the preparation or were the consequence of binding of the *O*-3 protein. If binding of *O*-3 protein does promote dissociation of the small subunit from the *EcoB* and *EcoK* holoenzymes, the reaction is apparently not very rapid under the conditions we used.

(b) Binding of *O*-3 protein at different stages of the reaction of *EcoK* with DNA

We investigated the binding of *O*-3 protein to *EcoK* at various stages in the reaction of *EcoK* with DNA. In order to bind specifically to its recognition sequence in DNA, *EcoK* must first be activated by binding AdoMet (Yuan *et al.*, 1975; Bickle *et al.*, 1978). Apparently, *O*-3 protein binds to free *EcoK* whether or not the *EcoK* has been activated by AdoMet, since the pattern of binding was the same whether or not AdoMet was present in the reaction mixture (Fig. 1, lanes 2 and 3). When DNA was added after the *O*-3-*EcoK* complexes had been allowed to form, there was no apparent effect on the pattern of label in the presence of AdoMet (lanes 2 and 5), but there was a significant shift of label from the position of holoenzyme to that of the small subunit in the absence of AdoMet (lanes 3 and 6). This shift of label suggests that addition of DNA in the absence of AdoMet may promote or accelerate dissociation of the small subunit and its bound *O*-3 protein from the holoenzyme.

EcoK that has been activated by AdoMet can bind to DNA specifically at its recognition site and

also non-specifically; but further reaction requires ATP (Bickle *et al.*, 1978). When such binding of *EcoK* to unmodified T7 DNA was allowed to take place before labeled *O*-3 protein was added, a substantial decrease in the amount of label at the holoenzyme position was observed (compare lane 2 with 8, and lane 13 with 14). This decrease was not accompanied by a comparable increase in label at the subunit position or near the origin of electrophoresis, where the *EcoK*-DNA complexes would be expected to migrate. This suggests that the binding of *EcoK* to unmodified DNA inhibits its ability to bind *O*-3 protein. The simplest assumption would be that the binding site for *O*-3 protein is masked when *EcoK* binds specifically to unmodified DNA. An excess of *EcoK* molecules over recognition sites was present in these experiments (estimated to be twofold in sample 8 and fourfold in sample 14). If only those *EcoK* molecules specifically bound at the recognition site were prevented from binding *O*-3 protein, and if only one *EcoK* could bind at each recognition site, the excess of *EcoK* over recognition sites would account for the label found at the holoenzyme position. Given the uncertainties in estimating the number of active *EcoK* molecules, the magnitudes of the observed decreases in label at the holoenzyme position seem to be in acceptable agreement with this idea.

The inhibition of binding of *O*-3 protein to *EcoK* found when *EcoK* binds to unmodified DNA is not observed with modified DNA. When *EcoK* was allowed to react with fully modified lambda DNA in the presence of AdoMet before *O*-3 protein was added, the labeling pattern was essentially identical to that obtained when no DNA was present (lanes 2 and 9). This suggests that binding of *EcoK* to modified DNA is weak enough for all of it to be dissociated and trapped by *O*-3 protein during the course of the reaction. It has been shown previously (Yuan *et al.*, 1975) that *EcoK* dissociates more rapidly from modified than from unmodified DNA. An alternative possibility is that *O*-3 protein can bind to *EcoK* that has complexed with modified DNA and cause it to dissociate, but cannot bind to *EcoK* that has complexed with unmodified DNA.

When ATP as well as AdoMet is added to *EcoK* and unmodified DNA, the DNA is rapidly degraded and there is extensive ATPase activity (Meselson & Yuan, 1968; Yuan *et al.*, 1972; Bickle *et al.*, 1978). Addition of labeled *O*-3 protein after the nuclease reaction was essentially complete (lanes 7 and 15) revealed yet another pattern of labeling: little or no label at the position of holoenzyme or at the origin of electrophoresis, but a substantial increase in label found at the subunit position. This pattern suggests that the subunit of *EcoK* that binds *O*-3 protein may dissociate from the *EcoK*-DNA complex at some stage during the ATP-stimulated reactions of the enzyme, but these experiments do not distinguish whether this dissociation is part of the normal reaction of *EcoK* with DNA or occurs only in the presence of *O*-3 protein.

(c) *Effect of 0.3 protein on ability of EcoB and EcoK to bind DNA to filters*

A simple assay for activity of *EcoB* or *EcoK* is ability to bind labeled DNA to nitrocellulose filters (Meselson & Yuan, 1971). We have examined the effect of 0.3 protein on the ability of *EcoB* and *EcoK* to form filter-binding complexes with unmodified T7 and lambda DNAs. The most extensive analyses were done with *EcoK*, but essentially equivalent results were obtained with *EcoB* in whatever reactions were tested. Representative results are given in Table 1.

As expected, *EcoK* in the presence of AdoMet and ATP reacted with DNA to form a complex that was retained by nitrocellulose filters (Table 1, experiments 3 and 5). Under the same conditions, 0.3 protein by itself was incapable of binding DNA to filters (Table 1, experiment 2), consistent with the experiments using labeled 0.3 protein, where no binding to DNA was observed (Fig. 1, lane 10). When 0.3 protein was added to the reaction mixture immediately before *EcoK*, it prevented filter binding (Table 1, experiment 4). Even when 0.3 protein was added immediately after *EcoK*, most of the DNA remained unbound (not shown).

As in the previous section, the effect of adding 0.3 protein at various stages of the reaction of *EcoK* with DNA, was analyzed. When *EcoK*-DNA complexes were formed in the presence of AdoMet, addition of a large excess of 0.3 protein (100 molecules per *EcoK*) prevented filter binding when ATP was added (Table 1, experiment 6). Other experiments (not shown) found that considerably lower ratios of 0.3 protein to *EcoK* (less than 10) were only partially effective in preventing pre-formed complexes from binding to filters when ATP

was added, even though they did prevent nuclease activity. However, when ATP was added just 20 seconds before 0.3 protein, DNA was bound to the filter about as efficiently as if no 0.3 protein had been added, even in the presence of a 100-fold excess of 0.3 protein (Table 1, experiment 7). Apparently, high levels of 0.3 protein can prevent the initial *EcoK*-DNA complexes from binding to filters, but once some reaction that requires ATP begins, even high levels of 0.3 protein cannot prevent filter binding.

These results are entirely consistent with those of the previous sections. The efficient binding of 0.3 protein to free holoenzyme prevents it from forming filter-binding complexes. Once *EcoK* has bound to DNA in the presence of AdoMet, binding of 0.3 protein is interfered with, and relatively high levels of 0.3 protein are needed to prevent the formation of filter-binding complexes. When added after ATP, even high levels of 0.3 protein do not prevent filter binding. This would be consistent with the observation that the subunit that binds 0.3 protein seems to dissociate in the presence of ATP. If the remaining subunits were still bound to the DNA and were capable of binding to filters, 0.3 protein would be unable to prevent filter binding.

(d) *Effect of 0.3 protein on binding of EcoK to DNA, as measured by gel filtration*

The association of *EcoK* and its subunits with unmodified T7 DNA and the effect of 0.3 protein on this association were also measured by gel filtration of DNA-protein complexes, followed by polyacrylamide gel electrophoresis of the column fractions in the presence of sodium dodecyl sulfate, as described

Table 1
Effect of 0.3 protein on the binding of DNA to filters by *EcoK*

Expt	First incubation	Second	Third	DNA bound to filters	
				(cts/min)	(%)
1	DNA + AdoMet + ATP	—	—	2570	5.6
				3030	6.6
2	DNA + AdoMet + ATP + 0.3	—	—	3410	7.4
				4560	9.9
3	DNA + AdoMet + ATP + <i>EcoK</i>	—	—	27,750	60.4
				30,000	65.4
4	DNA + AdoMet + ATP + 0.3 + <i>EcoK</i>	—	—	530	1.1
				560	1.2
5	DNA + AdoMet + <i>EcoK</i>	ATP	—	18,940	41.2
6	DNA + AdoMet + <i>EcoK</i>	0.3	ATP	1250	2.7
				1200	2.6
7	DNA + AdoMet + <i>EcoK</i>	ATP	0.3	29,100	63.3
				37,800	82.3

Reaction mixtures (50 μ l) contained 100 ng of 32 P-labeled unmodified lambda DNA (1.9×10^9 molecules) and 2 μ l *EcoK*-1 (1.7×10^{11} molecules), a ratio of 90 *EcoK* molecules per DNA molecule. Where indicated, 880 ng of 0.3 protein (1.9×10^{13} molecules of dimer) were added, giving a ratio of 110 molecules of 0.3 protein per *EcoK*. The first 4 reactions were incubated for 2 min at 30°C. The first incubation of experiments 5, 6 and 7, was for 3 min; addition of 200 μ M-ATP in the second or third steps was followed by a 20 s incubation, while addition of 0.3 protein was followed by a 1 min incubation.