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The Mechanism of Action of Ricin and Related Toxic Lectins on Eukaryotic Ribosomes

THE SITE AND THE CHARACTERISTICS OF THE MODIFICATION IN 28 S RIBOSOMAL RNA CAUSED BY THE TOXINS*

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Ricin is a potent cytotoxic protein derived from the higher plant *Ricinus communis* that inactivates eukaryotic ribosomes. In this paper we have studied the mechanism of action of ricin A-chain on rat liver ribosomes *in vitro*. Our findings indicate that the toxin inactivates the ribosomes by modifying both or either of two nucleoside residues, G_{4323} and A_{4324}, in 28 S rRNA. These nucleotides are located close to the α-sarcin cleavage site and become resistant to all ribonucleases tested. The examination of the lability of phosphodiester bonds of these nucleotides to both mild alkaline digestion and aniline treatment at acidic pH suggests that the base of A_{4324} is removed by the toxin. This unique activity of ricin A-chain was also observed when naked 28 S rRNA is used as a substrate, indicating that the toxin directly acts on the RNA. Similar activity on 28 S rRNA is also exhibited by abrin and modeccin, ricin-related toxins, suggesting a general mechanistic pathway for ribosome inactivation by lectin toxins.

There is a group of cytotoxic proteins acting on eukaryotic ribosomes including those from fungi (α-sarcin) and higher plants (ricin, abrin, and modeccin). These toxins have been known to catalytically and irreversibly inactivate 60 S ribosomal subunits affecting the activities in peptide elongation reaction (see Ref. 1 for a review). However, the molecular mechanisms of their action have not been elucidated except for the case of α-sarcin, which has been known to hydrolyze a single phosphodiester bond between G_{4325} and A_{4326} in an evolutionally conserved region of 28 S rRNA (2-5). Ricin and other plant lectins, *e.g.* abrin and modeccin, consist of two peptide chains, A and B, linked together by a disulfide bond while α-sarcin is a single peptide (6). The B-chain binds the toxins to receptors on the cell surface, and the A-chain enters the cytoplasm and inactivates the 60 S ribosomal subunits. Irrespective of the structural differences, the mode of action of ricin and the related lectins is known to be identical with that of α-sarcin in the following aspects: (i) they affect EF-1[1] and EF-2 associated functions of 60 S subunits and (ii) they do not require energy and any cofactors. These lines of evidence suggested to us that they also act on rRNA rather than on ribosomal proteins in 60 S ribosomal subunits like α-sarcin. The possibility that the toxins are endonucleases was once ruled out in 1976 by Mitchell *et al.* (7) who reported that ricin does not change the sizes of any rRNA species of L cell polysomes *in vitro*, but more recently it was suggested by Obrig *et al.* (8) who demonstrated that the lectins, ricin and phytolaccin, and Shiga toxin are able to hydrolyze naked 5 S and 5.8 S rRNAs.

Recently, to test whether ribonuclease activity of ricin, if any, is involved in the inactivation of ribosomes, we determined the sequences of 5′ and 3′ termini of each rRNA species after ricin treatment and found that even 100 times molar exess of the toxin over ribosomes did not hydrolyze any rRNA species both exo- and endonucleolytically,[2] consistent with the results of Mitchell *et al.* (7). Instead, we noticed that 28 S rRNA from ricin-treated ribosomes always migrates more slowly in gel electrophoresis than control and demonstrated that catalytic amounts of ricin and other related toxins modify probably one nucleotide of 28 S rRNA located adjacent to the α-sarcin site. The possible reactions catalyzed by the toxin are discussed.

EXPERIMENTAL PROCEDURES

Materials—The following enzymes were purchased from Pharmacia P-L Biochemicals: polynucleotide kinase and RNA ligase from the virus T4, ribonuclease T_1, ribonuclease U_2, ribonuclease Phy M from *Physarum polycephalum*, and ribonuclease from *Bacillus cereus*. Ribonuclease T_2 was obtained from Behring Diagnostics, and [γ-^{32}P] ATP (3000 Ci/mmol) and cytidine-3′,5′-[^{32}P]bisphosphate (3000 Ci/mmol) were from Amersham Corp. Ricin and abrin were purchased from E. Y. Laboratories, Inc. (CA) and modeccin was from Pierce Chemical Co. Ricin A-chain, the catalytic subunit of ricin, was purified by the method of Olsnes (9). Abrin and modeccin were activated with 2-mercaptoethanol before use (10).

Preparation of Ribosomes and Treatment with Toxins—Ribosomes were isolated from rat liver (11) and from *Escherichia coli* strain K-A19 (12); the preparation from rat liver contained mainly polysomes (11, 13). The ribosome preparations were suspended in buffer (25 mM Tris/HCl, pH 7.6, 25 mM KCl, and 5 mM $MgCl_2$) to give a concentration of 150 A_{260} units/ml. Treatment of rat liver ribosomes (1.5 A_{260} units) with ricin A-chain (1 ng), abrin (2 ng), or modeccin (20 ng) was carried out in 100 μl of buffer (25 mM Tris/HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$) incubated for 10 min at 37 °C. The activity of ribosomes was measured in poly(U)-directed polyphenylalanine synthesis by the same method as described by Olsnes *et al.* (14) except for a pretreatment of the ribosomes with puromycin KCl to make them run off (15). For the treatment of bacterial ribosomes with ricin A-chain, the toxin amount was raised up 10,000 times higher (10 μg) than that for rat liver ribosomes. The reaction was stopped by the addition of 0.5 ml of 0.5% sodium dodecyl sulfate in 50 mM Tris/HCl, pH 7.6.

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[1] The abbreviations used are: EF-1 and EF-2, eukaryotic elongation factors 1 and 2, respectively; α-fragment, the oligonucleotide of 488 bases that is cleaved from the 3′ end of 28 S rRNA by the action of α-sarcin; β-fragment, the polynucleotide of the remaining 5′ end of 28 S rRNA after the removal of the α-fragment.

[2] Y. Endo and K. Tsurugi, unpublished results.

For the treatment of naked rRNA with ricin A-chain, isolated rRNA was incubated with a high concentration of the toxin for a prolonged time period. Total rRNA (1.5 A_{260} units) was dissolved in 100 μl of the same buffer as for ribosomes and incubated with 10 ng of ricin A-chain for 60 min at 37 °C. Denaturation of rRNA was done in 25 mM Tris/HCl, pH 7.6, by heating at 95 °C for 2 min followed by quick chilling on ice.

Preparation and Analysis of rRNA—RNA was extracted from reaction mixtures with 0.5% sodium dodecyl sulfate and phenol (3) and analyzed by electrophoresis, either on 2.5% acrylamide-0.5% agarose composite gel (16) or on polyacrylamide gels (3).

Preparation of the 3′ Terminal Fragment of 28 S rRNA Modified by Ricin A-chain—RNA from ricin A-chain-treated ribosomes was dissolved in buffer (25 mM Tris/HCl, pH 7.6, 0.1 M NaCl, 0.2 mM EDTA) and layered on a 10–30% linear sucrose gradient in the same buffer. The several species of rRNA were separated by centrifugation in a VTi 50 rotor (Beckman Instruments) at 50,000 rpm at 4 °C for 2.5 h. The fractions containing the modified fragment, which has a sedimentation coefficient of approximately 10, were collected and precipitated with ethanol. The fragment was made radioactive at either the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase (17), or the 3′ end with cytidine-3′,5′-[^{32}P]bisphosphate and T4 RNA ligase (18). The radioactive oligonucleotide was separated from contaminants by 3.5% polyacrylamide gel electrophoresis (3). This modified fragment was localized by brief exposure of the gel to x-ray film, excised from the gel, and eluted from the polyacrylamide (19).

Determination of the Nucleotide Sequences of the Modified Fragment—The sequence at both the 5′ and 3′ termini of the fragment was determined with an enzymatic method (20, 21). The alkaline digestion was carried out in 40 mM sodium carbonate buffer, pH 9.0, at 90 °C for 15 min or, in some experiments, for 1 min. In some experiments, 5′-labeled RNA was treated with the solution of 1 M aniline/acetate, pH 4.5, at 60 °C for 20 min in the dark according to Peattie (22).

RESULTS AND DISCUSSION

The Effect of Ricin and Related Toxins on 28 S rRNA in Rat Liver Ribosomes—When rat liver ribosomes were treated with ricin A-chain at a molar ratio of 1:960 (ricin:ribosomes), the activity of the ribosomes was decreased by greater than 85% in poly(U)-directed polyphenylalanine synthesis (data not shown), confirming that the toxin catalytically inactivates ribosomes. To gather evidence that RNA is the toxin target, total rRNAs were extracted from the ricin-treated ribosomes and analyzed by composite gel electrophoresis. As shown in Fig. 1*A*, comparison of electrophoretic mobility of treated (*lane 2*) *versus* untreated (*lane 1*) rRNAs showed identical mobility of 18 S rRNA while a mobility difference between the two 28 S rRNAs was clearly visible, as indicated by the *arrow*. This small but definitive difference between the two 28 S rRNAs was reproducible from experiment to experiment. The same mobility shift of 28 S rRNA was observed when run-off ribosomes were used as a substrate (data not shown). This finding suggested that ricin A-chain modifies 28 S rRNA which results in slow migration on the gel, without cleaving the RNA. The resistance of RNA cleavage by ricin observed here is in disagreement with the previously reported data (8).

To identify the site of modification on the 28 S rRNA, we searched for a modified rRNA fragment among the many RNA fragments that are normally generated by the contaminated ribonuclease(s) which associate ribosomes during their preparation. A fragment of 550 nucleosides clearly had slower mobility compared to ricin-untreated ribosomes, as shown by an *arrow* in *lane 2* of Fig. 1*B*. This difference in rate of migration disappeared when both treated and untreated samples were analyzed by gel electrophoresis in the presence of 7 M urea (data not shown). This observation suggested that the mobility shift of the ricin-treated rRNA was due to the possible change in the conformational or chemical modification rather than change in length. Other ricin-related toxins, abrin and modeccin, also resulted in the similarly modified

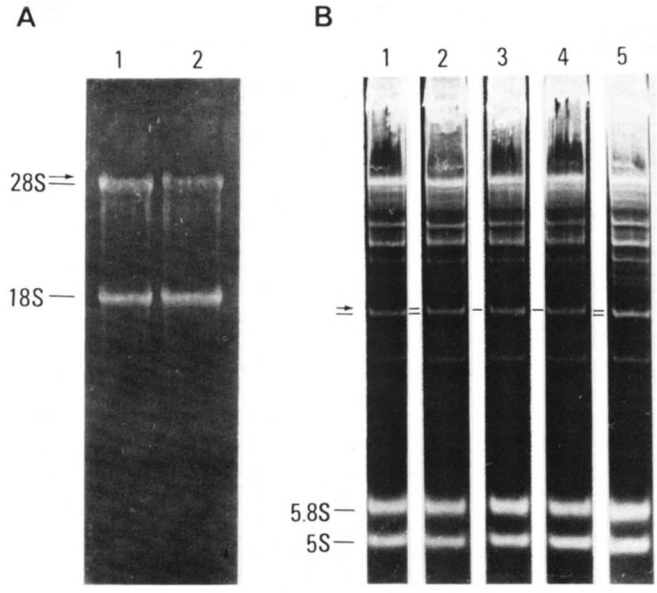

FIG. 1. **Analysis by gel electrophoresis of RNAs from toxin-treated rat liver ribosomes.** Rat liver ribosomes (3.49×10^{-7} M) were treated with either ricin A-chain (3.12×10^{-10} M), abrin (3.08×10^{-10} M), or modeccin (3.51×10^{-9} M) and the RNA was extracted with sodium dodecyl sulfate and phenol. *A*, 2 μg of RNAs from control (*lane 1*) and ricin-treated ribosomes (*lane 2*) were analyzed by 2.5% acrylamide-0.5% agarose composite gel electrophoresis. *B*, 20 μg of RNAs from control (*lanes 1* and *5*), and ricin- (*lane 2*), abrin- (*lane 3*) or modeccin-treated ribosomes (*lane 4*) were analyzed by 3.5% polyacrylamide gel electrophoresis. RNA bands were visualized with ethidium bromide. *Arrow* denotes 28 S rRNA (*A*) or the fragment (*B*) altered in its migration rate by ricin and other toxins.

550-nucleotide fragment as shown in *lanes 3* and *4* of Fig. 1*B*. Thus, it appears that related toxic lectins (ricin, abrin, and modeccin), isolated from different sources, exhibit the same rRNA-modifying activity. These results exclude the possiblity that the modifying activity is due to a contaminant in the toxin preparation because it is highly unlikely that the same contaminant was associated with these different toxin samples that were isolated from different sources. Furthermore, the 28 S rRNA-modifying activity was exhibited by the purified ricin A-chain but not by the purified B-chain (cell-binding subunit).

Characterization of the Altered Fragment—In order to determine the origin of the modified fragment, we isolated the fragment and determined the nucleotide sequences of both 5′ and 3′ terminal regions (Fig. 2). Both modified and unmodified 550-nucleotide fragments revealed identical 5′ and 3′ end sequences by the enzymatic digestion method and we localized this fragment to the 3′ terminal 553 nucleotides of the 28 S rRNA (Fig. 2). The occurrence of this fragment in a ribosomal preparation has been reported previously (24). This result indicated that a modified region may be located in the middle portion of the modified fragment. Further nucleotide sequence analysis revealed absence of bands corresponding to G_{4323} and A_{4324} in the modified fragment, whereas these bands were present in the unmodified fragments as shown by *arrows* in Fig. 3*A*. This striking observation immediately suggested that these two nucleotides have been modified by an unknown enzymatic activity of the ricin A-chain. A possibility of known type of modification in the residue A_{4324} can be excluded because of its resistance to hydrolysis by ribonuclease T_2 which is known to recognize most kinds of modified bases (25) (Fig. 3*A*, *lanes 1* and *2*). However, it is unclear about the G_{4323} residue which remained resistant to hydrolysis by ribonuclease T_2 before and after ricin treatment. The higher

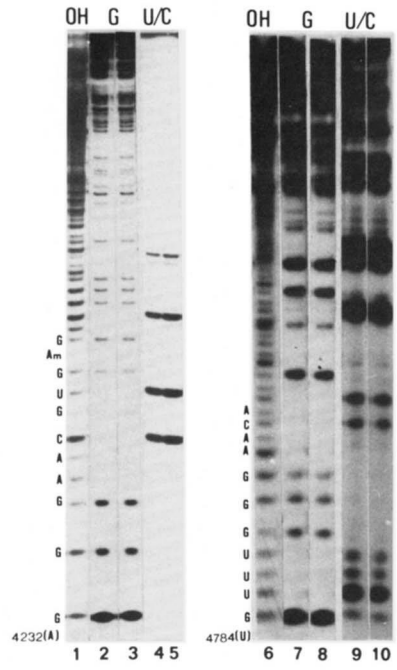

FIG. 2. **Radioautographs of sequencing gel of the 5′ and 3′ terminal regions of the modified fragment by ricin.** The RNA fragment was made radioactive by labeling with ^{32}P at the 5′ or 3′ terminus and was partially digested by an enzymatic method using five kinds of ribonucleases described in the legend to Fig. 3. The digests were analyzed by 20% polyacrylamide gel electrophoresis in the presence of 7 M urea. *Lanes*: 5′ terminal (*lanes 1–5*) or 3′ terminal sequences (*lanes 6–10*) of the fragments from control (*lanes 1, 2, 4, 6, 7,* and *9*) or ricin-treated ribosomes (*lanes 3, 5, 8,* and *10*). Only the lanes for the digests with ribonuclease T_1 (*G*) and ribonuclease from *B. cereus* (*U/C*) are shown here, as no differences were observed between the lanes of control and ricin-treated fragments for all nuclease digests. The alkaline digests are designated *OH*. 5′ and 3′ terminal bases, A_{4232} and U_{4784}, respectively, were identified by means of two-dimensional thin layer chromatography (data not shown). Nucleotides in 28 S rRNA are numbered from its 5′ terminal end according to Chan *et al.* (23).

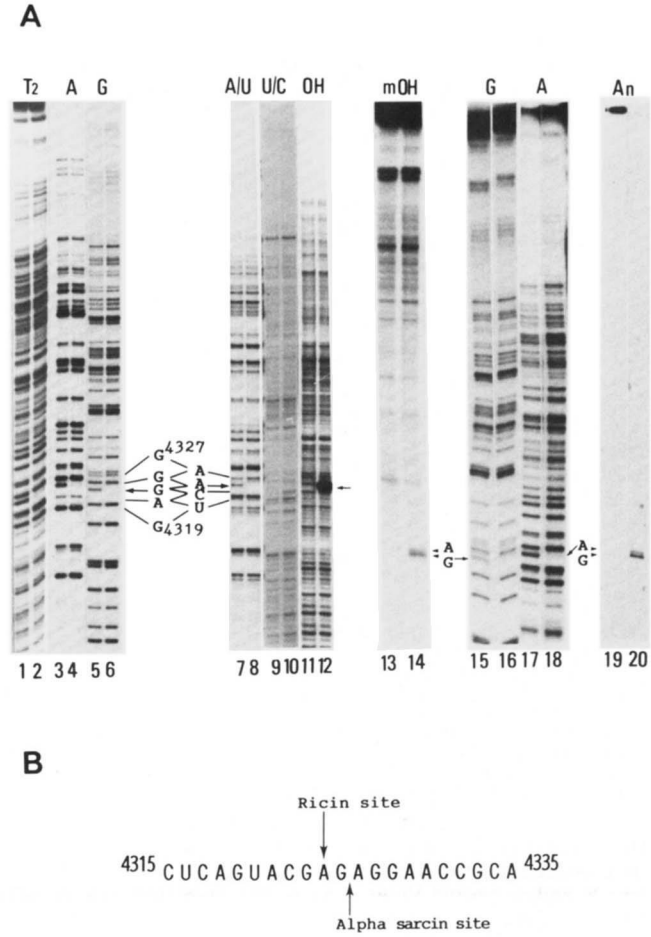

FIG. 3. **Radioautographs of sequencing gels of the modified region of the 3′ terminal fragment of 28 S rRNA.** *A*, the fragments from control and ricin-treated ribosomes were made radioactive at the 5′ terminus with [γ-^{32}P]ATP using polynucleotide kinase and were partially digested with ribonuclease T_1 (*G*), ribonuclease U_2 (*A*), ribonuclease Phy M (*A/U*), ribonuclease T_2 (*A/U/G/C*), or ribonuclease from *B. cereus* (*U/C*). The partial alkaline digests obtained under mild (1 min) and ordinary (15 min) conditions were designated *mOH* and *OH*, respectively. The aniline-induced products were designated *An*. The digests of the fragments from control (*odd-numbered lanes*) and ricin-treated ribosomes (*even-numbered lanes*) were separated by 10% acrylamide gel electrophoresis at 1.2 kV for either 4 h (*lanes 1–12*) or 6 h (*lanes 13–20*). The *arrows* denote the bases missing in ricin-treated RNA corresponding to G_{4323} and A_{4324}. The *arrowheads* denoted the bands developed by alkaline (*lane 14*) and aniline treatment (*lane 20*) which also apparently correspond to G_{4323} and A_{4324}. *B*, nucleotide sequence of 28 S rRNA around the site of ricin action. Nucleotides in 28 S rRNA are numbered from its 5′ terminal end according to Chan *et al.* (23).

susceptibility to hydrolysis of C_{4322} by *B. cereus* ribonuclease of the modified over unmodified fragment (Fig. 3*A*, *lane 10*) suggests that, as a result of the modification of either or both G_{4323} and A_{4324}, the neighboring C residue has been widely exposed to digestion by the ribonuclease.

Since it is possible that modification of either the G_{4323} or A_{4324} results in the ribonuclease resistance of both of the nucleotides, we examined the cleavage behavior of the modified fragment by the chemical methods. It is known that various amines and hydroxide ion cleave RNA strand by β-elimination reaction if the base of the nucleoside residue is removed, leaving the aldehyde radical at C1 of ribose (26). As shown in Fig. 3*A*, *lanes 13* and *14*, partial alkaline hydrolysis shows stronger radioactive bands corresponding to the G_{4323} and A_{4324} residues compared to other nucleotides (as shown by *arrowheads*). This result suggests that the modification imparts increased lability of the phosphodiester bonds surrounding A_{4324}, since the 5′ end of the fragment was radiolabeled. Treatment of the modified and unmodified fragments with aniline at acidic pH according to Peattie (22) also resulted in chain scission at positions apparently corresponding to the G_{4323} and A_{4324} residues. This result also indicated that the fragment was sensitive at both phosphodiester bonds surrounding the A_{4324} residue. If the G_{4323} residue is also modified, the band of C_{4322} will appear on the gel by these treatment. These results suggested that the base of A_{4324} is cleaved leaving the aldehyde radical at C1 of ribose rather than that the bases of both residues are missing or severely damaged.

Examination of the Site of Modification in Total rRNAs of the Toxin-treated Ribosomes—Examination of the total rRNAs for the ricin A-chain-induced modification by aniline treatment showed that only 28 S rRNA was modified (Fig. 4*A*). Results similar to ricin A-chain modification were observed when α-sarcin, abrin, and modeccin were used as the toxins as shown in Fig. 4*A*, *lanes 1–5*. These results demonstrated that 28 S rRNA is the only target RNA for all toxin treated and that in each case the site of modification is located close to the α-sarcin cleavage site. The data presented in Fig. 4*B* indicate that inactivation of ribosomes by ricin A-chain is specific for eukaryotic ribosomes because rRNA of *E. coli* ribosomes remained resistant to aniline treatment.

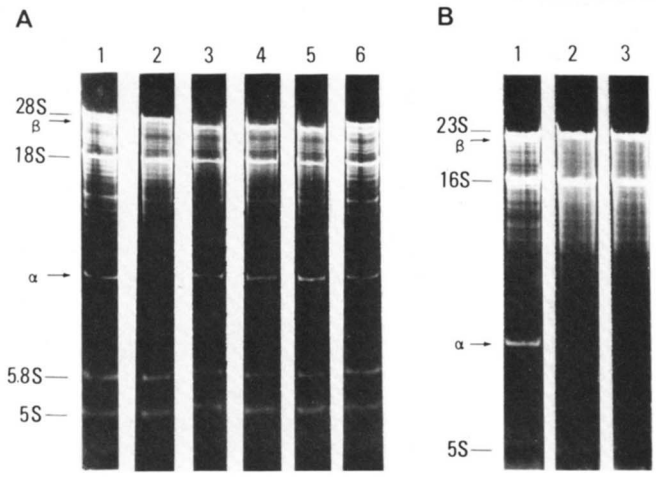

FIG. 4. **Analysis by gel electrophoresis of aniline-treated rRNA from toxin-treated rat liver** (*A*) and bacterial ribosomes (*B*). Rat liver ribosomes were treated with one of the three toxins as in Fig. 1, and *E. coli* ribosomes were treated with 10,000-fold amount of ricin A-chain used for rat liver ribosomes. Five μg of RNAs extracted from those ribosomes were treated with aniline and analyzed by the composite gel system. For the molecular weight marker, an rRNA sample containing α- and β-fragments cleaved at the α-sarcin site was prepared from rat liver and *E. coli* ribosomes treated with the toxin as described elsewhere (3). It should be noted that *E. coli* ribosomes require 100 times more α-sarcin to generate such fragments. *A*, *lanes 1* and *6*, RNAs from α-sarcin-treated rat liver ribosomes as molecular weight marker; *lane 2*, aniline-treated RNAs from rat liver ribosomes untreated with toxins (control); *lanes 3–5*, aniline-treated RNAs from rat liver ribosomes treated with ricin A-chain (*lane 3*), abrin (*lane 4*), and modeccin (*lane 5*). *B*, *lane 1*, RNAs from α-sarcin-treated *E. coli* ribosomes as molecular weight marker; *lane 2*, aniline-treated RNAs from *E. coli* ribosomes; *lane 3*, those from *E. coli* ribosomes treated with ricin A-chain. α and β denote the RNA fragments produced by α-sarcin action.

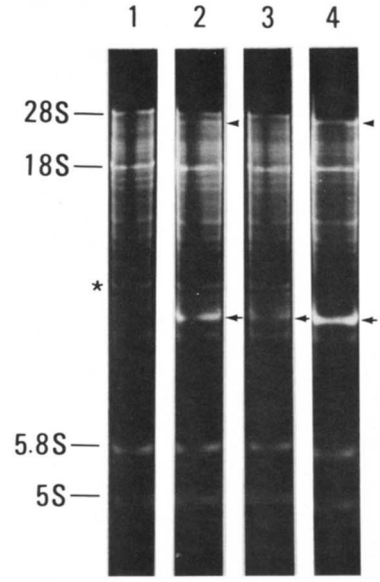

FIG. 5. **Analysis by gel electrophoresis of aniline-treated rRNA which was treated with ricin A-chain in its naked state.** Total rRNA (3.49×10^{-7} M) with or without denaturation was incubated with ricin A-chain (3.12×10^{-6} M) and then re-extracted as described under "Materials and Methods." Ten μg of aniline-treated rRNA was analyzed as in Fig. 4. *Lane 1*, control rRNA; *lane 2*, intact rRNA treated with ricin A-chain; *lane 3*, denatured rRNA treated with the A-chain; *lane 4*, rRNA from ricin A-chain-treated ribosomes as a reference. *Arrowheads* and *arrows* denote the 5′- and 3′-side fragment of 28 S rRNA, respectively. *Asterisk* marks the fragment of 553 bases.

Effect of Ricin A-chain on Naked 28 S rRNA—To understand the mechanism of these toxins and the role of ribosomal proteins in allowing toxins to modify the rRNA, we examined the effect of ricin A-chain on naked 28 S rRNA. The native total rRNA was isolated (27) and incubated with ricin A-chain followed by treatment with aniline. Analysis of the reaction mixture by gel electrophoresis, showed the presence of the same size fragment (553 nucleotides, Fig. 5, *lane 2*) as generated from the ribosomes on treatment with ricin A-chain. This result clearly shows that the site of modification of the naked rRNA by ricin A-chain is similar to when rRNA is part of the ribosomes. However, the rate and the amount of modification of the naked RNA was slow and far less than the RNA present in the ribosomal particle. This may be partly caused by the fact that the isolated rRNA may have lost some of the native secondary structure resulting in less than quantitative modification of the 28 S rRNA. This study strongly suggested an important role of ribosomal proteins in inducing and maintaining the secondary structure recognized by the ricin A-chain. This suggestion was further supported by the fact that denatured 28 S rRNA was not modified by the ricin A-chain (Fig. 5, *lane 3*). Furthermore, the 553-nucleotide fragment alone did not serve as a substrate for modification by the ricin A-chain (compare *lanes 1*, *2*, and *4*).

The question of the nature of the specific modification by the ricin A-chain was examined next. A previous study dealing with the ricin A-chain-induced inactivation of ribosomes proposed that ricin A-chain acts as a ribonuclease (8). Our data presented above, however, show that the RNA chain remains intact after treatment with ricin A-chain. In other words, ricin A-chain does not catalyze hydrolysis of the RNA but instead modifies a specific nucleotide which renders the surrounding phosphodiester bonds highly susceptible to hydrolysis. The nature of modification could involve removal of adenine from the A_{4324} in a manner, similar to *N*-glycosidases act, which causes its phosphodiester bonds to be sensitive to chemical hydrolysis. However, rRNA *N*-glycosidases have not yet been described but ricin A-chain acting as a specific *N*-glycosidase appears to be a very attractive possibility.

These studies strongly suggest that inactivation of eukaryotic ribosomes by toxins such as ricin A-chain occurs by specific modification of the A_{4324} residue of 28 S rRNA. Although the nature of the modification has not yet been established, it is very likely that ricin A-chain acts as a specific *N*-glycosidase. The results also show that this specific *N*-glycosidase activity is not nucleotide sequence-specific because the ricin A-chain target sequence AGUACGAGAGGAAC, which is conserved between eukaryotes (rat, yeast, etc.) and prokaryotes (*E. coli*), is only modified when this sequence is part of the eukaryotic ribosomes. This observation emphasizes the role of the ribosome particles in the toxin-induced inactivation of the ribosomes.

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