Proc. Nat. Acad. Sci. USA
Vol. 68, No. 5, pp. 964–968, May 1971

Specific Inactivation of 16S Ribosomal RNA Induced by Colicin E3 *In Vivo**

C. M. BOWMAN, J. E. DAHLBERG†, T. IKEMURA†, J. KONISKY‡, AND M. NOMURA

Institute for Enzyme Research and Departments of Biochemistry, Genetics, and †Physiological Chemistry, University of Wisconsin, Madison, Wis. 53706

Communicated by Folke Skoog, February 23, 1971

ABSTRACT Treatment of sensitive *Escherichia coli* cells with colicin E3 leads to inactivation of 30S ribosomal subunits. *In vitro* reconstitution of 30S subunits indicates that the E3-induced defect lies solely in the 16S RNA. 16S RNA from E3-treated cells lacks several T_1 RNase oligonucleotides of normal 16S RNA, including the one from the 3′-end of the 16S RNA, as analyzed by the fingerprint technique of Sanger. An RNA fragment about 50 nucleotides long has been isolated from E3-treated cells. This RNA contains the original 3′-terminal oligonucleotide and other oligonucleotides missing in the E3-16S RNA. The results show that colicin E3 treatment causes the cleavage of 16S RNA at a specific position near the 3′-terminus.

Certain strains of *Escherichia coli* carry extrachromosomal genetic elements for the production of bacteriocidal proteins called colicins (for a review, see ref. 1). Although the colicins have several common characteristics, their apparent modes of action are different, depending on the kind of colicin (2, 3). We have previously shown that one of the colicins, colicin E3, specifically inhibits protein synthesis in sensitive *E. coli* cells, while allowing nucleic acid synthesis to continue (2, 3). We found that ribosomes isolated from E3-treated cells, though physically intact, are functionally defective. Although it has been established that the alteration induced by E3 is in the 30S ribosomal subunit (4), the molecular component(s) within the 30S subunits responsible for this inactivation has not been identified. Using the ribosome reconstitution technique (5), we now can show that the protein fraction retains full activity, and that the 16S RNA is the altered component. Furthermore, we find that the 16S RNA in the inactivated ribosomes is specifically cleaved at a site near the 3′-terminus.

MATERIALS AND METHODS

E. coli strain Q13, which lacks RNase I, was used as the colicin-sensitive strain. Colicin E3 was obtained from *E. coli* strain CA38 by induction with mitomycin C, and was partially purified by precipitation with ammonium sulfate (4, 6).

The sensitive cells were grown in tryptone broth (1.3% tryptone–0.7% NaCl) at 37°C to a cell density of 5×10^8/ml. Colicin E3 was added at a multiplicity of about 15 (1, 2), and the incubation was continued for 25 min. By that time, less than 1% of the bacteria were viable. Cell extracts and 70S ribosomes, as well as 30S and 50S ribosomal subunits, were prepared as described (4). ^{32}P-Labeled ribosomes (and RNA) were prepared from cells grown in a phosphate-deprived tryptone medium (18) containing $[^{32}P]PO_4^{3-}$. Usually 2 mCi of ^{32}P was added to 30 ml of the medium.

Preparation of 16S RNA and total 30S proteins, as well as the method of reconstitution of 30S subunits, was described previously (5, 8). Ribosomal RNA was analyzed on 3% acrylamide–0.5% agarose column gels or 10% acrylamide slab gels in Tris–EDTA–borate buffer, pH 8.3 (9).

The purified RNAs were studied by the fingerprint technique of Sanger *et al.* (10, 11) after they were digested with T_1 RNase and alkaline phosphatase. The fingerprints obtained were compared with those obtained by Fellner and his coworkers (12), in order to assign numbers to the various oligonucleotides. The molar yield of each product was determined by counting, in a scintillation counter, the area of paper containing it. Particular oligonucleotides were characterized after elution from the paper by digestion with 5 μl of pancreatic RNase (0.1 mg/ml in 10 mM Tris (pH 7.6)–1 mM EDTA plus 2 mg/ml of carrier RNA) for 30 min at 37°C and electrophoresis at pH 3.5 on DEAE-cellulose paper (13).

RESULTS

Reconstitution of 30S particles with components from E3-inactivated 30S subunits ("E3-30S")

We have used the ribosome reconstitution technique (5) to determine which component is responsible for the inactivity of E3-30S. As shown in Table 1, 16S RNA ("E3-16S RNA") from E3-30S did not reconstitute to active 30S subunits

TABLE 1. *Activity of 30S ribosomes reconstituted from control and E3-16S RNA and 30S ribosomal proteins (TP30)*

30S Ribosomal components			
16S RNA	TP30	Incorporation (cpm)	%
Control	Control	3812	100
E3	E3	195	5
Control	E3	3920	103
E3	Control	337	9

Reconstitution mixtures, 0.75 ml in the reconstitution buffer in 0.34 M KCl, contained 10 A_{260} units of 16S RNA and about 13 equivalent units of TP30. The mixtures were incubated at 42°C for 60 min and were then chilled. Duplicate 40-μl aliquots were removed and assayed for poly(U)-directed protein synthesis activity, in the presence of 1 A_{260} unit of 50S subunit (7).

* Paper no. 1455 of the Laboratory of Genetics and paper 5 in the series, "Interaction of colicins with bacterial cells". Paper 4 in this series is ref. 32.

‡ Present address: Department of Microbiology, University of Illinois, Urbana, Ill. 61801.

when it was incubated with proteins from control 30S subunits. In contrast, the mixture of total proteins from E3-30S was fully active in reconstituting functional 30S subunits when it was combined with 16S RNA from control 30S subunits. We conclude that 16S RNA is altered after E3 treatment *in vivo,* and that this alteration is responsible for the inactivity of E3-30S. 30S ribosomal protein components are neither inactivated nor lost from the ribosomes. This conclusion is also consistent with the failure to detect any difference in ribosomal-protein composition between E3-30S and control 30S subunits, as analyzed by both column chromatography on carboxymethyl cellulose (14) and polyacrylamide gel electrophoresis (ref. 15 and our unpublished results).

Cleavage of 16S RNA in E3-30S

Our previous analysis of the sedimentation behavior of E3-16S RNA and control 16S RNA indicated that if a difference exists, it is very slight (4). We have also done sucrose gradient sedimentation analyses of a mixture of E3-16S RNA and (radioactive) reference 16S RNA after formamide treatment (16) or heating in 3% formaldehyde (17) to destroy their secondary structure. No difference was detected between the two. This indicates that at least a major part of the polynucleotide backbone of E3-16S RNA is intact. Using the more sensitive polyacrylamide gel electrophoresis technique, however, we have been able to show that E3-16S RNA clearly moves faster than control 16S RNA (Fig. 1*a*). It is interesting to note that RNA with the mobility of E3-16S RNA exists in the control preparation in a very small, but clearly recognizable, amount.

The mobility difference between control 16S and E3-16S RNA could not be abolished by denaturation of the RNAs in 3% formaldehyde at 63°C for 15 min, which suggests that the size of E3-16S RNA is, in fact, slightly smaller than control 16S RNA. We therefore looked for small RNA fragments produced by cleavage(s) of 16S RNA, after E3 treatment, that might still be bound to the ribosomes and would be undetected in the experiment in Fig. 1*a*. For this purpose, ^{32}P-labeled cells were treated with E3, and RNAs were extracted from isolated 70S ribosomes ("E3-70S"). Electrophoresis on a 10% polyacrylamide gel slab was used to separate small molecular weight RNAs, which were then detected by autoradiography. As shown in Fig. 1*b*, we detected a fragment ("E3-fragment" RNA) in the RNA preparation from E3-70S that moved faster than tRNAs and that was not present in the preparation from control 70S ribosomes. Analysis of the ribosome supernatant fractions ("S-100") revealed that the fragment is present in the soluble portion of the treated, but not of the untreated, cells (unpublished data).

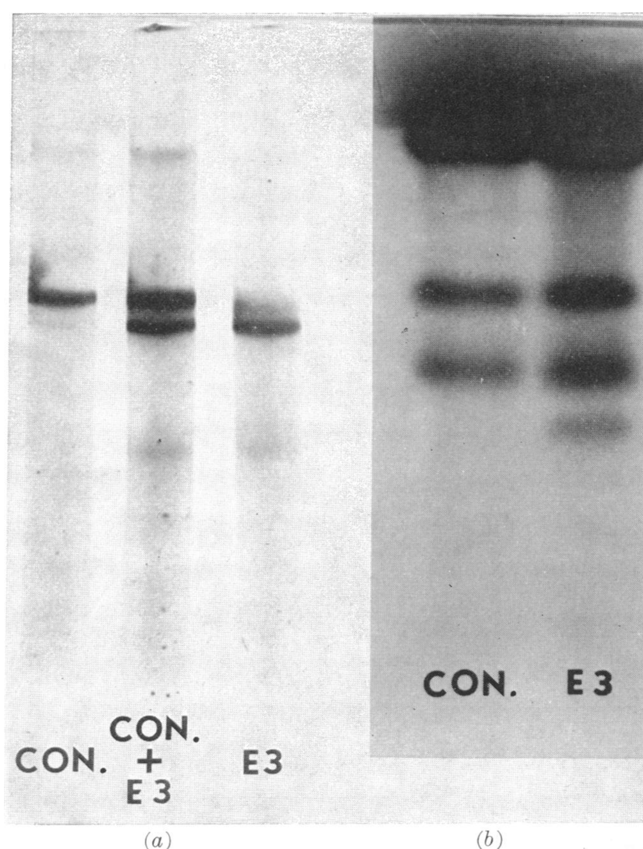


FIG. 1. Polyacrylamide gel electrophoresis of 16S, E3-16S, and E3-fragment RNAs. In (*a*), samples were 16S RNA isolated from control 30S subunits (*con.*), E3-16S RNA isolated from E3-30S (*E3*), and a mixture of both (*con.* + *E3*). Gel analysis was on polyacrylamide–agarose columns (70 V for 7-cm columns, 6 hr) and RNAs were stained with "Stains-all" (9). In (*b*), samples were [^{32}P]RNAs isolated from control70S ribosomes (*con.*) and [^{32}P]RNAs isolated from E3-70S (*E3*). Electrophoresis was on a 10% polyacrylamide gel (200 V for a 20-cm-long slab, 4 hr) and the figure is a photograph of the autoradiogram of the gel.

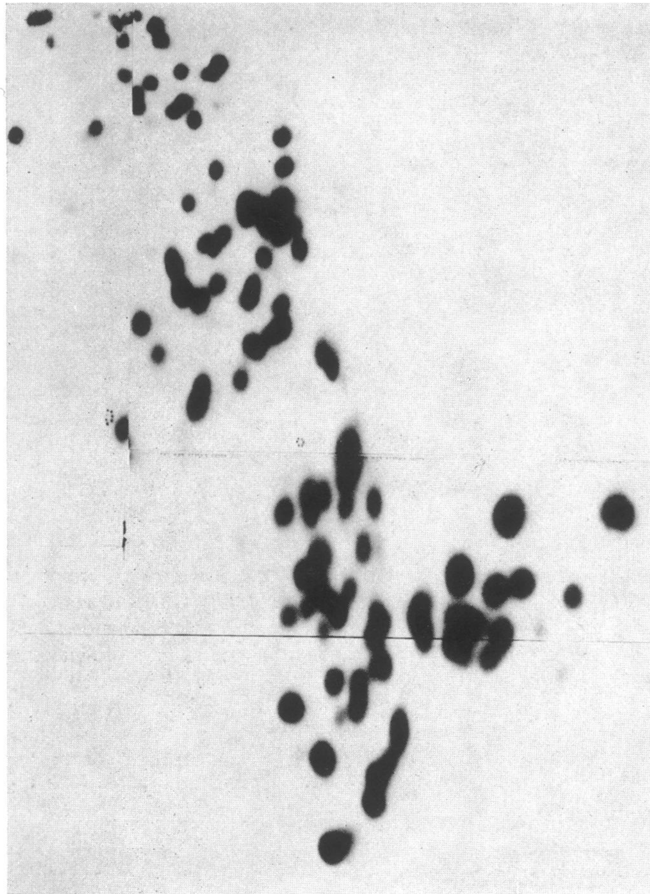
Fingerprint analysis of 16S, E3-16S, and E3-fragment RNAs

The fingerprints of the 16S and E3-16S RNAs after digestion with T_1 RNase and alkaline phosphatase are presented in Figs. 2*a* and *b*. Fig. 2*d*, a line diagram, shows the various oligonucleotides that are in both the 16S and E3-16S in approximately equal amounts. Those oligonucleotides that are represented by the *filled circles* are present in 16S RNA, but are significantly reduced in amount in the E3-16S RNA (>0.8 mol lost per mole of RNA).

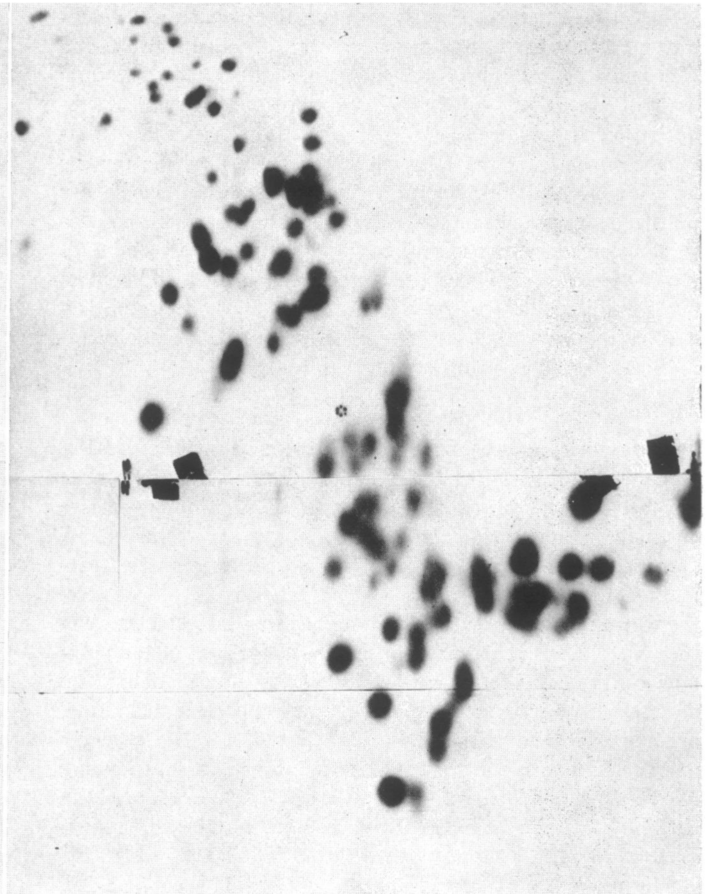
The missing oligonucleotides are listed in Table 2. The E3-16S RNA has the normal 5′-oligonucleotide (No. 23), pAAAUUG, but it lacks the normal 3′-terminal oligonucleotide (No. 10*b*). Thus, the smaller size of the E3-16S RNA must be a result of the cleavage of the molecule at a site near its 3′-OH terminus. It is noteworthy that, besides the 3′-terminal oligonucleotide, several others are missing, including one (No. 71) that contains two N^6-dimethyladenosine residues, and one that contains a methyluracil (No. 57*b*). We find that the 3′-terminal oligonucleotide has a composition at variance with that originally reported by Fellner *et al.* (12) but is in agreement with his revised structure (P. Fellner, personal communication).

Fingerprint analysis of the E3-fragment RNA is shown in Fig. 2*c*. It is clear from a comparison of the fingerprint of E3-fragment RNA and the missing spots in the line diagram (*filled circles*) that E3-fragment RNA contains all of the oligonucleotides that are absent from the E3-16S RNA. Pancreatic RNase digestion showed that the two sets of oligonucleotides are probably identical (Table 2). Since small oligonucleotides may be present in 16S RNA a large number of times, the absence of one copy of each from the E3-16S RNA would not be detectable.

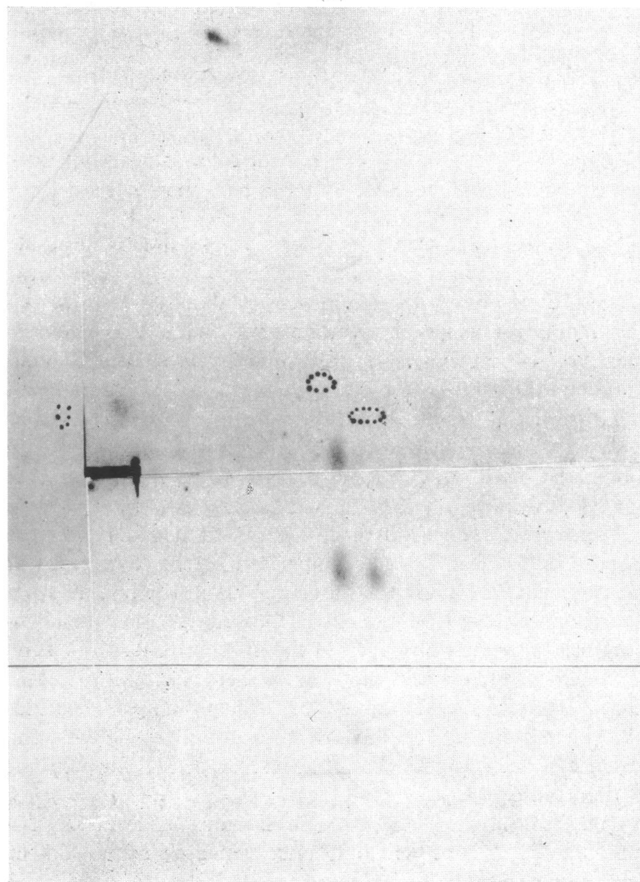
Quantitation showed that each oligonucleotide in the E3-fragment RNA is present only once. Since we cannot detect any of spot No. 71, or its unmethylated precursor No. *p*-71



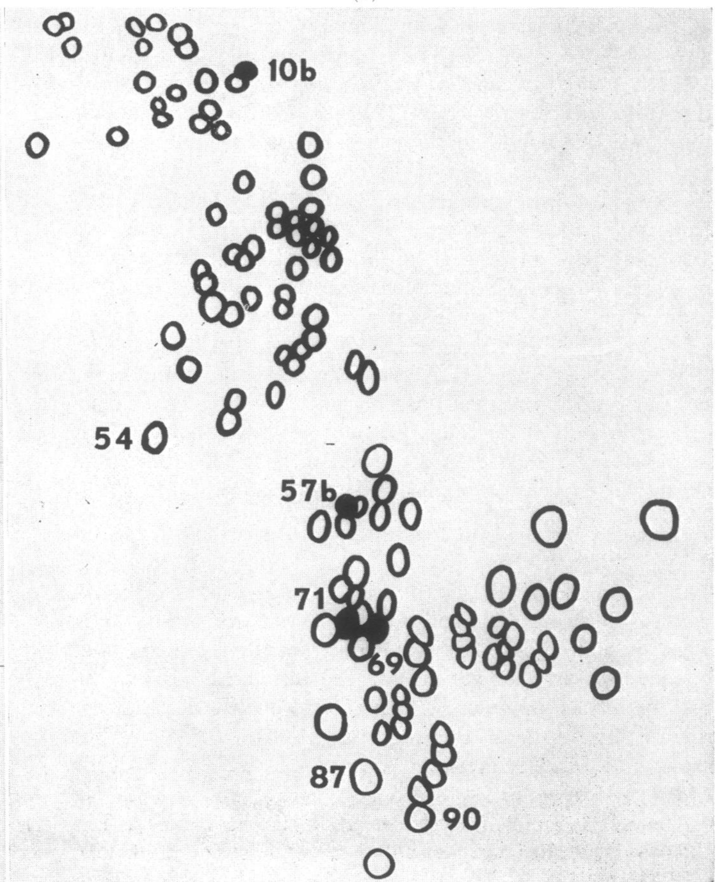
(a)



(b)



(c)



(d)

TABLE 2. *Oligonucleotide differences between 16S, E3-16S, and E3-fragment RNAs*

Oligonucleotide number (Boldface indicates its presence in the E3-fragment)	Reported sequence (12)	Pancreatic RNase products of oligonucleotides from 16S RNA	Pancreatic RNase products of oligonucleotides from comparable E3-16S regions	Pancreatic RNase products of oligonucleotides from E3-fragment RNA
10*a*	AACCUUACCU,G	AAC,AC,C,U	AAC,AC,C,U	Absent
10b	[AAAU,(AU)$_2$,(AC)$_2$,3C,U]A$_{OH}$	AC,AU,4C,3U	Absent	AC,AU,C,U
54	UUG	U	U	U
57*a* or **57b**	AAACUG or (A$_3$,mU)CG or mUAACAAG (18)	AAAC, "slow AAU", AAG$_{OH}$,U	AAAC	"slow AAU", AAG$_{OH}$
69 or 72	CUAACG or UCAAG	AAC,AAG$_{OH}$,C, U	AAG$_{OH}$,C, U	AAC,C, U
71	m$^6_2$Am$^6_2$ACCUG	"fast AAC",C,U	Absent	"fast AAC",C,U
87	UAG	U,AG$_{OH}$	U,AG$_{OH}$	U,AG$_{OH}$
90	UCG	C,U	C,U	C,U

The oligonucleotides were assigned numbers by comparison with the fingerprint pattern obtained by Fellner *et al.* (12). The absence of a product from E3-16S RNA was determined by scintillation counting of the region on the E3-16S paper comparable to the position of the spot in the 16S fingerprint. Quantitation of the pancreatic RNase products was done visually (10, 11), except for spot No. 10*b*, in which all products were counted. Spots 57*a* and *b* were not separated from each other, nor were spots 69 and 72. Inclusion of spots 57*b* and 69 in the E3-fragment RNA is based upon a reduction in the total radioactivity of these spots and the pancreatic RNase products obtained from these oligonucleotides in E3-16S and E3-fragment RNA. The absence of G$_{OH}$ from spot 10*b* was confirmed by digestion with snake-venom phosphodiesterase. Spots 54, 87, and 90 were studied when their presence in the E3-fragment was recognized. Those oligonucleotides that are boldface in the left-hand column are found in the E3-fragment RNA. Preliminary evidence, based upon T$_1$ RNase digestion of E3-fragment, indicates that UG, CG, AG, and about 8 Gs may also be present.

(18), in the E3-16S RNA, there probably is only one mole of this sequence in normal 16S RNA (12, 21). The total number of nucleotides in the E3-fragment RNA is about 40 (plus 8 GMPs that are undetectable after the phosphatase digestion).

Although the exact point of cleavage is not known, some evidence about it does exist. After digestion of E3-fragment RNA with T$_1$ RNase alone, no product with a 5′-terminal phosphate was found (our unpublished data). Thus, cleavage probably produces a 3′-phosphate and a 5′-OH. If the site of cleavage is in the middle of a T$_1$ RNase oligonucleotide, that oligonucleotide is probably present in multimolar amounts, such as a trinucleotide, since we can account for all of the missing unique sequences in the E3-fragment RNA. In addition, all of the oligonucleotides in the E3-16S and the E3-fragment RNA are also in 16S RNA.

Therefore, we conclude that colicin E3 treatment causes a specific endonucleolytic break in the 16S RNA molecule at a position near its 3′-OH end. The cleavage point is probably about 50 nucleotides from the 3′-terminus, as judged from the size of E3-fragment RNA. However, the possibility exists that smaller oligonucleotides, in addition to the E3-fragment RNA studied here, were also produced after E3 treatment.

DISCUSSION

The present results have delineated a specific biochemical change in *E. coli* cells treated with colicin E3, a cleavage of the 16S rRNA molecule at a position near the 3′-terminus. Since the protein fraction retains full activity in the reconstitution assay, and since the cleavage of 16S RNA is the only observed change in the RNA molecule, it is safe to conclude that the cleavage reaction is responsible for the inactivation of the ribosomes and for the inhibition of protein synthesis (4). A fragmentation of 16S RNA after E3 treatment has also been observed by Senior and Holland (19).

The E3-fragment RNA containing the original 3′-terminal oligonucleotide was found on the E3-70S ribosomes, but in a small amount. Preliminary experiments suggest that most of the E3-fragment RNA is, in fact, in the soluble fraction. It is not established whether the cleavage itself, or the loss of the fragment, is responsible for inactivation. E3-30S and E3-70S usually show a variable (5–30%) residual activity (4, see also ref. 20). Since cleavage appears to be nearly complete (Figs. 1 and 2), this residual activity of E3-ribosomes might be related to the presence of a small fraction of E3-ribosomes that still have the fragment RNA attached.

Our results suggest that both E3-16S and E3-fragment RNAs are homogeneous. These fragments should be useful in rRNA sequencing, as well as in studies of the structure–function relationship of ribosomal RNA. In the experiments described in Table 1, inactive particles that were reconstituted with E3-16S RNA were examined. These particles showed a sedimentation pattern very similar to the control reconstituted 30S particles, but lacked at least one protein, P15 (unpublished experiments). Further studies should reveal the

←

FIG. 2. T$_1$ RNase plus phosphatase fingerprints of (*a*) 16S, (*b*) E3-16S, (*c*) E3-fragment RNA, and (*d*) a line diagram of the fingerprints. The [^{32}P]RNAs were digested and fingerprinted according to the methods of Sanger *et al.* (10, 11). The line diagram (*d*) indicates the number assignments for various oligonucleotides. Digestion products that are present in 16S RNA, but absent from E3-16S RNA, are indicated by the *filled circles*. Electrophoresis in the first dimension, at pH 3.5 in 8 M urea, was from *right* to *left*; in the second dimension, in 7% formic acid, it was from *top* to *bottom*.

functional role played by the E3-fragment portion of the 16S RNA. In this connection, it is interesting to note that the E3-fragment (about 50 nucleotides long) contains 3 of the 12 methylated bases found in the entire 16S RNA (about 1700 nucleotides long) (21).

Fellner has recently isolated a large (39-nucleotide) fragment from the 3′-end of 16S RNA by digestion of 30S subunits with ribonuclease (P. Fellner, personal communication). The sequence of this piece contains only oligonucleotides that we find in the E3 fragment (numbers 10*b*, 57*b*, 69, 71, and 87).

It is not clear whether the E3-induced cleavage is due to the activation of a specific RNase or to an induced structural alteration of the ribosomes *in vivo* that renders this site particularly susceptible to a preexisting RNase. In any event, the E3 molecule itself cannot induce such cleavage *in vitro*, nor is there an increase in the activity of RNase in the soluble fraction ("S-100") obtained from E3-treated cells (unpublished experiments; see also ref. 4). As we showed previously, colicins, in general, stay at their receptor sites and act from outside the cell membrane (22–24). The specific cleavage of the 16S RNA molecule induced by E3 must also involve participation of membrane components as intermediaries. The possible involvement of conformational changes of some membrane components as a general property of the action of all colicins was discussed previously (24, 26, 27).

We found that colicin E2, which shares a common receptor with E3 (23, 25), causes DNA degradation (2). The initial event in this degradation appears to be a scission of single strands of the DNA duplex (28). Thus, even though the target molecules are entirely different, there is a common feature in the biochemical effects of E2 and E3: induction of an endonucleolytic cleavage of polynucleotide chains. Specific nucleolytic cleavages of DNA and their repairs are known and implicated in various physiological phenomena such as restriction (29, 30) and recombination (31). No comparable cases are known with respect to RNA. The present finding of a specific cleavage of the 16S RNA molecule poses a question of the significance of such a cleavage in normal cellular physiology, as well as a question of the possible existence of RNA repair systems.

We thank Mrs. B. Stark for technical help. This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, Madison, and by grants from the National Institute of General Medical Sciences (GM-15422) and National Science Foundation (GB-6594 and GB-15671). J.E.D. holds a National Institutes of Health Research Career Development Award (1-K4-GM-32,770). C.M.B. was supported by a Medical Scientist Training Grant from the National Institute of General Medical Sciences (GM-01932).

1. Nomura, M., *Annu. Rev. Microbiol.*, **21**, 257 (1967).
2. Nomura, M., *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 315 (1963).
3. Nomura, M., and A. Maeda, *Zentralbl. Bakeriol. Parasitenk.*, **196**, 216 (1965).
4. Konisky, J., and M. Nomura, *J. Mol. Biol.*, **26**, 181 (1967).
5. Traub, P., and M. Nomura, *Proc. Nat. Acad. Sci., USA*, **59**, 777 (1968).
6. Herschman, H. R., and D. R. Helinski, *J. Biol. Chem.*, **242**, 5360 (1967).
7. Traub, P., and M. Nomura, *J. Mol. Biol.*, **34**, 575 (1968).
8. Nomura, M., P. Traub, and H. Bechmann, *Nature*, **219**, 793 (1968).
9. Dahlberg, A. E., C. W. Dingman, and A. C. Peacock, *J. Mol. Biol.*, **41**, 139 (1969).
10. Sanger, F., G. G. Brownlee, and B. G. Barrell, *J. Mol. Biol.*, **13**, 373 (1965).
11. Brownlee, G. G., and F. Sanger, *J. Mol. Biol.*, **23**, 337 (1967).
12. Fellner, P., C. Ehresman, and J. P. Ebel, *Nature*, **225**, 26 (1970).
13. Adams, J. M., P. G. N. Jeppesen, F. Sanger, and B. G. Barrell, *Nature*, **223**, 1009 (1969).
14. Osawa, S., E. Otaka, T. Itoh, and T. Fukui, *J. Mol. Biol.*, **40**, 321 (1969).
15. Leboy, P. S., E. C. Cox, and J. G. Flaks, *Proc. Nat. Acad. Sci., USA*, **52**, 1367 (1964).
16. Helmkamp, G. K., and P. O. P. T'So, *J. Amer. Chem. Soc.*, **83**, 138 (1961).
17. Boedtker, H., *J. Mol. Biol.*, **35**, 61 (1968).
18. Lowry, C., and J. Dahlberg, paper submitted to *Nature*.
19. Senior, B. W., and I. B. Holland, *Proc. Nat. Acad. Sci. USA*, **68**, 959 (1971).
20. Senior, B. W., J. Kwasniak, and I. B. Holland, *J. Mol. Biol.*, **53**, 205 (1970).
21. Fellner, P., *Eur. J. Biochem.*, **11**, 12 (1969).
22. Nomura, M., and M. Nakamura, *Biochem. Biophys. Res. Commun.*, **7**, 24 (1962).
23. Maeda, A., and M. Nomura, *J. Bacteriol.*, **91**, 685 (1966).
24. Nomura, M., *Proc. Nat. Acad. Sci. USA*, **52**, 1514 (1964).
25. Fredericq, P., *Symp. Soc. Exp. Biol.*, **12**, 104 (1958).
26. Luria, S., *Ann. Inst. Pasteur*, **107**, 67 (1964).
27. Changeux, J. T., and J. Thiery, *J. Theor. Biol.*, **117**, 315 (1967).
28. Ringrose, P., *Biochim. Biophys. Acta*, **213**, 320 (1970).
29. Meselson, M., and R. Yuan, *Nature*, **217**, 1110 (1968).
30. Arber, W., and S. Linn, *Annu. Rev. Biochem.*, **38**, 467 (1969).
31. Howard-Flanders, P., *Annu. Rev. Biochem.*, **37**, 175 (1968).
32. Levisohn, R., J. Konisky, and M. Nomura, *J. Bacteriol.*, **96**, 811 (1967).