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Ribosome inactivation by ricin A chain: a sensitive method to assess the activity of wild-type and mutant polypeptides

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When recombinant ricin A chain transcripts are translated in a rabbit reticulocyte lysate the ribosomes are rapidly inactivated as shown by their inability to support translation of yeast preproalpha factor or chicken lysozyme transcripts added subsequently. In contrast, ribosomes which have translated transcripts encoding non-toxic polypeptides such as ricin B chain, readily translate the second transcript under identical conditions. Ribosome inactivation is accompanied by a highly specific modification of 28S rRNA which occurs at the same position as the *N*-glycosidic cleavage of an adenine residue and which is thought to cause inactivation of the ribosomes. Protein synthesis by wheat germ ribosomes was not inhibited under the conditions which inhibit reticulocyte ribosomes confirming earlier observations that plant cytoplasmic ribosomes are much less sensitive to inhibition by ricin A chain than are mammalian ribosomes. Using the same assay we have shown that deleting an internal hexapeptide, which shares homology with hamster elongation factor-2, completely abolishes catalytic activity. Deleting a second pentapeptide conserved between ricin A chain and the ribosome-inactivating plant toxin trichosanthin, had no effect. Deleting the first nine residues from the N-terminus of A chain did not affect toxicity whereas deleting a further three residues inactivated the polypeptide. Point mutations which individually converted arginine 48 and arginine 56 of ricin A chain to alanine residues or which deleted arginine 56 were also without effect on the catalytic activity of the toxin.

Key words: in vitro transcription and translation/mutant A chain/primer extension/ribosome inactivation/ricin A chain

Introduction

The extreme potency of the cytotoxic plant protein ricin (from the seeds of *Ricinus communis*), was first recognized a century ago (Stillmark, 1888). Only recently, however, has the catalytic activity which confers this potency been identified (Endo *et al.*, 1987). Ricin comprises two structurally and functionally distinct *N*-glycosylated polypeptides, each of mol. wt ~30 kd, covalently joined by a single disulphide bond (Olsnes and Pihl, 1982). One of these polypeptides (the A chain), inhibits protein synthesis on eukaryotic ribosomes by catalytically inactivating the 60S ribosomal subunit. This is apparently achieved by an *N*-glycosidic cleavage which releases a specific adenine base from the sugar–phosphate backbone of 28S rRNA (A4324 in rat liver 28S rRNA) (Endo *et al.*, 1987; Endo and Tsurugi, 1987). The second polypeptide (the B chain), is a lectin which binds to galactose residues on the surface of cells and which is believed to have a role in transporting the A chain into the target cell cytoplasm (Youle *et al.*, 1979; Vitetta, 1986). Ricin A chain is the best known example of a group of plant proteins which are able to depurinate specifically 28S rRNA or 26S rRNA. The group includes the A chain of other cytotoxic plant lectins such as abrin and modeccin (Endo *et al.*, 1987) and single chain ribosome-inactivating proteins from plants such as gelonin and trichosanthin (Stirpe *et al.*, 1988). The group can now be extended to include *Shigella dysenteriae* type 1 (Shiga) toxin and *Escherichia coli* Shiga-like toxin (Endo *et al.*, 1988). The protein sequence data available at present show that these toxic proteins share considerable homology (Xuejun and Jiahuai, 1986; Calderwood *et al.*, 1987; DeGrandis *et al.*, 1987) suggesting that the prokaryotic and eukaryotic toxins have evolved from a common ancestral gene (Ready *et al.*, 1984).

Ricin has been sequenced (Funatsu *et al.*, 1978, 1979), cloned (Lamb *et al.*, 1985; Halling *et al.*, 1985) and its X-ray structure has been determined (Montfort *et al.*, 1987). It is, therefore, an ideal candidate for further study of the mechanism of 28S rRNA depurination and for identification of amino-acid residues essential for catalytic activity. To facilitate this work, we have developed a simple and sensitive *in vitro* method to assess the activity of ricin A chain. The effects of amino-acid deletions and substitutions on biological activity are also assessed.

Results

Construction of transcription vectors carrying ricin A chain cDNAs

The construction of pSP64XβM, a transcription vector carrying the *Xenopus* β-globin gene, has been described elsewhere (Krieg and Melton, 1984). For our purposes, most of the *Xenopus* β-globin coding sequence, its 3′ untranslated region and the multiple cloning site was removed by *Pvu*II digestion. A *Bam*HI linker was ligated to re-circularize the plasmid. This generates pSP64△Bam (Figure 1). Alternatively, the entire β-globin sequence with the exception of the initiation codon was deleted by digestion with *Nco*I, end-filling and *Pst*I digestion to generate a vector fragment suitable for ligation with 5′ deletions of A chain cDNA (Figure 1).

Ricin A chain cDNA was derived from a preproricin cDNA (Lamb *et al.*, 1985) into which a translation stop codon has been introduced immediately after the codon for the carboxy-terminal residue of mature A chain (O'Hare *et al.*, 1987). The 5′ end of this A chain cDNA begins at

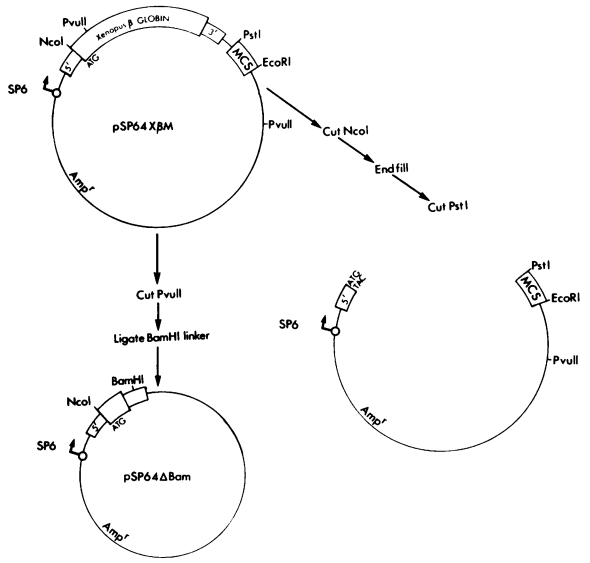


Fig. 1. Transcription vectors. The bulk of *Xenopus* β-globin coding sequence, the 3′ untranslated region and MCS was removed by digesting pSP64XβM with *Pvu*II. A *Bam*HI linker (CCCGGATCGGG) was ligated to recircularize the vector (pSP64ΔBam). Alternatively for the analysis of 5′ deletions of A chain, the entire β-globin coding sequence with the exception of the initiation codon, was deleted by *NcoI digestion, end-filling and Pst*I digestion.

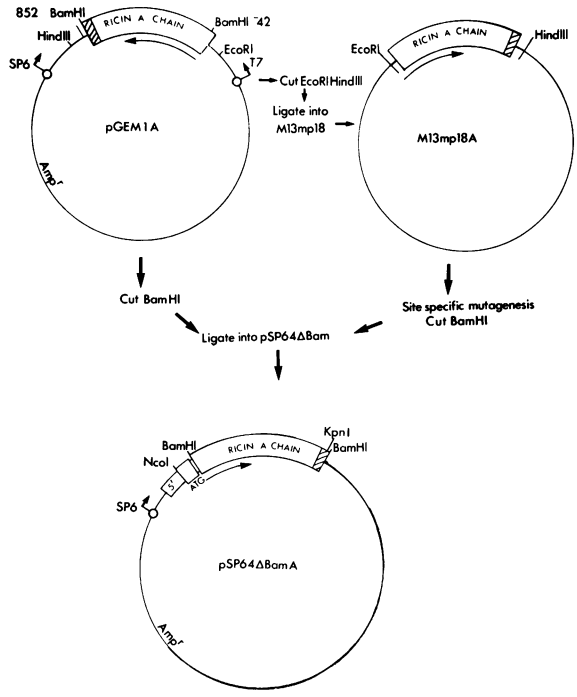


Fig. 2. Transcription vectors carrying ricin A chain cDNAs. For *in vitro* transcription the ricin A chain coding sequence was excised from pGEM1A as a *Bam*HI fragment and ligated into pSP64ΔBam. For oligonucleotide site directed mutagenesis, a *Hin*dIII−*Eco*RI fragment was isolated and ligated into M13mp18. After mutagenesis the altered A chain sequences were excised as *Bam*HI fragments and ligated into pSP64ΔBam. The hatched region represents the stretch of ricin sequence downstream of the inserted A chain stop codon.

base position −74, according to the previous designation (Lamb *et al.*, 1985), and is preceded by an *Xho*I linker (CCTCGAGG). The DNA fragment containing the entire ricin A chain coding sequence was excised from an earlier clone as an *Xho*I−*Sal*I fragment and ligated into the *Sal*I site of pGEM1 to give pGEM1A (Figure 2). For *in vitro* transcription experiments here, ricin A chain DNA was cleaved from pGEM1A as a *Bam*HI fragment and ligated into the corresponding site in pSP64△Bam to generate pSP64△BamA (Figure 2) which can be used to transcribe efficiently A chain RNAs. The construct pSP64△BamA encodes a fusion protein of 290 amino acids in which the eight N-terminal residues of *Xenopus* β-globin (MGLT-AHDR−) are joined by a proline (from the linker) to the last 14 residues of the N-terminal extension peptide of preproricin (−GSTSGWSFTLEDNN−). This in turn precedes the 267 residues of mature A chain. The effects of internal deletions and substitutions were analysed by transcription and translation of corresponding constructs in pSP64ΔBam. For analysis of N-terminal deletions, the 5′ ends of A chain sequences in pGEM1A were deleted with exonuclease III. After end-filling and *Pst*I digestion, the deleted fragments were ligated into the vector fragment generated from pSP64XβM (Figures 1 and 3).

In-vitro toxicity assay

The principle of the method is to allow ribosomes which are very sensitive to ricin A chain, namely rabbit reticulocyte ribosomes, to translate mRNA encoding recombinant A chain. Assuming that the A chain folds into a catalytically active conformation, the newly synthesized toxin should immediately begin to inactivate ribosomes and should rapidly render them incapable of translating a second transcript added subsequently. The feasibility of this approach is shown in Figure 4. When ribosomes which have been translating *in vitro* synthesized transcripts encoding non-toxic yeast

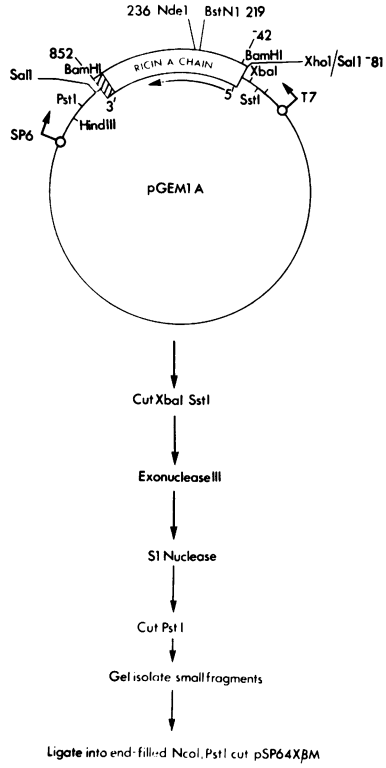


Fig. 3. 5′ deletions of ricin A chain DNA. pGEM1A was digested with *Xba*I and *Sac*I and the 5′-end deleted using exonuclease III. After treatment with S1 nuclease and *Pst*I digestion, gel-isolated DNA fragments were ligated into pSP64XβM cleaved with *Nco*I, end-filled and recleaved with *Pst*I.

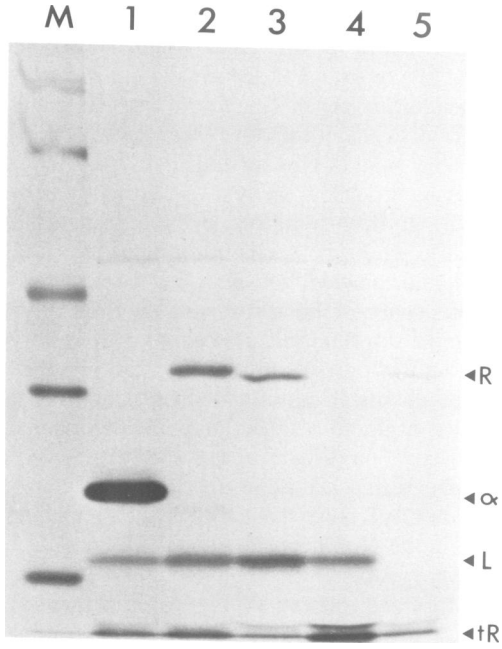


Fig. 4. Ricin A chain inactivation of reticulocyte ribosomes. [^{35}S]-Methionine-supplemented reticulocyte lysates were allowed to translate, for 1 h, transcripts encoding yeast preproalpha factor (**lane 1**), ricin B chain (**lane 2**), ricin A chain from which a hexapeptide sharing homology with EF-2 had been deleted (**lane 3**), truncated ricin A chain where a stop codon had been introduced into the coding sequence to give a product of M_r ~10 kd (**lane 4**) and wild-type ricin A chain (**lane 5**). A second transcript encoding chicken lysozyme was added to each mix and translation was continued for a further 30 min before the products were separated by SDS–PAGE and visualized by fluorography. R indicates the ricin subunit bands; α, yeast preproalpha factor; L, chicken lysozyme; tR, the truncated ricin A chain. **Lane M** shows mol. wt markers indicating the positions, in order of increasing mobility, of polypeptides of molecular size 92, 68, 45, 30 and 14 kd.

preproalpha factor (Figure 4, lane 1) or non toxic ricin B chain (Figure 4, lane 2) are supplemented with a transcript encoding lysozyme, they synthesize both polypeptides. In contrast, ribosomes which have been translating *in vitro* transcripts of ricin A chain under identical conditions, are incapable of subsequently translating transcripts encoding lysozyme (Figure 4, lane 5). Figure 5 shows that the inhibition observed (Figure 4, lane 5), is correlated with the catalytic activity of the A chain on its rRNA substrate. Ricin A chain and functionally related toxins can depurinate 28S or 26S rRNA at a specific site close to the 3′ end of the molecule. Depurination renders isolated rRNA susceptible to amine-catalysed hydrolysis of the phosphodiester bonds on either side of the modification site. This cleavage generates a small RNA fragment of ~390 ribonucleotides from reticulocyte 28S rRNA. This is very similar in size to that predicted for rat liver 28S rRNA derived from the sequence of Chan *et al.* (1983) and Endo *et al.* (1987). This fragment is therefore diagnostic of ricin A chain inactivation. Reticulocyte ribosomes incubated with biochemically purified ricin A chain, release the characteristic RNA fragment upon aniline treatment of isolated rRNA (Figure 5, lane 2). Ribosomes at zero time (Figure 5, lane 3) or after incubation (Figure 5, lane 6) in the complete translation mix in the absence of added transcripts, do not release the fragment after aniline treatment. However, ribosomes which have been translating ricin A chain transcript clearly do (Figure 5, lane 5). Ribosomes which have translated yeast preproalpha factor

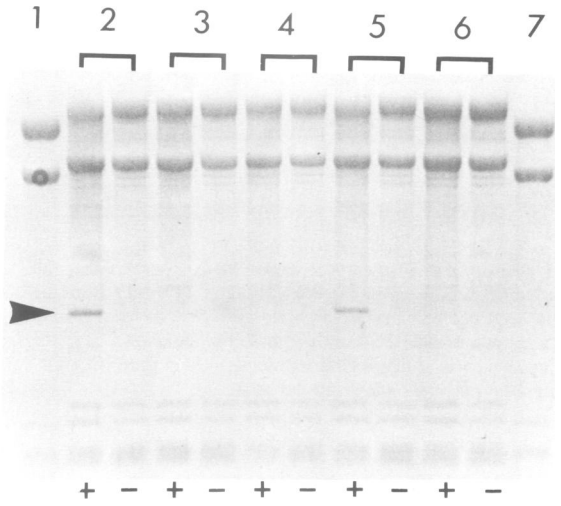


Fig. 5. Modification of reticulocyte rRNA by ricin A chain synthesized *in vitro*. Translation reaction mixtures were identical to those used for the experiments shown in Figure 4 except that the reticulocyte lysate had not been treated with micrococcal nuclease. Following 60-min incubation RNA was extracted, aniline treated where appropriate and fractionated as described in Materials and methods. **Lanes 1** and **7**, *E.coli* rRNA markers; **lane 2**, no added transcript but 100 ng of biochemically purified ricin A chain added; **lane 3**, no added transcript, zero time control; **lane 4**, transcript encoding ricin A chain from which a hexapeptide sharing homology with EF-2 had been deleted; **lane 5**, transcript encoding wild-type ricin A chain; **lane 6**, no added transcript, after 60 min incubation. + indicates aniline treatment; − indicates no aniline treatment. The arrow indicates the 390-nucleotide fragment released by aniline treatment of modified rRNA.

or ricin B chain RNAs do not release the RNA fragment upon aniline treatment (data not shown). Under the conditions of aniline treatment and RNA denaturation used, release of the ~390 ribonucleotide fragment from 28S rRNA was virtually complete (data not shown).

Deletion and substitution of internal sequences of ricin A chain

It has been observed that sensitive ribosomes can be partially protected against inactivation by ricin A chain by prior treatment with elongation factor (EF)-2 (Fernandez-Puentes *et al.*, 1976), or by adding high concentrations of EF-2 together with the A chain (Carrasco *et al.*, 1975). This suggests that EF-2 and ricin A chain may compete for the same binding site on the ribosome and prompted us to search for primary sequence homology between these two proteins. Figure 6 shows a short stretch of hamster EF-2 (Kohno *et al.*, 1986) and ricin A chain which display significant homology. The sequence of the corresponding region in trichosanthin is also shown. Trichosanthin is a single chain ribosome inactivating protein from *Trichosanthes kirilowii maxim* which shows considerable homology with ricin A chain (Xeujun and Jiahuai, 1986). By deleting DNA between two convenient restriction sites in the A chain encoding cDNA, six codons were removed (encoding DVTNAY) to perturb this region of homology with hamster EF-2 (Figure 6). Transcripts prepared from a clone containing this deletion translated a mutant A chain that did not inhibit translation in a rabbit reticulocyte lysate (Figure 4, lane 3; Figure 5, lane 4; Figure 7, lane 3). A second pentapeptide (SEAAR), corresponding to residues 176–180 in ricin A chain, is also completely conserved in trichosanthin. This pentapeptide

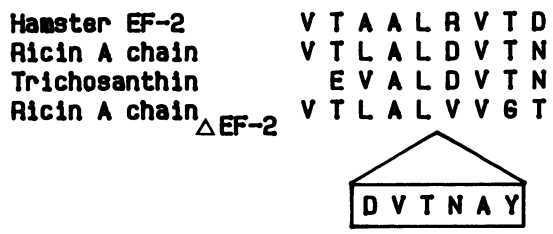


Fig. 6. Primary sequence comparison of EF-2, ricin A chain and trichosanthin. Sequences representing residues 115–123 from the primary sequence of hamster EF-2, residues 71–79 from ricin A chain and residues 59–66 from trichosanthin are shown as is the resulting sequence in this region for ricin A chain (ΔEF-2) after deleting a hexapeptide (shown in the box).

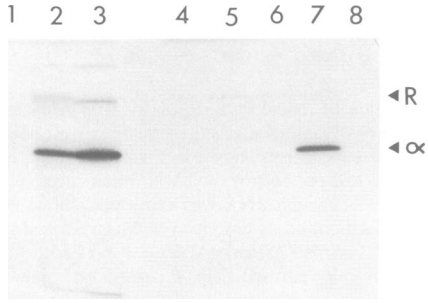


Fig. 7. Effect of wild-type and mutant ricin polypeptides on reticulocyte ribosomes. [^{35}S]Methionine-supplemented reticulocyte lysates were allowed to translate for 1 h, transcripts encoding wild-type ricin A chain (**lane 1**), ricin B chain (**lane 2**), ricin A chain from which a hexapeptide sharing homology with EF-2 had been deleted (**lane 3**), mutant ricin A chain in which arginine 48 had been substituted with alanine (**lane 4**), mutant ricin A chain from which arginine 56 had been deleted (**lane 5**), mutant ricin A chain from which a pentapeptide (SEAAR) had been deleted (**lane 6**), mutant ricin A chain from which the first 12 N-terminal residues had been deleted (**lane 7**), and mutant ricin A chain from which the first nine N-terminal residues had been deleted (**lane 8**). A second transcript encoding yeast preproalpha factor was added to each mix and translation was allowed to continue for a further 30 min, before the products were separated by SDS–PAGE and visualized by fluorography. R indicates the ricin subunit bands, and α indicates yeast preproalpha factor.

contains three residues (E_{177}, A_{178} and R_{180} which are conserved in all toxins sequenced to date which catalytically cleave the *N*-glycosidic bond in adenine 4324 in 28S rRNA. These residues, together with a further four conserved residues, have been implicated as potential active-site constitutents (Hovde *et al.*, 1988). In addition to ricin A chain and trichosanthin, these residues are conserved in *E.coli* Shiga-like toxins SLT-1 and SLT-11, and in a ribosome inactivating protein from barley (Hovde *et al.*, 1988). Further, when E_{167} of SLT-1 (equivalent to E_{177} of ricin A chain) was changed to D_{167} by oligonucleotide-directed mutagenesis of the SLT-1 structural gene, the mutant polypeptide produced was 1000-fold less toxic than wild-type SLT-1, suggesting that E_{167} is critical for activity (Hovde *et al.*, 1988). In contrast ricin A chain transcripts carrying the SEAAR deletion produced a mutant protein which displayed inhibitory activity (Figure 7, lane 6), and depurinated 28S rRNA (data not shown).

Obviously the method used here provides only a qualitative assessment of the presence or absence of toxicity for the expressed polypeptides. As such any reduction in the specific activity of ricin A chain resulting from the deletion may not be evident. We did not consider it worthwhile to compare the rate or extent of depurination of 28S ribosomal RNA in reticulocyte lysates which were translating wild-type or mutant ricin A chain mRNA. Meaningful comparisons are impossible in this type of suicide system since any mutation giving reduced toxicity might therefore permit the synthesis of significantly higher levels of mutant A chain with respect to wild-type A chain translated over the same period. It should be noted, however, that we never observed increased production of mutant carrying the SEAAR deletion (or the arginine substitutions, see below) over wild-type on any of the autoradiographs produced in the present study.

Reversible chemical modification of ricin A chain has indicated that arginine residues play an important role in ribosome inactivation (Watanabe and Funatsu, 1986). There are 20 arginine residues in ricin A chain (Lamb *et al.*, 1985) but recent work has shown that the essential arginine residues are present in the N-terminal region which contains a total of six arginines. A comparison of the N-terminal sequences of ricin A chain and trichosanthin reveals that three of these arginines are conserved in both proteins. The conserved residues are at positions 29, 48 and 56 in ricin A chain. Of these, it is arginine 48 and arginine 56 that appear to lie within a three-dimensional cleft proposed as a possible active site (Montfort *et al.*, 1987). Accordingly we mutated the A chain cDNA such that in the expressed polypeptides, arginine 48 or 56 had been converted to alanine 48 or 56. Translation products from the transcripts of these substitutions retained full activity and therefore prevented translation of a second transcript (Arg48 → Ala, Figure 7, lane 4; Arg 56 → Ala data not shown). This was accompanied by depurination of the translating ribosomes (Figure 8, lanes 1 and 2). In a third mutant, arginine 56 was deleted. Once again, the polypeptide product was fully active towards ribosomes in a reticulocyte lysate assay (Figure 7, lane 5). Clearly neither arginine 48 nor arginine 56 alone are crucial for the catalytic activity of ricin A chain.

N-terminal deletions of A chain

In a preliminary attempt to define an active A chain polypeptide of minimum size, we have generated proteins with deletions at the N-terminus. Deletions were accomplished using exonuclease III in the strategy described in Figure 3. In-frame deletions were transcribed and translated and those of interest, in terms of the activity of expressed products, are shown in Figure 7. Deleting the first nine residues from the N-terminus of A chain appeared to have no effect on the activity of the polypeptide (Figure 7, lane 8), whereas deleting the first 12 residues completely abolished activity (Figure 7, lane 7).

Primer extension

Primer extension was used to investigate in more detail the precise position of the rRNA modification catalysed by ricin A chain and mutant derivatives synthesized *in vitro*. The rationale for this is based on the observation of Hagenbuchle *et al.* (1978) and Youvan and Hearst (1979), that reverse transcriptase is unable to read certain chemically modified bases in an RNA template. Pauses or stops give rise to bands corresponding to the length of the cDNA from the 5′ end of the primer to the nucleotide immediately preceding the modified position. The primer used here was complementary to a region to the 3′ side of the ricin modification site

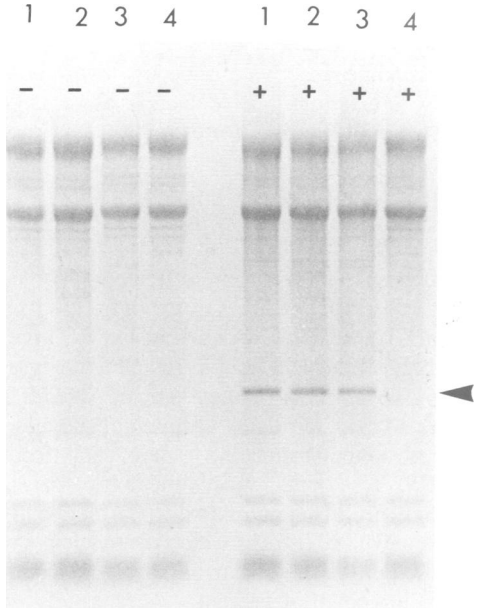
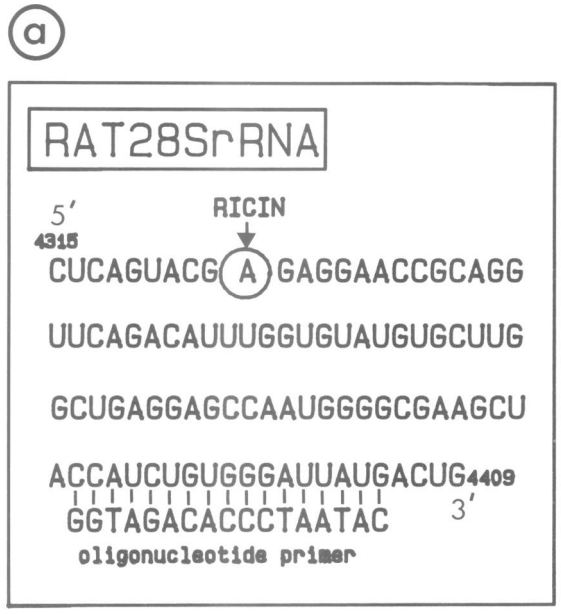


Fig. 8. Effect of converting arginine residues at positions 48 and 56 to alanine on the rRNA modifying activity of ricin A chain. Translation reaction mixtures were identical to those used for the experiments shown in Figure 7 except that the reticulocyte lysates had not been treated with micrococcal nuclease. Following 1 h translation, RNA was extracted, aniline treated where appropriate and fractionated as described in Materials and methods. **Lane 1**, Arg 48 → Ala; **lane 2**, Arg 56 → Ala; **lane 3**, wild-type ricin A chain; **lane 4**, control transcript encoding plastocyanin ferredoxin reductase. + indicates aniline treatment; − indicates no aniline treatment. The arrow indicates the 390-nucleotide fragment released by aniline treatment of modified rRNA.

identified for rat liver rRNA (Endo *et al.*, 1987; Endo and Tsurugi, 1987), and is shown in Figure 9a. Figure 9b shows a sequencing gel of the products of a primer extension, reverse-transcribed from unmodified rRNA, rRNA modified by wild-type A chain synthesized *in vitro* and two substitution mutants (Arg 48 → Ala, Arg 56 → Ala) described above. The product from wild-type A chain shows a major termination site (indicated by the arrow in Figure 9b, lane 2), corresponding to G4325 in the RNA sequence, i.e. one nucleotide to the 3′ side of A4324 identified by Endo *et al.* (1987) as the base catalytically removed by ricin A chain. This fragment is absent when unmodified rRNA is used as template (Figure 9b, lane 1), but is present in rRNA modified by the substitution mutants (Figure 9b, lanes 3 and 4). The arginine mutations do not therefore appear to have altered the specificity of the modification reaction. We have observed another strong termination site at position ~4250 in the rRNA (indicated by the asterisk in Figure 9b) but this is independent of ricin action since it is present in all samples and possibly represents a natural methylation site.

Translation in wheat germ extracts

Transcripts encoding wild-type ricin A chain and both active and non-active mutants are all translated efficiently in a wheat germ extract and accumulate in comparable amounts. The hexapeptide and pentapeptide deletions (Figure 10a, lanes 2 and 6 respectively) translate as polypeptides detectably smaller than wild-type A chain (Figure 10a, lane 1) and the arginine modifications (Figure 10a, lanes 3, 4 and 5). In all cases wheat germ ribosomes which have been translating



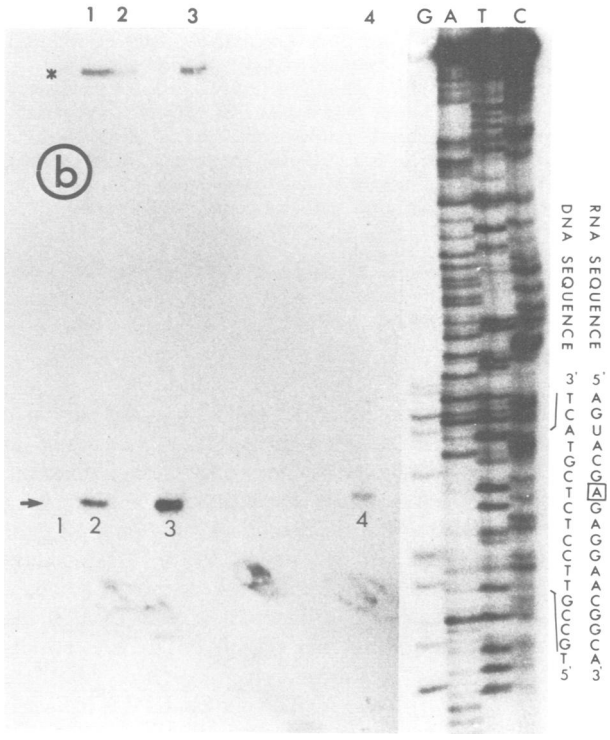


Fig. 9. Location of the ricin A chain modification site in 28S rRNA. **(a)** Part of the sequence of rat liver 28S rRNA showing the position of the adenine catalytically removed by ricin A chain, and the oligonucleotide used for primer extension of reticulocyte 28S rRNA. **(b)** Primer extension of reticulocyte rRNA. **lane 1**, untreated rRNA; **lane 2**, recombinant ricin A chain-modified rRNA; **lanes 3** and **4**, rRNA modified by two substitution mutants of ricin A chain, Arg 48 → Ala and Arg 56 → Ala, respectively. The arrow indicates the termination site caused by ricin A chain and the asterisk indicates a natural termination site on primer extension. Lanes labelled GTAC are the products of primer extension of unmodified rRNA synthesized in the presence of dideoxy-nucleotides as described by Moazed *et al.* (1986).

ricin A chain for 1 h remain equally capable of the further translation of a second mRNA added subsequently (Figure 10b). It appears therefore that wheat germ ribosomes are

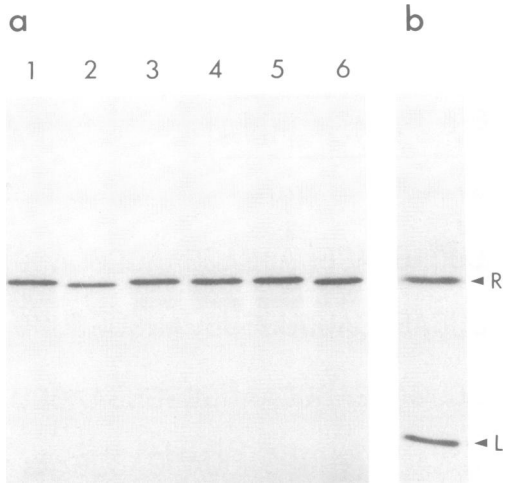


Fig. 10. Translation of transcripts encoding wild-type and mutant ricin A chains in wheat germ extracts. (**a**) [^{35}S]Methionine supplemented wheat germ extracts were allowed to translate, for 1 h, transcripts encoding wild-type ricin A chain (**lane 1**), ricin A chain from which a hexapeptide sharing homology with EF-2 had been deleted (**lane 2**), ricin A chain in which Arg 48 had been converted to Ala (**lane 3**), ricin A chain in which Arg 56 had been converted to Ala (**lane 4**), ricin A chain from which Arg 56 had been deleted (**lane 5**), and ricin A chain from which a pentapeptide conserved in trichosanthin had been deleted (**lane 6**). (**b**) A wheat germ lysate which had translated a transcript encoding wild-type ricin A chain for 1 h was supplemented with a transcript encoding chicken lysozyme and translation was allowed to continue for a further 30 min. The products were separated by SDS–PAGE and visualized by fluorography. R indicates the position of ricin A chain; L indicates the position of lysozyme.

much less sensitive to ricin than mammalian and yeast ribosomes.

Discussion

In the present study we have developed a simple and sensitive procedure in which expression and the assessment of biological activity of the ribosome-inactivating protein ricin are combined. This is feasible because ricin A chain is an extremely potent inhibitor of mammalian ribosomes (k_{cat} for ribosomes of 25 s^{-1}, Olsnes *et al.*, 1975). When rabbit reticulocyte ribosomes translate wild-type ricin A chain transcripts the translation product rapidly inactivates the ribosomes indicating that the protein is folded into its catalytically active conformation. *In vitro* ribosome inactivation by newly made A chain polypeptides is indicated by the failure of ribosomes to translate a second mRNA and is confirmed by the aniline cleavage assay (Figures 4 and 5). Further, primer extension and dideoxy sequencing has located the base in 28S rRNA cleaved by ricin A chain (Figure 9b) confirming it to be at or near to A4324 as reported previously for rat liver 28S rRNA. It should be noted that while the synthesis of a polypeptide with detectable catalytic activity in a eukaryotic cell free system is unusual, there is a precedent for this (Glass *et al.*, 1987).

We assume the *in vitro* assay would function effectively with transcripts encoding any ricin-like plant or bacterial toxin which specifically modifies 28S rRNA. The major requirement is that the translating ribosomes are sensitive at the concentrations used and are therefore rapidly inactivated by the amount of toxin generated. With sensitive ribosomes, such as those from rabbit reticulocytes or yeast (data not shown), the potency of ricin A chain is such that complete ribosome inactivation occurs even before the translation product can be detected as a significant radiolabelled band (Figure 4, for example). It is known that plant ribosomes are less sensitive to ricin A chain than their mammalian or yeast counterparts (Cawley *et al.*, 1977; Endo *et al.*, 1988; M.Hartley, unpublished observations). This is also shown by the data shown in Figure 10 where all the transcripts appear to be translated efficiently in wheat germ extracts. This observation allows us to confirm that unaltered ricin A chain transcripts, which translate to give barely detectable polypeptides in rabbit reticulocyte lysates, do translate to give proteins of the expected size. It may be significant that wheat (*Triticum aestivum*) produces its own single-chain ribosome-inactivating protein, tritin (Roberts and Stewart, 1979). It is quite conceivable that plants, many of which accumulate this type of toxin (Barbieri and Stirpe, 1986), have evolved ribosomes which are much less sensitive to them, although the molecular basis for the difference in sensitivity is unknown at present.

We have constructed several ricin A chain mutants and used the assay described above to assess their toxicity. The deletion of a hexapeptide which shares homology with hamster EF-2 and trichosanthin (Figure 6) resulted in a polypeptide completely devoid of activity towards ribosomes. The deleted peptide may cause a deleterious conformational change in a region which is important for activity. This may be due to a perturbation of the catalytic site itself or to a domain which normally interacts with a receptor on the ribosome. In contrast to the effects of the hexapeptide deletion, we found that deleting a pentapeptide-SEAAR- (also conserved in trichosanthin) from another region of the A chain had no effect on the ability of the polypeptide to inhibit protein synthesis (Figure 7).

Chemical modification studies have previously shown that the catalytic activity of ricin A chain (Watanabe and Funatsu, 1986) and the A chain-like protein gelonin from *Gelonium multiflorum* (Srinivasan *et al.*, 1985), are dramatically reduced after modification of certain arginine residues. In the case of ricin A chain, the key arginine residues were located in an N-terminal peptic fragment. Three of these (Arg 29, 48 and 56) are conserved in trichosanthin suggesting they may be functionally important. Two of these (Arg 48 and 56) are also appropriately placed in the putative active site region (Montfort *et al.*, 1987). However, the single conversion of Arg 48 → Ala or Arg 56 → Ala, and the deletion of Arg 56, are without effect on the depurination activity of ricin A chain. It is possible that the simultaneous substitution of several arginine residues may be necessary before toxicity is eliminated.

All A chains exhibiting an inhibitory effect on protein synthesis were shown to cause modification of reticulocyte 28S rRNA at a specific site lying in a conserved stretch of rRNA sequence which is identical to that shown by Endo *et al.* (1987) and Endo and Tsurugi (1987) as being the target site for ricin A chain (Figure 9). The exceptional specificity of these A chains synthesized *in vitro*, appears to be the same as that shown by non-recombinant, native A chain. The finding that polypeptides carrying the arginine substitutions show the same rRNA modification confirms that their inhibitory effect on protein synthesis (Figure 8) is the result of rRNA modification rather than a non-specific event. The

demonstration that biologically active expressed toxin specifically depurinates 28S rRNA also eliminates the possibility that inhibition of translation was due to low levels of double-stranded RNA contaminants which might have been generated during the transcription reaction.

It is not known whether the position of the target adenine is identical in rabbit reticulocyte 28S rRNA and in rat liver 28S rRNA, since the former has not been completely sequenced. However, it is likely that the two rRNAs are extensively homologous. Certainly the cDNA sequence from primer extension is exactly complementary to rat liver 28S rRNA as far as it can be read in the gel shown in Figure 9b. The corresponding conserved sequence in yeast 26S rRNA forms a surface exposed loop thought to be involved in elongation factor (EF)-1-dependent binding of aminoacyl tRNA to the ribosomal A site (Veldman *et al.*, 1981). This is consistent with the finding that ricin A chain, and other ribosome-inactivating proteins, inhibit EF-1-dependent binding of aminoacyl tRNA to sensitive ribosomes (Fernandez-Puertes and Vazquez, 1977; Endo *et al.*, 1988). The importance of the structural integrity of this loop in the rRNA for protein synthesis is demonstrated further by the finding that the fungal toxin, α sarcin, also acts here, but its action differs from that of ricin A chain as it is a ribonuclease (Endo *et al.*, 1893). The ribosomes of many organisms show rRNA backbone cleavages which occur as normal physiological events during ribosome ageing, but are nevertheless still active in protein synthesis (Cahn *et al.*, 1970; Ellis and Hartley, 1974). Cleavages or modifications in the rRNA loop region of interest here however, clearly lead to functional inactivation of the ribosome.

The experimental approach described here should prove useful in identifying residues and domains essential for the biological activity of ricin A chain and related toxins.

Materials and methods

Materials

All radioisotopes and enzymes for DNA manipulations and transcription were purchased from Amersham Corp. except for S1 nuclease which was from Sigma, AMV reverse transcriptase from Life Sciences, exonuclease III from New England Biolabs and mungbean nuclease and calf intestinal phosphatase form Boehringer, RNasin was from Premega Biotech Ltd and the cap analogue ^{7}m(5′)Gppp(5′)G was from Pharmacia. Aniline and formamide were from BDH, Poole, UK. Aniline was twice redistilled before use. Oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer and purified by HPLC. Ricin A chain was purified from *Ricinus communis* seeds as described previously (Butterworth and Lord, 1983).

Bacterial strains and plasmids

E.coli K12 strains DH1 and JM101 were used except for oligonucleotide directed mutagenesis when 71.18 mutL was used (Kramer *et al.*, 1984). Plasmids were kindly provided by the following: plastocyanin ferrodoxin reductase DNA (Smeekens *et al.*, 1986) by Dr C.Robinson (Warwick, UK); pGEM2α36 containing preproalpha factor coding sequence (Rothblatt *et al.*, 1987) by J.Rothblatt, EMBL, FRG; and pspLYS$^+$, containing the chicken lysozyme coding sequence (Drummond *et al.*, 1985), by A.Colman, Birmingham, UK. pGEM1 was purchased form Promega Biotech Ltd. pGEM1A contains a DNA fragment which includes the entire A chain coding sequence preceded by a truncated signal sequence (to −74) and an *Xho*I linker fused with a *Sal*I site, and followed by a translation stop codon, the natural linker sequence and a short stretch of ricin B chain sequence to the *Bam*HI site at +852. The base numbering is from the preproricin cDNA described by Lamb *et al.* (1985).

DNA manipulations

Recombinant DNA techniques were carried out essentially as described by Maniatis *et al.* (1982). In order to delete a stretch of six A chain codons which have homology with hamster EF-2, pGEM1A was cleaved with *Nde*I and the two base overhang removed using mungbean nuclease. The blunt-ended DNA was cleaved with *Eco*RI and the large fragment, including vector DNA, was isolated. pGEM1A was cleaved with *Bst*NI which cuts the DNA once in the A chain DNA and at three positions within the vector DNA. The reaction mixture was end-filled using Klenow and cleaved with *Eco*RI. The appropriate fragment of 315-bp, which carries the 5′ end of A chain, was isolated and ligated with the blunt ended *Eco*RI fragment isolated previously to generate pGEM1AΔEF-2. A 894 bp *Bam*HI fragment was excised from pGEM1AΔEF-2 and recloned into pSP64ΔBam. Other deletions (SEAAR and Arg 56) and substitutions were made by oligonucleotide site-directed mutagenesis of a *Hin*dIII−*Eco*RI fragment containing A chain cDNA ligated into M13mp18, following standard procedures (Zoller and Smith, 1982). 5′ deletions of pGEM1A were performed by firstly cleaving 5 μg of DNA with *Sac*I and *Xba*I and then by incubating at 25°C in 50 mM Tris−HCl pH 8.0, 5 mM $MgCl_2$ and 1 mM DTT in the presence of 50 U of exonuclease III. Samples were taken at 20-s intervals, heated at 65°C for 5 min and added to a 10-fold volume of S1 nuclease mix (30 mM Na acetate pH 4.6, 4 mM $ZnSO_4$, 250 mM NaCl, 0.2 mg/ml tRNA and 2.5 U S1 nuclease). After 20 min at 37°C samples were phenol extracted, precipitated in ethanol and the DNA finally digested with *Pst*I. Fragments of a mixed size range were isolated and ligated into pSP64XβM which had been cleaved with *Nco*I, end-filled and recleaved with *Pst*I. The extent of deletions which ultimately gave rise to translation products was determined by plasmid sequencing (Murphy and Kavanagh, 1988).

In vitro transcription and translation

Approximately 2 μg of plasmid DNA was linearized with *Kpn*I. This cleaves at +826 in the ricin cDNA, 23 bp downstream from the A chain translation stop codon. Linearized DNA was incubated in a final volume of 20 μl with 2 mM spermidine, 40 mM Hepes−KOH pH 7.5, 6 mM Mg acetate, 10 mM DTT, 100 μg/ml BSA, 0.5 mM each NTP except GTP (0.1 mM), 0.25 mM ^{7}mG(5′)ppp(5′)G, 20 U RNasin and 15 U SP6 RNA polymerase. Reactions were performed at 40°C for 30 min at which time GTP in 20 mM Hepes−KOH pH 7.5 was added to 0.4 mM and the reaction continued for a further 30 min. Electrophoretic analysis of aliquots from transcription reactions supplemented with [^{32}P]UTP followed by fluorography, confirmed that in all cases a single major transcript of the expected size was generated.

In vitro transcription mix (1 μl) was incubated for 60 min at 30°C with 10 μl of nuclease-treated rabbit reticulocyte lysate and 1.5 μl [^{35}S]-methionine. To assay the translation product of an SP6 polymerase-generated transcript for activity, 1 μl of a second transcript mix (yeast preproalpha factor or chicken lysozyme transcript) was added to the translation after 1 h and incubated for a further 30 min. Translation products were analysed by SDS−PAGE and visualized by fluorography as described previously (Roberts and Lord, 1981). Translation of 1 μl of transcription mix in a 12.5 μl wheat germ extract was carried out according to the procedure of Anderson *et al.* (1983).

Aniline cleavage of rRNA and gel fractionation

Total RNA was extracted from reticulocyte lysate translation mixtures as described by Hartley *et al.* (1975). RNA (3 μg) was incubated at 60°C for 3 min in 20 μl of 1 M aniline pH 4.5 (D'Alessio, 1982). Ethanol-precipitated RNA samples were dissolved in 20 μl of 60% formamide in 0.1 × E buffer (3.6 mm Tris, 3 mM NaH_2PO_4, 0.2 mM EDTA) (Loening, 1969), and incubated at 65°C for 5 min before cooling. Gels were electrophoresed in 1.2% agarose, 0.1 × E buffer and 50% formamide.

Primer extension

Oligonucleotide (100 ng) was end-labelled and annealed with 4 μg of total RNA from reticulocyte lysates in a final volume of 7.5 μl of 50 mM Hepes KOH pH 7.0, 5 mM Na borate and 0.1 M KCl. This annealing mix (1 μl) was incubated in a final volume of 5 μl containing 50 mM Tris−HCl pH 8.5, 50 mM KCl, 10 mM DTT, 10 mM $MgCl_2$, 50 μM of each dNTP and 1 U AMV reverse transcriptase for 30 min at 37°C (Moazed *et al.*, 1986). Dideoxy sequencing reactions were performed exactly as described by Moazed *et al.* (1986). Extension reactions were stopped by ethanol precipitation and DNA was electrophoresed on 8% acrylamide (1:20 bis, 7.5 M urea TBE gels (Maniatis *et al.*, 1982).

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