The A-chain of ricin is a cytotoxic RNA N-glycosidase that inactivates ribosomes by depurination of the adenosine at position 4324 in 28 S rRNA. Of the 267 amino acids in the protein, 222 could be deleted, in one or another of 74 mutants, without the loss of the capacity to catalyze hydrolysis of a single specific nucleotide in rRNA (Morris, K. N., and Wool, I. G. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4869–4873). The 45 amino acids that could not be omitted when the deletions were in sets of 20, 5, or 2 residues have now been deleted one at a time; 9 of these deletion mutants retained activity. A RNP-like structural motif in ricin A-chain that may mediate binding to ribosomal RNA has been identified.

Ricin is a cytotoxic RNA N-glycosidase that is synthesized in the castor bean Ricinus communis. Prorcin is a polypeptide of approximately 65 kDa which is processed by removal of 12 amino acids to form an A-chain of 267, and a B-chain of 262, residues linked by a disulfide bond (1). The toxic ricin A-chain (RA) inhibits protein synthesis by inactivating ribosomes; the inhibition is the result of the hydrolysis of the bond between the base and the ribose of the adenosine at position 4324 (A4324) in 28 S rRNA (2, 3). RA is extraordinarily toxic: a single molecule will inactivate 1500 ribosomes in 1 second (4). RA catalyzes depurination inactivates the ribosome implies that this region of 28 S RNA is crucial for the function of the particle and there is evidence that the ricin domain is involved in elongation factor-1 dependent binding of aminoacyl-tRNA to the ribosomal A-site and elongation factor-2 catalyzed GTP hydrolysis and translocation of peptidyl-tRNA to the P-site (4).

A substantial effort has been made to relate the structure of RA to its mechanism of action spurred in part by the use of the protein in the construction of immunotoxins for cancer therapy (5). The amino acid sequences of ricin and of several homologous proteins have been determined (6–15) and an atomic structure of the single molecule will inactivate 1500 ribosomes min⁻¹, indeed, a single molecule resident in a cell is sufficient to kill it (1); finally, this one covalent modification accounts entirely for the cytotoxicity.

A value of an analysis of the mechanism of action of antibiotics and of ribotoxins is that it directs attention to components of the ribosome where efforts to comprehend functional correlates of structure are likely to be rewarded. That this one RA-catalyzed depurination inactivates the ribosome implies that this region of 28 S RNA is crucial for the function of the particle and there is evidence that the ricin domain is involved in elongation factor-1 dependent binding of aminoacyl-tRNA to the ribosomal A-site and elongation factor-2 catalyzed GTP hydrolysis and translocation of peptidyl-tRNA to the P-site (4).

A substantial effort has been made to relate the structure of RA to its mechanism of action spurred in part by the use of the protein in the construction of immunotoxins for cancer therapy (5). The amino acid sequences of ricin and of several homologous proteins have been determined (6–15) and an atomic structure of ricin has been obtained from x-ray diffraction of crystals (16–18). In the three-dimensional structure there is a prominent cleft that was proposed to be the active site of RA before the mechanism of action of the toxin had been defined, indeed, before the substrate had been identified (16). Strong support for the suggestion has come from the observation that most of the amino acids that are invariant in related plant and bacterial toxins are clustered at the bottom of the putative active site cleft (18); from site-directed mutagenesis (19–20); and from an analysis by systematic deletion of amino acids (25).

Three series of amino acid deletions (of 20, 5, or 2 residues) that scanned RA were constructed: of 138 mutants, 74 retained the capacity to catalyze depurination of A4324 and in the latter 222 of the 267 residues in RA were omitted (25). Four additional amino acids could be omitted if care was taken to construct the deletions so the amphiphilic character of helix D was preserved (26). The resolution of the deletion map has now been extended as far as one can go by constructing single amino acid omissions encompassing the 45 residues that had produced inactive mutants when deleted as parts of larger sets.

**EXPERIMENTAL PROCEDURES**

Materials—Kanamycin and ampicillin were obtained from Sigma; T4 polynucleotide kinase, T4 DNA ligase, and ribonucleotides from Boehringer Mannheim; deoxyribonucleotides and dideoxyribonucleotides from Pharmacia Biotech Inc.; T7 DNA polymerase (Sequenase 1.0) from U. S. Biochemical Corp.; Taq DNA polymerase from Perkin-Elmer Corp.; restriction endonucleases from Biolabs Laboratory; and reticuloocyte lysate from Promega. The RA cDNA was isolated from Escherichia coli C36 cells transformed with pRAIB130 (25).

Mutagenesis—A BamHI-HindIII restriction fragment of 860 base pairs containing a RA cDNA was excised from the plasmid pRAp229 (27), provided by L. L. Houston of the Perkin-Elmer Corp., and subcloned in the plasmid pBl30 to form pRAIB130. This plasmid contains a gene for ampicillin resistance, the fl origin of replication, and the start codon of the RA cDNA 12 base pairs downstream from a T3 RNA polymerase promoter. E. coli strain C36 was transformed with pRAIB130 and the single-stranded replicative form of the plasmid was isolated by infecting these cells with the helper phage M13K07 (28).

Growth was for 16 h at 37°C in LB media containing kanamycin (10 μg/ml) to allow selection of infected cells. Cells were collected by centrifugation and the phage were precipitated with 300 mM NaCl and 6% polyethylene glycol 8000. Single-stranded DNA was isolated from the phage by multiple extractions with phenol and chloroform; the DNA was precipitated with ethanol and resuspended in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The single-stranded plasmid packaged by the helper phage contains uracil in place of some thymidines because C36 carries dUT, dU- mutations (29).

Deletions were made in the RA cDNA using synthetic oligodeoxyribonucleotides with bases complementary to the 5’ and 3’ sequences flanking the nucleotides to be deleted (25). The synthetic oligodeoxyribonucleotides (166 ng) were annealed to single-stranded pRAIB130 DNA (2 μg) in 10 μl of 20 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, and 50 mM NaCl. The reaction mixture was heated at 70°C for 5 min, allowed to cool to room temperature, and placed in an ice-water bath. The complementary DNA strand was synthesized with the annealed oligodeoxyribonucleotides as primers by adding reagents to the original annealing reaction to give the following concentrations in 13 μl: 23 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 35 mM NaCl, 1.5 mM dithiothreitol, 0.4 mM dATP, dCTP, dGTP, and dTTP, 0.75 mM ATP, 77 units/ml T4 DNA ligase, and 23 U/ml T4 DNA polymerase.
Ricin A-Chain Deletion Mutants

Analysis of Single Amino Acid Deletions in RA—A set of 45 deletions of 3 base pairs each was constructed in RA cDNA; each deletion encoded an amino acid (Table I) whose omission was not tolerated when part of larger deletions (25). The wild-type and mutant RA cDNAs were transcribed in vitro with viral T3 RNA polymerase. Transcription yielded a single RNA, i.e. a single band on electrophoresis in agarose gels (results not shown). The RNA from the transcription of approximately 70 ng of wild-type or mutant RA cDNA was translated in a rabbit reticulocyte lysate that had been depleted of endogenous mRNA (24).

Translation of the RNA was for 2 h at 37°C, incubation was purposely long so that depurination by the least active mutants might be detected. If a mutant is active, translation of the mRNA yields a protein that inactivates the reticulocyte ribosomes. Inactivation of ribosomes was assessed by formation of a fragment of 460 nucleotides after treatment of the extracted RNA with aniline which causes scission of the phosphoribose backbone at the site of depurination; scission is at A4324 in 28S RNA (see Fig. 1, lane 2). This fragment was produced when RA (Fig. 1, lane 2) or wild-type RA cDNA transcripts (Fig. 1, lane 3) were added to the lysate. However, depurination did not occur if no transcript (Fig. 1, lane 1) was added. The RNAs encoding each of the 45 deletion mutants were translated. The mutant polypeptides were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (results not shown). Generally there was an inverse correlation between the amount of the mutant RA synthesized, judged by the intensity of the RNA fragment, and the activity of the mutant, judged by the intensity of the RNA fragment band. However, there were too many exceptions for this to be a reliable means for quantifying the activity of the mutants. Of the 45 single amino acid deletions, 9 retained activity: ΔAsn-122, ΔTyr-123, ΔGly-140, ΔAsn-141, ΔMet-174, ΔPro-200, ΔAsp-201, ΔVal-204, and ΔIle-205 (Table I).

Control Experiments—The assay assesses only the presence or absence of residual activity of RA mutants. A decrease in the activity of the enzyme of 10–4 but not of 10–5 would be detected and scored. This estimate was derived from an assessment of the activity of decreasing amounts of wild-type transcript in

| Mutant | Activity | Mutant | Activity |
|--------|----------|--------|----------|
| Phe-24 | I        | Ile-175| I        |
| Ile-25 | I        | Ser-176| I        |
| Val-28 | I        | Glu-177| I        |
| Arg-29 | I        | Ala-178| I        |
| Val-81 | I        | Ala-179| I        |
| Val-82 | I        | Arg-180| I        |
| Gly-83 | I        | Phe-181| I        |
| Tyr-84 | I        | Gin-182| I        |
| Asn-122| A        | Tyr-183| I        |
| Tyr-123| A        | Ile-184| I        |
| Gly-140| A        | Pro-200| A        |
| Asn-141| A        | Asp-201| A        |
| Glu-146| I        | Pro-202| I        |
| Ala-147| I        | Ser-203| I        |
| Ile-148| I        | Val-204| A        |
| Ser-149| I        | Ile-205| A        |
| Phe-168| I        | Thr-206| I        |
| Ile-169| I        | Leu-207| I        |
| Ile-170| I        | Ser-210| I        |
| Cys-171| I        | Trp-211| I        |
| Ile-172| I        | Gly-212| I        |
| Gin-173| I        | Arg-213| I        |
| Met-174| A        |        |          |
the translation reaction and from a determination of the limit amount that gives detectable depurination (see below).

Because of the extraordinary activity of RA (1), and because of the exquisite sensitivity of the translation assay, it was important to rule out the possibility that the apparent enzymatic activity of the mutants was due to contamination with wild-type RA. This requires assurance that the mutant transcripts are not contaminated with wild-type mRNA. The smallest amount of contaminating wild-type DNA that would yield enough transcript to result in detectable depurination of 28 S rRNA upon translation in the reticulocyte lysate was determined. Transcripts were prepared from mixtures having various ratios of the DNAs (a total of 250 ng) encoding an inactive mutant (ΔArg-213) and the wild-type; one-fourth of the total was translated in the lysate. Depurination was observed when the amount of wild-type DNA was 25 pg (only 0.04% of the total in the mixture) but not when it was 10 pg. Thus translation of transcripts from extremely small amounts of cDNA can catalyze sufficient depurination to be detected and this amount of cDNA would not be discerned on sequencing gels. Therefore, the quality of the results is conditioned by the purity of the mutant clones.

Single colonies were selected from among transformants and grown overnight; the DNA was isolated and the sequence of nucleotides determined. The colonies with mutant DNA were streaked on agar plates so that single colonies could be selected easily. This is the colony purification procedure. These cells were grown, the DNA purified, and the sequence of nucleotides determined again. As a further precaution, cells were transformed with the DNA that had been isolated after colony purification from the nine active mutants and an additional cycle of selection and colony purification was carried out. None of these mutants lost their activity in the process.

The polymerase chain reaction was used to detect possible contamination of active mutants with wild-type DNA. Pairs of oligodeoxynucleotides were synthesized; one complementary to a sequence in both mutant and wild-type DNA, the second only to the wild-type. In control experiments with mixtures of wild-type and mutant DNA as little as 1 pg of the former could be detected after amplification. No wild-type DNA was detected in the reaction with the mutants specified above. Therefore, contamination is with less than 1 pg of wild-type DNA, much less than the amount (between 10 and 25 pg) required after transcription and translation to give detectable depurination. We conclude that the activity of the mutants cannot be accounted for by contamination with wild-type DNA.

**DISCUSSION**

Inspection of the amino acid sequences of 11 ribosome inactivating proteins that have the same mechanism of action as ricin led to the identification of 13 invariant residues (35). In RA they are: Tyr-21, Phe-24, Arg-29, Tyr-80, Tyr-123, Gly-140, Ala-165, Glu-177, Ala-178, Arg-180, Glu-208, Asn-209, and Trp-211 (Fig. 2). Most of these amino acids are congregated in or near the putative active site cleft; three are in helix E. The identification of the invariant residues supported the assumption from the three-dimensional structure that the cleft was the active site and provided the first indication of which amino acids might be important for the function of the toxin.

Several of the invariant residues were subsequently the object of site-directed mutagenesis. For example, mutations were made in Glu-177 and Arg-180 (19, 20, 36). These residues were chosen not only because they are invariant but also because they were thought to be directly involved in catalysis. The details of the chemistry of the hydrolysis of the N-glycosidic bond of A4324 in 28 S rRNA by RA are not known, however, a mechanism has been proposed based on an analogy with the cleavage by a nucleosidase of the bond between adenosine and ribose in AMP, a reaction that has been studied in some detail (37). For RA the proposal (38) is that Arg-180 forms a hydrogen bond with N-3 of the substrate adenosine in a strained syn configuration approximating the transition state; this interaction may promote the adenine ring transiently and thereby facilitate bond breakage. Glu-177 is thought to stabilize the developing oxycarbonium character of the ribose ring and, perhaps, also to polarize the water molecule that may be the nucleophile; the nitrogen-carbon bond is then displaced by nucleophilic attack of the water molecule on the C-1 of ribose. The conversion of the invariant Glu-177 to aspartic acid or to alanine reduced, by 0.08 and 0.18, respectively, but did not abolish enzymatic activity (19). To rationalize the disappointingly trivial affect of the E177A mutation, it was suggested that either the carboxylate group at position 177 is not important for catalysis (19), which seemed unlikely, or that Glu-208, which is also at the bottom of the cleft, can substitute for the missing functional group (20). The E177D mutant may be less active than wild-type RA because the carboxylate of the shorter aspartic acid side chain is unable to reach an optimum position for catalysis and because it prevents Glu-208, by electrostatic repulsion, from moving into an effective position. The neutral alanine side chain in the E177A mutant, on the other hand, might not interfere with the positioning of the carboxylate of Glu-208. This possibility was tested by constructing an E177A,E208D double mutant which had, as predicted, virtually no activity; a result that supports the original explanation.
that Glu-208 can substitute for Glu-177 in the E177A mutant and affirms that a carboxylate function is important for catalysis. A control mutant, E208D, with no change at position 177, had enzymatic activity equal to the wild type and affirms that a carboxylate function is important for catalysis. The contribution of the side chain of Arg-180 was assessed by converting it to lysine and histidine (20). These mutations had no effect on enzymatic activity (22, 23), presumably because phenylalanine can also contribute to binding, as tyrosine and tryptophan are thought to contribute by intercalating its aromatic ring between the bases of RNA. The conversion of Tyr-80 and Tyr-123 to serine, on the other hand, decreased enzymatic activity by 0.005 and 0.01, respectively (36). The conversion of the invariant Asn-209 which is at the bottom of the cleft to serine had only a relatively small effect on enzymatic activity (23). Arg-56 is near invariant (it is either arginine or lysine in the 11 toxins), is near the cleft, and might interact with bases in rRNA or with the phosphate backbone; however, an R56A mutation had no effect on the ability of the toxin to depurinate A4324 (24). In this vein, modification of arginine residues at positions 193, 196, 213, and 234, and 235 with phenylglyoxal had no effect on RA activity either (40).

Thus, apart from the implication of Glu-177 and Arg-180 in the chemistry of catalysis, site-directed mutagenesis had not provided a great deal of help in the identification of functionally important residues. Perhaps for this reason another approach was adopted: yeast were transformed with an expression vector containing randomly mutated RA cDNA (41). The assumption was that only yeast harboring a RA cDNA with an inactivating mutation would continue to grow. The sequence of nucleotides was determined for cDNAs isolated from seven yeast colonies that survived induction of expression of the toxin. The mutations were: E177D and E177K; W211R; G212W and G212E; S215P; and I252R. These results must be interpreted with caution since cells apparently can survive even if the mutant toxin is still active. For example, the E177D mutant retains activity (0.08 of wild-type) when assayed on ribosomes in a reticulocyte lysate (19); in addition, Ser-215 and Ile-252 can be deleted from RA without complete loss of activity (25) which leads to a strong presumption that their conversion to proline and arginine would not lead to a complete loss of activity either, a presumption strengthened by the fact of their being distant from the putative active site.

Another approach is to include all of the RA amino acids in the screen. In systematic deletion analysis one starts with a set of contiguous deletions, for example, of 20 amino acids as was done with RA (25), each deletion adjacent to the preceding one. Subsequent sets increase the resolution of the deletion map by analyzing smaller omissions, first of 5 and then of 2 residues, and finally as here of one amino acid; each set after the first encompassing all of the residues whose omission had produced inactive mutants in the previous set. What recommends this approach is that it is blind to prejudices or preconceived ideas as to where the active site is and to which residues are important, one simply surveys them all. In the present study 9 of 45 single amino acid deletions retained activity (Fig. 3A). Thus in the four sets of amino acid deletions, we have identified 32 amino acids that cannot be dispensed with/without loss of activity. The 32 residues are congregated in or near the putative active site cleft (Fig. 3B). The 32 include 6 of the 13 invariant amino acids.

Helix E forms the backbone of RA (18) and has the active site residues Glu-177 and Arg-180 which are at the bottom of the cleft (Figs. 2A and 3). The largest concentration of amino acids whose omission inactivates the enzyme are in helix E and the adjacent unstructured region, 16 of 17 residues between positions 168 and 184; this is half of all the essential residues and it is noteworthy that 11 of the 17 are hydrophobic. The single residue in this sequence that can be omitted is Met-174 which is difficult to rationalize since it is very close to Glu-177; the distance from Cα of Glu-177 to the Cα of Met-174 is only 6.25
Moreover, Met-174 appears to contribute to the stabilization of the catalytic center. E is the longest helix in RA and has a distinct bend of roughly 30° near its carboxyl-terminal end. This bend positions Glu-177 and Arg-180 in the active site cavity. The bend disrupts the local hydrogen bonding pattern in the helix (18) near the carboxyl carbon of Met-174; the latter is rotated away from the helix axis by roughly 50° thereby allowing it to hydrogen bond to the hydroxyl group of Tyr-21. Thus, the bend does not depend on the Met-174 side chain and in the absence of the amino acid the carboxyl carbon of Ile-175 may be able to compensate for the loss of Met-174 and for a perturbation in the structure of helix E.

Phe-168 and Ile-172 in helix E are important (they cannot be deleted), perhaps because they stabilize the core through hydrophobic contacts with residues in the β-sheet (Tyr-84, Phe-93, and Phe-117) and in helix C (Tyr-123 and Leu-126) (18). Phe-181 is another essential nearby amino acid that interacts with the invariant Trp-211 and presumably their close packing is important to the geometry of the active site. The essential Ile-184 also contacts Phe-181 and the methylene carbons of the active site Glu-177 further stabilizing the active center. Thus, the cluster of essential hydrophobic residues in or near helix E appear to be important for the packing and hence the geometry of the active site.

All of the amino acids in helices B, C, and D are dispensable, although helix D is a special case (25, 26). Each of the amino acids in helix D can be deleted from one mutant or another provided care is taken that the deletion does not disrupt the amphipathicity of the helix (26). The hydrophobic surface of helix D abuts helix E and the hydrophilic surface shields helix E from solvent. Thus it is not surprising that single amino acid deletions in helix D (Glu-146, Ala-147, Ile-148, and Ser-149) abolished RA activity (Table I).

Many of the residues in helices A, G, F, and H can be deleted, although a number are essential (Fig. 2 and Table I). For example, Phe-24, Ile-25, Val-28, and the invariant Arg-29 in helix A cannot be omitted. These amino acids are relatively distant from Glu-177 and Arg-180 so it is unlikely they participate directly in the chemistry of catalysis; this suggests, but only because there is no other explanation, that they participate in substrate binding. Five of the nine residues in helix G (Pro-202, Ser-203, Thr-206, Leu-207, and Ser-208) are essential (Fig. 2 and Table I); the role of these five residues in the structure and function of RA cannot be defined. The activity of RA does not survive deletion, even one at a time, of three residues (Trp-211, Gly-212, and Arg-213) in helix H (Table I); however, Arg-213 can be chemically modified with phenylglyoxal, and presumably inactivated, without loss of RA activity (40). The invariant Trp-211 is thought to participate in the binding of the adenosine in the RNA substrate (39).

A relatively large number of proteins that bind to RNA have a domain, referred to as the RNP motif (cf. Ref. 42 for citations and discussion). The element has 90 to 100 amino acids and its most characteristic feature is two conserved sequences of 6 and 8 residues, RNP1 and RNP2; there are also a number of conserved, mostly hydrophobic, residues distributed in the motif. It is important that some RNA binding proteins lack consensus RNP1 and RNP2 amino acid sequences but have similar, structurally significant, residues. The three-dimensional structures of the RNP domains of two proteins, U1A (43) and hnRNP C (44), have been determined and they share a ββαββαβ fold in which the four β-strands form an antiparallel β-sheet packed against the two perpendicularly oriented α helices. In the figure, amino acids that could not be deleted in any of the mutants without loss of RA activity are in red.
folded domain the amino acids of RNP1 and RNP2 are juxtaposed in the central strands (β1 and β3) of the β-sheet and the charged and aromatic side chains of these amino acids are thought to interact with bound RNA through hydrogen bonds and stacking of rings. The conserved residues of RNP1 and RNP2 are critical for the association with RNA but do not confer specificity on binding; specificity is believed to reside in the variable regions especially in the unstructured loops. Binding studies (Ref. 42 and references therein) support this interpretation in that they indicate that the β-sheet of the RNP motif can make nucleotide sequence independent, and hence nonspecific, contacts with RNA.

RA has an RNP-like structural motif, different from the canonical but with sufficient similarity to justify a comparison to the domains in U1A (Fig. 4). (We chose U1A for the comparison because the coordinates for the structure are available whereas those for hnRNP C are not.) The RNP-like motif of RA is composed of the following secondary structure elements: βααββββββ...αE; this is to be contrasted with the related elements in U1A, βααββββββ...αE. Thus the motif in RA differs from the canonical, and particularly from that in U1A, in the order of, and in the lack of continuity in the sequence of amino acids in the secondary structure elements; and perhaps most importantly in that RA has neither RNP1 nor RNP2 consensus amino acid sequences. Recall, however, that RNP1 and RNP2 sequences are not absolutely essential to RNP motifs.

Despite these differences the tertiary structure of the RNP domain in RA is in large measure congruent with the RNA binding domain of U1A (Fig. 4). In particular the β-sheet of RA closely approximates that of U1A; the root mean square deviation of the Cα backbones is about 1 Å. The structural relationship of the strands in the two β-sheets are as follows: βα of RA to β1 (RNP2) of U1A; βγ of RA to β3 (RNP1) of U1A; and βδ of RA to β2 of U1A. Indeed, there is only one significant difference in the β-sheets: βα of RA is parallel to βα, whereas in U1A βα is antiparallel to β3.

In the comparison of the structures, helix αA of RA is displaced from the related helix α2 of U1A by 4 Å although they are parallel. It is possible that the binding of RNA induces a change in the position of helix αA of RA bringing it closer to βα and thereby to more nearly approximate the relationship of α2 and β4 of U1A. The long loop between βα and βδ of RA might facilitate this change in structure. Helix αE of RA corresponds to α1 of U1A, in the superimposition of the structures the two helices intersect at an angle of about 20°. The intersection is at Met-174 of RA which is the site of a bend in helix E (see earlier); this bend in αE brings the carboxyl-terminal regions of the two helices close together.

We judge that there is sufficient structural similarity of the RNP-like domain of RA to the structure in U1A to suggest that the former is involved in binding to 28 S rRNA. The β-sheet of the motif might mediate an initial, nonspecific, low affinity association of RA with ribosomal RNA; this initial interaction might facilitate subsequent homing in on a high affinity, specific site and the positioning of A4324 in the catalytic center. This interpretation is supported by the observation that a number of hydrophobic and aromatic residues in βδ (VVGY), in αA (F1...VR), and in αE (F11C1Q1SEAR) are essential for RA activity, indeed, these 3 structural elements have more than half (20 of 32) of the essential residues in RA.

Two hundred and three deletion mutants of RA have been constructed and 96 retain activity. In those 96, 235 of the 267 amino acids (88%) in the protein were omitted. This is an indication that many of the residues are neither absolutely essential for folding into some effective conformation nor for catalysis. We recognize that in any single mutant the deletion that was tolerated was short, only in one instance as long as 20 residues and in the others 5 amino acids or less. Nonetheless, it is surprising that residues that are hydrophobic and are buried in the interior of the protein, or that are part of α-helices or of β-strands, can be deleted without loss of activity. This unexpected outcome reinforces the conviction that deletion analysis is a valuable procedure for defining the minimal co-hort of amino acids necessary for substrate recognition and catalysis. The findings from deletions of amino acids and from site-directed mutagenesis indicate that RA has a great capacity for compensatory change in structure and, hence, a great ability to preserve function; in short, the protein displays considerable plasticity.

Acknowledgment—We are grateful to our colleagues Yuen-Ling Chan and Anton Glück for advice on experimental procedures and for critically reading the manuscript. We received material assistance with the molecular graphics from Zhong Ren and Xiaojing Yang. Arlene Timo-siek provided help in the preparation of the manuscript.
Rutenber, E., Xuong, N. H., Hamlin, R., and Robertus, J. D. (1987) J. Biol. Chem. 262, 5398–5403.

17. Rutenber, E., Katzin, B. J., Ernst, S., Collins, E. J., Mlsna, D., Ready, M. P., and Robertus, J. D. (1991) Proteins 10, 240–250.

18. Katzin, B. J., Collins, E. J., and Robertus, J. D. (1991) Proteins 10, 251–259.

19. Schlossman, D., Withers, D., Welsh, P., Alexander, A., Robertus, J., and Frankel, A. (1989) Mol. Cell. Biol. 9, 5012–5021.

20. Frankel, A., Welsh, P., Richardson, J., and Robertus, J. D. (1990) Mol. Cell. Biol. 10, 6257–6263.

21. Ready, M. P., Katzin, B. J., and Robertus, J. D. (1988) Proteins 3, 53–59.

22. Bradley, J. L., and McGuire, P. M. (1990) Int. J. Peptide Protein Res. 35, 365–366.

23. Ready, M. P., Kim, Y., and Robertus, J. D. (1991) Proteins 10, 270–278.

24. May, M. J., Hartley, M. R., Roberts, L. M., Krieg, P. A., Osborn, R. W., and Lord, J. M. (1989) EMBO J. 8, 301–308.

25. Morris, K. N., and Wool, I. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4869–4873.

26. Morris, K. N., and Wool, I. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7530–7533.

27. Piatak, M., Lane, J. A., Laird, W., Bjorn, M. J., Wang, A., and Williams, M. (1988) J. Biol. Chem. 263, 4837–4843.

28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

29. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382.

30. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492.

31. Holmes, D. S., and Quigley, M. (1981) Anal. Biochem. 114, 193–197.

32. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

33. Morris, C. E., Klement, J. F., and McAllister, W. T. (1986) Gene (Amst.) 41, 193–200.

34. Evans, S. V. (1993) J. Mol. Graphics 11, 134–138.

35. Funatsu, G., Islam, M. R., Minami, Y., Sung-Sil, K., and Kimura, M. (1991) Biodemis 73, 1157–1161.

36. Kim, Y., and Robertus, J. D. (1992) Protein Eng. 5, 775–779.

37. Mench, F., Parkin, D. W., and Schramm, V. L. (1987) Biochemistry 26, 921–930.

38. Monzingo, A. F., and Robertus, J. D. (1992) J. Mol. Biol. 227, 1136–1145.

39. Watanabe, K., Honjo, E., Tsukamoto, T., and Funatsu, G. (1992) FEBS Lett. 304, 249–251.

40. Watanabe, K., Dansako, H., Asada, N., Sakai, M., and Funatsu, G. (1994) Biochim. Biophys. Acta 1208, 716–721.

41. Frankel, A., Schlossman, D., Welsh, P., Hertler, A., Withers, D., and Johnston, S. (1988) J. Biol. Chem. 263, 4837–4843.

42. Burd, C. G., and Dreyfuss, G. (1994) Science 265, 615–621.

43. Oubridge, C., Ito, N., Evans, P. R., Teo, C. H., and Nagai, K. (1994) Nature 372, 432–438.

44. Wittekind, M., Gorlach, M., Friedrichs, M., Dreyfuss, G., and Mueller, L. (1992) Biochemistry 31, 6254–6265.
