Proc. Natl. Acad. Sci. USA
Vol. 73, No. 11, pp. 3989–3993, November 1976
Biochemistry

Colicin E2 is a DNA endonuclease

(colicin-E2-immunity protein/colicin E3/colicin-E3-immunity protein)

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Communicated by Henry Lardy, September 10, 1976

ABSTRACT **Colicin E2 purified by conventional methods contains a tightly bound low-molecular-weight protein, as has been found with purified colicin E3 [Jakes, N. & Zinder, N. D. (1974) *Proc. Natl. Acad. Sci. USA* 71, 3380–3384]. Such E2 preparations do not cause DNA cleavage *in vitro*. After separation from the low-molecular-weight protein, colicin E2 retained the original *in vivo* killing activity, and in addition showed a high activity *in vitro* in cleaving various DNA molecules, such as a ColE1 hybrid plasmid and DNAs from *Escherichia coli*, λ phage, φX174 phage, and simian virus 40. The low-molecular-weight protein ("E2-immunity protein") specifically prevented this *in vitro* DNA cleavage reaction, i.e., had an "immunity function." The results demonstrate that colicin E2 itself is a DNA endonuclease and explain the *in vivo* effects caused by E2 in sensitive cells as well as the mechanism of immunity in E2-colicinogenic cells.**

Colicin E2 (E2) is a protein antibiotic synthesized by certain strains of coliform bacteria that carry the ColE2 plasmid, and is classified in the E group of colicins along with colicins E1 and E3 (1, 2). Although the physical properties of E2 and E3 are similar (3) and both colicins share the same receptor (1, 2), their apparent mode of action is different. It was first discovered that E2 causes degradation of DNA, while colicin E3 (E3) causes specific inhibition of protein synthesis in sensitive *Escherichia coli* cells treated with these colicins (4). Subsequent work on the mode of action of E3 has demonstrated that E3 inactivates ribosomes in treated cells (5) by causing the cleavage of a fragment from the 3′ end of the 16S RNA (6, 7). The same cleavage reaction has also been demonstrated *in vitro* using "purified" E3 protein (see below) and purified ribosomes (8, 9). This *in vitro* demonstration of E3 action has strongly indicated that E3 acts directly on ribosomes rather than indirectly and that E3 itself is an RNase with a very stringent substrate specificity.

In contrast to E3, the mechanism of action of E2 has not been clear. Since the initial discovery of DNA degradation in E2-treated sensitive cells (4), several authors have confirmed and extended the original observations on DNA cleavage reactions (10–13). However, others have suggested that the primary action of E2 is not on the cellular DNA, but on some other targets such as membranes (14) or tRNA (15).

Since E2 has properties similar to E3 (3), and E3 causes ribosome inactivation *in vitro*, one might expect some kind of effects of E2 on cellular DNA *in vitro*, if DNA is in fact the primary target of E2. Several attempts have been made to demonstrate such effects *in vitro* using purified E2 (e.g., refs. 16–19). However, published results were either negative or too uncertain to allow a definitive conclusion regarding such direct effects.

Recently, Jakes and Zinder observed that the "purified" E3 preparations obtained with the conventional purification methods (3) contained about one molar equivalent of the E3-immunity protein (20). These authors demonstrated that the immunity protein could be separated from the E3 protein by preparative electrophoresis in sodium dodecyl sulfate/polyacrylamide gels and that E3 protein (called "E3*") free of immunity protein was much more active than the "complexed" E3 preparation in ribosome inactivation *in vitro* (20). They also noted the presence of a small-molecular-weight protein in highly purified E2 preparations (cited in ref. 20). From the analogy of the colicin E3–immunity protein complex, they inferred that this small-molecular-weight protein is probably the E2-immunity protein. We have now succeeded in separating colicin E2 (called "E2*") from the low-molecular-weight protein and demonstrated that E2* thus obtained is highly active in degrading DNA *in vitro* and that the small-molecular-weight protein prevents this DNase activity, i.e., has an "immunity function."

MATERIALS AND METHODS

E. coli PR13, which lacks RNase I, was used as the colicin-sensitive strain. Colicins E3 and E2 were purified from mitomycin-C-induced cultures of *E. coli* CA38 and W3110 (ColE2), respectively, according to the method described by Herschman and Helinski (3). The colicins obtained were still complexed with immunity proteins (cf. ref. 20). E3-immunity protein was previously purified by J. Sidikaro in this laboratory (21). Pure E2-immunity protein was obtained as described below.

Colicin E2* was separated from the immunity protein by dissociation of the complexed E2 with guanidine·HCl at about 4°. Lyophilized E2 was dissolved in dissociation buffer (1 mg/ml). The dissociation buffer contained 6 M guanidine·HCl (Grade I, Sigma Chemical Co.), 0.2 M NaCl, 1 mM dithiothreitol, and sufficient 1 M K_2HPO_4 to adjust the pH to 6.8. After the incubation for 1 hr at 4°, the solution was subjected to one of two methods to obtain a complete separation of E2* from the immunity protein. In the first method, the solution was applied to a Sephadex G-100 "superfine" column equilibrated with the dissociation buffer and the two proteins were separated. In the second method, the solution was filtered through a PM30 membrane (Amicon) using a 50 ml stirred cell. The protein left in the Amicon cell (E2*) was diluted with the dissociation buffer and the filtration process was repeated. After five such processes, the E2* preparation was diluted in a solution of 6 M urea and 0.2 M NaCl, concentrated again to about 2 mg/ml of protein, and dialyzed against 0.1 M sodium phosphate, pH 7.0, containing 0.2 M NaCl. The filtrate containing the E2-immunity protein was concentrated using a UM2 filter (Amicon) and treated in the same way as E2*. The proteins could be stored in the dialysis buffer at 4° in polypropylene tubes for at least 2 months without loss of *in vivo* or *in vitro* activity. Colicin E3* was prepared in a similar way.

Colicin activity *in vivo* was determined by spot-testing dilutions of colicin in 10 mM potassium phosphate containing

Abbreviations: E2 and E3, colicins E2 and E3, respectively; Col, a colicinogenic plasmid; kb, kilobase (1000 base pairs); SV40, simian virus 40.

Table 1. *In vivo* killing activity of colicins

E3	E3*	E2	E2*
1 ± 0.5	1 ± 0.5	1 ± 0.5	1 ± 0.5

Values given are activity units per ng of colicin as defined by Helinski (3).

2 mg/ml of bovine serum albumin on an agar layer of sensitive cells (3). To follow the *in vitro* DNA cleavage reaction, E2* was incubated with DNA (usually covalently closed circular pLC 21-9 plasmid DNA) at 37°, and samples were analyzed using slab gel electrophoresis with 0.9% agarose (22). The incubation mixture contained 20 mM Tris·HCl, pH 8.0, 80 mM NaCl, and 10 mM $MgSO_4$.

pLC 21-9 plasmid DNA was prepared by M. Kenerley in this laboratory from a derivative of *E. coli* strain MV12 (F^+, *recA*$^-$, *thr*$^-$, *leu*$^-$, *thi*$^-$, Δ*trp* E5) which carries this plasmid. The plasmid was originally constructed by Clarke and Carbon by connecting an *E. coli* DNA fragment to ColE1 DNA (23). The method used to prepare this plasmid DNA was similar to that used to make ColE3 DNA (21). Electron microscopic studies showed that the plasmid DNA is 12,400 base pairs (12.4 kb) in length and consists of ColE1 DNA (6.5 kb) and bacterial DNA (M. Kenerley and M. Nomura, unpublished experiments). The DNA was mostly in the covalently closed circular form (see, e.g., Figs. 2 and 4). *E. coli* DNA (24) and λ phage DNA (25) were prepared as described previously. Both simian virus 40 (SV40) DNA and hamster liver DNA were gifts from B. Weisblum, and phage φX174 DNA was a gift from R. D. Wells. Phage R17 RNA was prepared according to (26).

RESULTS

Preparation of colicin E3* free of the E3-immunity protein using guanidine·hydrochloride

We originally developed a method to dissociate the E3-immunity complex and to recover active E3*. The method involves dissociation of the complex by 6 M guanidine·HCl as described in *Materials and Methods*. Jakes and Zinder were unable to recover active E3* after 6 M guanidine·HCl treatment and hence used sodium dodecyl sulfate to obtain E3* (20). Using E3* prepared by our method (compare Fig. 1b), we have confirmed the observation made by Jakes and Zinder (20) that E3* is much more active *in vitro* (about 40-fold) than the original E3 preparation. In addition, we have observed that the killing activity of E3* was similar to that of the original E3 preparation when tested *in vivo* by the spot-test method (Table 1). In contrast to our results, Jakes and Zinder noted that their E3* is 50–500 times less active *in vivo* than the original E3 (20). We conclude that the difference between our results and their results is probably due to the difference in the method of preparation of E3* and that the E3-immunity protein is not required for killing of intact cells by E3* (see *Discussion*).

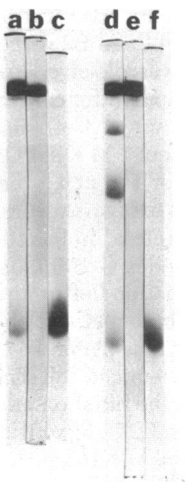

FIG. 1. Electrophoresis of colicin E3 and E2. Purified colicin E3 and E2 were electrophoresed on 10% polyacrylamide disc gels containing 0.1% sodium dodecyl sulfate and 6 M urea in 0.1 M sodium phosphate, pH 7.2 (32). The samples were E3 (a), E3* (b), E3-immunity protein (c), E2 (d), E2* (e), and E2-immunity protein (f). In the preparation shown in gel (d), we observed, in addition to E2* and the E2 immunity protein, two protein bands that presumably represent impurities. During the subsequent purification process these "impurities" were apparently removed.

Degradation of DNA by E2*

We have applied the guanidine·HCl dissociation method to prepare E2* free of the presumed E2-immunity protein. Fig. 1 shows the results of polyacrylamide/urea gel analysis of the original E2 preparation (gel d), E2* (gel e), and the presumed immunity protein (gel f). We have found that E2* thus prepared degrades various DNAs *in vitro* as analyzed by agarose gel electrophoresis. In addition, we also demonstrated that the presumed E2-immunity protein has in fact the ability to inhibit the DNase activity of E2*.

In the experiment shown in Fig. 2, pLC 21-9 plasmid DNA (mostly in the form of covalently closed circular DNA) was incubated with various amounts of E2* for 1 hr at 37° and reaction mixtures were analyzed by gel electrophoresis. It can be seen that at lower concentrations of E2* (gels a to e compared with the control, gel m), conversion of covalently closed DNA to open circles (and possibly linear "whole" molecules) is apparent (see also below). With 0.25 μg of E2* (gel e), it is clear that this conversion is almost complete, and yet no significant production of smaller DNA fragments was observed. This indicates that under these conditions E2* probably caused mainly single-strand DNA scissions. At higher E2* concentrations, the amount of open circular DNA of high molecular weight decreased and the production of smaller DNA fragments with random size distribution was observed (gels f–i).

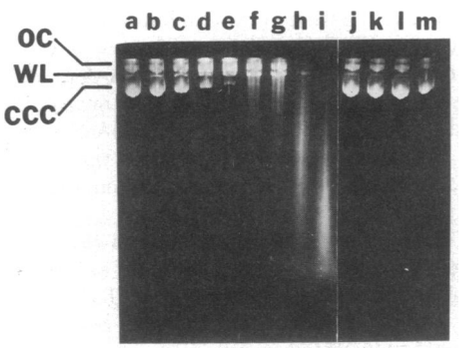

FIG. 2. Degradation of pLC 21-9 plasmid DNA by E2* *in vitro*. Plasmid DNA (10 μg) was incubated with various amounts of E2* in a total volume of 100 μl for 1 hr at 37°. Twenty-five microliters from each reaction mixture was analyzed by agarose gel electrophoresis. The different amounts of colicin E2* (in μg) were 0.012 (a), 0.025 (b), 0.05 (c), 0.12 (d), 0.25 (e), 0.5 (f), 1.25 (g), 2.5 (h), 3.25 (i); 2.5 μg of E2* preincubated with 2.5 μg of E2-immunity protein for 30 min at 37° (j); 2.5 μg of E2-immunity protein without E2* (k), 10 μg of complexed E2 (l); and no addition, i.e., of untreated plasmid DNA (m). Three arrows indicate the positions of open circular (OC), whole linear (WL), and covalently closed circular (CCC) plasmid DNA molecules. For WL, see also Fig. 4.

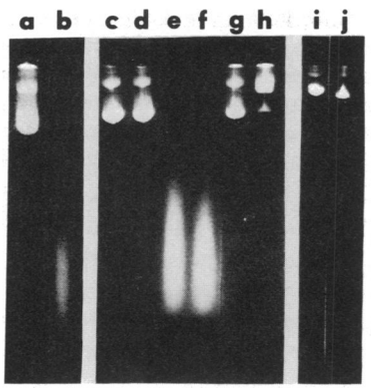

FIG. 3. Specific inhibition of the E2*-induced *in vitro* DNA cleavage reaction by E2-immunity protein. E2* (1 μg) was preincubated for 30 min at 37° with various amounts of E3-immunity protein [5.0 μg (e), 2.5 μg (f)] or E2-immunity protein [0.5 μg (g), 0.25 μg (h)] in 40 μl of reaction mixture. pLC 21-9 plasmid DNA (6.5 μg in 10 μl) was then added, and incubation was continued for one additional hour. Samples were then analyzed as described in the legend to Fig. 2. Controls contained DNA only (a), E2* without immunity protein (b), 0.5 μg of E3-immunity protein (c), and 0.5 μg of E2-immunity protein (d). In a separate experiment the plasmid DNA was incubated with 1 μg of E3* (j) and without (i), and DNAs were analyzed in a similar way. The results shown here indicate that the amount of E2-immunity protein needed to titrate E2* is between 0.25 (gel h) and 0.5 μg (gel g) per 1 μg of E2*. From the molecular weights of E2* and E2-immunity protein, this corresponds to about 1.2 to 2.4 immunity protein molecules per E2* molecule. Further studies are required to determine the exact stoichiometry for the inhibition of E2* by the immunity protein.

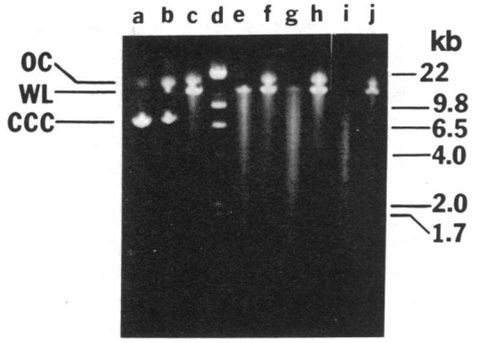

FIG. 4. Effect of E2-immunity protein on the second stage of E2*-induced DNA degradation *in vitro*. A 300 μl reaction mixture containing 34 μg of pLC 21-9 plasmid DNA and 0.07 μg of E2* was incubated at 37° (molar ratio of DNA to E2* 3:1). After 1 hr 100 μl were removed and incubated separately with 0.05 μg of E2-immunity protein. The remainder was incubated without immunity protein. Samples (30 μl each) were taken at different timepoints from both mixtures and cooled to 0°. A control with DNA only is shown in (a). Portions were taken from the reaction mixture without E2-immunity protein at "zero time" (b), at 1 hr incubation (c), 3 hr (e), 6 hr (g), and 12 hr (i). From the reaction mixture with E2-immunity protein added at 1 hr, samples were taken at 3 hr (f), 6 hr (h), and 12 hr (j). λ DNA digested with *Hin*dIII restriction endonuclease provided the size standards (d). The sizes of the fragments (33) are given in kilobases (kb). OC, WL, and CCC are defined in the legend to Fig. 2. In this experiment its position indicates that WL is about 14 kb in size. Using other DNA fragments as standards, the size of WL was estimated to be about 12.5 kb. These values agree with 12.4 ± 0.7 kb length of the intact plasmid DNA determined by electron microscopic measurements.

In contrast to E2*, the original "purified" E2 preparation failed to cause any significant nucleolytic activity on the plasmid DNA (gel l). This can be explained by the presence of the presumed E2-immunity protein in the preparation, since the latter protein inhibited the nucleolytic activity of E2* completely (gel j compared to gel h). We call this protein "E2-immunity protein." The E2-immunity protein itself has no significant DNase activity (compare gel k).

Specificity and general features of *in vitro* DNA cleavage reaction by E2*

As described above, plasmid DNA is hydrolyzed by E2*. However, E3*, which is highly active in ribosomal RNA cleavage, has no such nucleolytic activity on pLC 21-9 DNA (Fig. 3, gels i and j). Both E2* and E3* appear to be pure (see Fig. 1b and e). In addition, since the method of preparation of E2* is almost identical to that of E3*, it seems unlikely that the DNA cleavage reaction induced by E2* *in vitro* is due to some nucleases such as endonuclease I contaminating E2* preparations. Moreover, E3-immunity protein, which has the ability to inhibit the ribosome inactivation induced by E3 *in vitro* (21, 27), is unable to inhibit the DNA cleavage reaction induced by E2 *in vitro* even at an amount 10 times higher than that effective with E2-immunity protein (Fig. 3, gels e and f compared to gels b, g, and h). We have also shown that partially purified endonuclease I degrades the plasmid DNA, and that the E2-immunity protein failed to inhibit this degradation (data not shown). Therefore, the specific inhibition of the E2*-induced DNA cleavage reaction by the purified E2-immunity protein also argues against the possibility that endonuclease I contamination of E2* preparations is responsible for the observed DNA cleavage reaction. Transfer RNA, which is known to inhibit endonuclease I (ref. 28; and our own unpublished experiments), also failed to inhibit the DNA cleavage reaction induced by E2* *in vitro* (data not shown). We conclude that the observed *in vitro* DNA cleavage reaction is due to the E2* protein itself and not due to contaminating nonspecific nucleases such as endonuclease I.

As described above (compare Fig. 2; and also experiments shown in Fig. 4), the first event in the *in vitro* degradation of pLC 21-9 plasmid DNA by E2* appears to be a single-strand scission to convert the double-stranded covalently closed circular DNA to an open circle. The first reaction ("the first stage") is followed by subsequent DNA cleavage ("the second stage") resulting in the production of linear DNAs and gradual decreases in the average size of the DNA (Fig. 4; see also Fig. 2). The cleavage reaction in this second stage is also due to the E2* protein itself, since addition of E2-immunity protein during

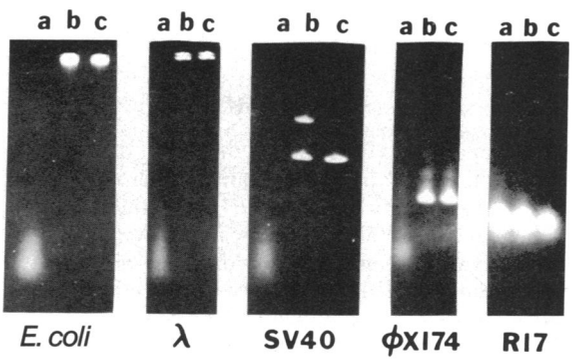

FIG. 5. Action of colicin E2* on different nucleic acids. E2* (1 μg) was preincubated for 30 min at 37° without (a) and with (b) 0.5 μg of E2-immunity protein. Various nucleic acid preparations (as indicated in the figure) were then added and incubated for one additional hour. Sample (c) was an incubation of the nucleic acid alone. The amounts of substrate in 50 μl reaction mixture were 2.65 μg of *E. coli* DNA, 5 μg of λ DNA, 1 μg of SV40 DNA, 5 μg of φX174 DNA, and 5 μg of R17 RNA. Samples were analyzed as before.

the second stage prevents further DNA cleavage (Fig. 4, gels f, h, and j compared to gels e, g, and i, respectively).

Catalytic nature of the DNA cleavage reaction induced by E2*

In the experiment described in Fig. 4, the molar ratio of E2* to DNA was 1 to 3. In this experiment, all the DNA molecules underwent at least two and probably several cleavage events caused by E2*. [The size of the original plasmid DNA was 12.4 kb. The degradation products showed broad size distribution from 10 kb to less than 2 kb (Fig. 4, gel i compared to the reference DNA fragments, gel d).] Moreover, because of the possibility that many inactive molecules could be present in our E2* preparation, the actual number of cleavages by one active E2* molecule might be much higher than that indicated in our experimental results. Therefore, it is apparent that one molecule E2* is able to cleave DNA several, and probably many, times, hence colicin E2* acts in a catalytic way like an enzyme.

Substrate specificity of the DNA cleavage reaction *in vitro*

The above experiments on the E2*-induced cleavage *in vitro* were done mainly using pLC 21-9 plasmid DNA as a "substrate." We have also demonstrated that E2* can degrade the following DNAs *in vitro: E. coli* DNA, λfus3 transducing phage DNA, λDNA, SV40 DNA, hamster liver DNA, and φX174 DNA. In every case, DNA degradation was inhibited by E2-immunity protein (Fig. 5; and other data not shown). In contrast to these DNAs, RNA prepared from phage R17 was not degraded (Fig. 5). We conclude that E2* can degrade a variety of DNAs, both double-stranded and single-stranded DNAs as well as both prokaryotic and eukaryotic DNAs, but not RNA. Exact nucleotide sequence specificity of the cleavage sites has not been studied. We note that the apparent broad substrate specificity of the E2*-induced DNA cleavage reaction is in marked contrast to the more stringent substrate specificity observed in the *in vitro* RNA cleavage reaction of E3*.

Absence of DNA exonuclease activity in E2* preparations

The results described above demonstrate that E2* has a DNA endonuclease activity. However, E2* does not appear to have any exonuclease activity. For example, we incubated radioactive *E. coli* DNA with E2* and found that no significant amount of acid-soluble radioactive oligonucleotides was produced even after a long period of incubation, when endonucleolytic cleavages of the DNAs were extensive (data not shown).

DISCUSSION

Experimental results described in this paper demonstrate that purified colicin E2* free of E2-immunity protein has a DNA endonuclease activity and that the E2-immunity protein specifically prevents this activity. These *in vitro* results can account for the *in vivo* biochemical effects of E2 on sensitive cells, and the phenomenon of the immunity in E2-colicinogenic cells. Although various biochemical and physiological changes in addition to the DNA degradation were reported in E2-treated cells (see *introduction*), none of these changes take place in the E2-colicinogenic cells. Therefore, we conclude that the primary target of colicin E2 is DNA. Any other changes observed, such as slow degradation of RNA (29) or inhibition of cell division (14), must be secondary consequences of the DNA cleavage in E2-treated cells. Our experiments also exclude several suggested mechanisms for the indirect action of E2 on DNA, such as the one involving an activation of cellular endonuclease I by E2 (15).

Saxe (19) previously reported a very weak endonucleolytic activity of E2 on supercoiled λ DNA *in vitro* (less than 10^{-4} times the activity shown in our experiments). The possibility could not be excluded that the observed nucleolytic activity was due to some nuclease copurified with E2 [see the Discussion in the paper by Saxe (19)]. Since Saxe used a colicin E2 preparation which was complexed with the E2-immunity protein, the weak activity could be explained by the inhibitory action of the E2-immunity protein on the nuclease activity of E2* as demonstrated in this paper. In fact, we failed to detect any nucleolytic activity with purified E2 complexed with the immunity protein (see Fig. 2, gel 1).

Earlier *in vivo* studies by Ringrose (11) showed that there are three distinguishable stages in the degradation of DNA in E2-treated *E. coli* cells: stage I, corresponding to single-strand DNA cleavage; stage II, double-strand DNA breakage; and stage III, formation of acid-soluble DNA breakdown products. Our *in vitro* experiments indicate that DNA breakdown corresponding to both stages I and II involves the direct action of E2* on DNA, but that cellular nuclease(s), and *not* colicin E2, is responsible for stage III.

The present studies have shown that the basic mechanism of immunity to E2 in E2-colicinogenic cells is analogous to the mechanism elucidated for the colicin E3 system and discussed in the previous papers (9, 21, 27). The E2-immunity protein used in the present studies was previously isolated by Jakes and Zinder (cited in ref. 20) and its role in E2 immunity was hypothesized. This hypothesis has now been proven experimentally.

Jakes and Zinder observed that the *in vivo* killing activity of the E3* prepared by them was much weaker than that of E3 complexed with the E3-immunity protein, and considered the possibility that E3-immunity protein may have a role in the attachment of E3 to receptors on sensitive cells (20). However, as described in this paper, our experiments show that E3- (and E2-) immunity protein is not required for the *in vivo* killing action of E3 (and E2), and hence for the attachment of the colicins to their receptors. The binding of E3* to isolated E3-receptors was also demonstrated (our unpublished experiments).

Finally we note that our *in vitro* system for E2* is simpler than the *in vitro* system for E3 studied previously. In the case of *in vitro* inactivation of ribosomes by E3, both 30S and 50S subunits are required, and free 16S rRNA cannot serve as a substrate (9, 30, 31), even though the reaction is a cleavage of 16S rRNA. Thus, one cannot completely exclude the possibility that E3 itself is not the enzyme, but activates a latent ribonuclease (e.g., a ribosomal protein) contained in the ribosomes. Our E2* system consists of protein-free DNA and purified E2* protein, and we have been able to conclude that E2* itself has a nuclease activity. Thus, we believe that the possibility that E3* activates a ribosomal RNAse is extremely unlikely and that E3* protein itself has a nucleolytic activity that results in the 16S rRNA cleavage.

We thank Dr. D. L. Nelson and L. Post for reading the manuscript. This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the United States National Science Foundation (GB-31086) and the National Institutes of Health (GM-20427). K.S. is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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