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Polynucleotide Sequence Analysis by Sequential Base Elimination: 3′-Terminus of Phage Qβ RNA

Abstract. *The nucleotide sequence of a 3′-terminal fragment obtained by ribonuclease T_1 hydrolysis of the ribonucleic acid from bacteriophage Qβ has been determined by an improved method of sequence analysis which involves sequential removal of bases by periodate oxidation and beta-elimination. The results obtained from ten such oxidation-elimination cycles and from the alkaline hydrolysis of the remaining oligonucleotide indicate that the first 16 nucleotides at the 3′-terminus of this ribonucleic acid have the sequence:*
-G-C-C-C-U-C-U-C-U-C-C-U-C-C-C-A.

The initial phase in the sequence analysis of RNA molecules (*1*) of high molecular weight has involved the development of methods for the investigation of the primary structures near their terminals. Although these methods are expected to produce only a limited amount of sequence information relative to that contained in the whole molecule, it is considered that such information will be of some value in studies on structure-function relations in RNA. In the case of viral ribonucleic acids, the interest in terminal structures results from the expectation that they will be found to contain specific nucleotide sequences which signal for the initiation and termination of both the replication and translation of these molecules. The terminal sequence -G(C_9U_4)C-A of the RNA from the bacteriophage Qβ has already been shown to differ from the corresponding sequences in the phages f2 and MS2, and it has been suggested that this difference in sequence may constitute the molecular basis of the observed template specificities displayed by the Qβ and MS2 polymerases (*2*).

For the specification of the actual order of the 14 pyrimidines at the Qβ terminus we have studied further the periodate-beta-elimination method that was developed for the determination of the terminal sequence -G-U-U-A-C-C-A-C-C-C-A of the f2 RNA (*3*). In this method the bases are removed sequentially from the 3′ end of the polynucleotide by cycles of operations consisting of (i) periodate oxidation of the terminal glycol group; (ii) treatment of the resulting dialdehyde with a primary amine to effect the removal of the terminal base by a beta-elimination reaction; and (iii) the removal of the terminal phosphate group, so produced, by phosphatase to regenerate the polynucleotide in a form ready for the next cycle. Previously we had used a cyclohexylamine solution buffered with di-*n*-propylmalonic acid for the beta-elimination step, because this mixture gave essentially quantitative yields when applied to the cleavage of small oligonucleotides. However, the results of experiments with this amine solution and larger oligonucleotides have indicated that, in these cases, the yields of the beta-elimination reaction fall somewhat short of the desired 100 percent. A new amine mixture containing cyclohexylamine and tetramethylglycinamide is now being used for this reaction, because, in experiments with both dinucleotides and hexanucleotides, this solution has been found to effect over 97 percent removal of the oxidized terminal nucleosides. The tetramethylglycinamide also serves another purpose in that it is used as the buffer for the subsequent enzymatic dephosphorylation step in each cycle. A second major modification in this method of sequence analysis resulted from the observation that the DEAE-Sephadex chromatography used to separate the liberated base from the polynucleotide fragment resulted in the incomplete recovery of the fragment in each cycle. This problem has now been solved by substituting, for the chromatographic matrix, DEAE-cellulose from which the recoveries are rapid and essentially quantitative.

In a typical oxidation-elimination cycle the polynucleotide (10 to 100 nmole) in water (250 μl) is treated with EDTA (free acid, 2.0 mg) to inactivate any phosphatase which is carried over from the previous cycle. The mixture is allowed to stand for 1 hour at 20°C with occasional shaking and then the amine solution (1*M* cyclohexylamine—2*M* *N,N,N′,N′*-tetramethylglycinamide-HCl; 100 μl) is added. When the EDTA is completely dissolved, 0.2*M* sodium periodate (50 μl) is added, and the mixture is kept at 45°C for 2 hours. The solution is then treated with 0.4*M* ribose (50 μl) to remove any unreacted periodate. After incubation of the solution for 15 minutes at 20°C, water (500 μl) and alkaline phosphatase [2 units (*4*) in 100 μl of water] are added (final *p*H = 8.0 at 37°C). The mixture is incubated at 37°C for 2 hours and then adjusted to *p*H 9.0 by the addition of 2*M* ammonium hydroxide (200 μl), and finally applied to a DEAE-cellulose column (Whatman DE23, HCO_3^- form, *p*H 9.0; 0.5 by 50 cm). The column is eluted with water to recover the liberated base and then treated with a linear gradient of triethylammonium bicarbonate (200 ml, 0.1 to 1.0*M*) at a flow rate of 15 ml/hour to recover the polynucleotide fragment. The combined fractions containing the fragment are then concentrated to dryness ready for the next cycle.

The 3′-terminal fragment of Qβ RNA was isolated by the method previously described (*5*). In this method the RNA is first digested with ribonuclease T_1 and then treated with sodium periodate. The oxidized terminal fragment is selectively absorbed on an aminoethyl-cellulose column and subsequently recovered from the column by treatment with *n*-propylammonium bicarbonate. This treatment releases the fragment by a beta-elimination reaction, and the fragment is obtained in a form that lacks its terminal nucleoside. The fragment from Qβ RNA obtained in this way had the composition (C_9U_4)Cp (*2*). This polynucleotide was dephosphorylated, and the product was submitted to ten cycles of periodate oxidation-beta-elimination. The order of the bases released from the 3′ end was: C,C,C,U,C,C,U,C,U,C. The identity of the base released in each degradation cycle was determined by its position of elution from the DEAE-cellulose column and by its ultraviolet spectrum. In a number of cases the entire water eluate from the column was analyzed on a small column of Dowex 50 × 8 (200 to 400 mesh, 0.4 by 60 cm) (*6*) to confirm that only one base was released at each degradation cycle. The recovery of the base from each cycle was more than 80 percent, based on the amount of oligonucleotide introduced into the cycle. For example, in cycle five, 4.25 O.D.U. at 260 nm of oligonucleotide gave 0.32 O.D.U. at 265 nm of cytosine.

The remaining part of the sequence of the terminal fragment was determined by the alkaline hydrolysis of the oligonucleotide that remained after the ten degradation cycles. This oligonucleotide (0.45 O.D.U. at 260 nm) was hydro-

lyzed with 0.25*N* sodium hydroxide (0.2 ml) for 18 hours at 37°C. The mixture was then neutralized with carbon dioxide and applied to a DEAE-Sephadex column (HCO_3^- form, *p*H 9.0; 0.4 by 100 cm). Elution with water gave uridine (16 nmole) as the only nucleoside, and subsequent elution with 0.2*M* ammonium bicarbonate (*p*H 9.0) gave cytidine 2′(3′)-phosphate (49 nmole) as the only nucleotide. The ratio of Cp to U was 3.02 to 1.0, indicating that the structure of the oligonucleotide was C-C-C-U. These results and the results of the ten degradation cycles taken together with the known specificity of ribonuclease T_1 and the results of the terminal base determination of the intact Qβ RNA (*2*) demonstrate that the 3′-terminal hexadecanucleotide sequence of the RNA is as shown above.

Recently, Dahlberg has also studied the 3′ terminus of Qβ RNA and has proposed (*7*) a nucleotide sequence which, like the present work, was based on the compositional formula -G(C_9U_4)C-A reported earlier (*2*). However, this sequence differs from that reported here in four positions (residues 7, 8, 9, and 10 from the 3′ terminus). A revised sequence has also been communicated by the same author (*8*), and this sequence differs in two positions (residues 5 and 6) from that derived here. At the moment the reasons for these conflicting results are not clear because the methods of sequence analysis used by Dahlberg differ quite markedly from ours and the experimental details of that analysis are not yet available for comparison with those reported here.

While the periodate-beta-elimination method yields results which appear to be unambiguous, it can be seen that the experimental operations suffer from the fact that they are rather time-consuming. However, because the method employs a cyclic process of degradation it is open to improvement by automation. Initial experiments have shown that it is possible to carry out all of the steps required in each degradative cycle while the polynucleotide is bound to a solid support. We are presently using this principle to develop a sequencing machine which will carry out the degradative cycles automatically, and this approach should reduce the time of analysis to about 2 hours per base.

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References and Notes

1. Abbreviations: RNA, ribonucleic acid; DEAE, diethylaminoethyl; O.D.U., optical density unit; Cp, cytidine 2′(3′)-phosphate; U, uridine; C, cytidine; A, adenosine; and G, guanosine. Sequences are presented in the standard way by the linear array of nucleosides, with the 3′-hydroxyl terminus to the right. Nucleosides in parentheses are of still-undetermined sequence.
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Single Chain Alkali Resistance in Hemoglobin Rainier: β 145 Tyrosine → Histidine

Abstract. *The mechanism of alkali resistance in hemoglobin Rainier, an adult human hemoglobin variant (β 145 tyrosine → histidine), has been investigated. Alkali denaturation kinetics, electrophoretic and hybridization study, and ultracentrifuge analysis provided evidence for a monomeric β Rainier chain resisting denaturation at alkaline pH. These data provide evidence for a previously unrecognized effect of a single amino acid substitution, that is, the change of the alkali denaturation properties of monomeric chains.*

Increased resistance to denaturation by alkali is a characteristic of human fetal hemoglobin ($\alpha_2\gamma_2$) and several adult animal hemoglobins ($\alpha_2\beta_2$). The structural basis of this property is not known. Dissociation studies and the first-order alkali denaturation kinetics in human Hb $\alpha_2\gamma_2$ and in bovine Hb $\alpha_2\beta_2$ support the postulate that the increased alkali resistance is confined to symmetrical dimers ($\alpha\beta$) rather than asymmetrical subunits (α_2 and β_2) (*1*). Among many variant hemoglobins caused by single amino acid substitutions, only Hb Rainier (β 145 Tyrosine → Histidine) exhibits resistance to alkali denaturation (*2*). Thus, the structural alteration causing alkali resistance is known. The mechanism, however, by which an amino acid substitution near the carboxyl terminal of the β chain alters the alkali denaturation properties of the hemoglobin is not apparent. Therefore, we tested whether in Hb Rainier alkali resistance is a property of the $\alpha\beta$ Rainier dimer, the β_2 Rainier dimer, or the monomeric β Rainier chain.

Alkali denaturation of Hb Rainier differs strikingly from that of Hb $\alpha_2\gamma_2$ (Fig. 1). In contrast to the first-order kinetics of fetal hemoglobin, approximately 50 percent of Hb Rainier is denatured within the first minutes of the reaction, while denaturation of the remaining hemoglobin fraction proceeds significantly slower than the denaturation of Hb F (Fig. 1). These findings are inconsistent with alkali resistance of symmetrical $\alpha\beta$ Rainier dimers, because first-order kinetics would be expected in that case. The observed biphasic alkali denaturation is more consistent with alkali resistance of only one type of hemoglobin chain. To test this possibility, hemoglobin Rainier was treated with 0.06*N* NaOH from 2 to 30 minutes. The hemoglobin solution was then neutralized, concentrated by vacuum dialysis, and studied by starch-gel electrophoresis in a buffer system of tris(hydroxymethyl)aminomethane(tris), ethylenediaminetetraacetate (EDTA), and borate at *p*H 8.6 (Fig. 2). After 2 minutes in 0.06*N* NaOH, only traces of hemoglobin with the electrophoretic migration of Hb Rainier were observed. However, a new fast-moving hemoglobin fraction with a mobility expected for the β Rainier chain appeared on electrophoresis (Fig. 2). The electrophoretic migration of this fraction was identical to that of the β Rainier chain prepared by treatment of Hb Rainier with *p*-chloromercuribenzoate (PCMB) (*3*), an indication that it consists of β Rainier chains. The β Rainier chains recovered after treatment of Hb Rainier with 0.06*N* NaOH were mixed with equal concentrations of αA chains prepared by treatment with PCMB; the mixture was incubated for 2 hours at 4°C and examined by starch-gel electrophoresis in the tris-EDTA-borate buffer system at *p*H 8.6. The β Rainier chain recombined with the αA chain to form hemoglobin Rainier (Fig. 2). These findings indicate that the hemoglobin recovered after alkali treatment is free β Rainier chain; therefore, the biphasic alkali denaturation kinetics of Hb Rainier are due to the original rapid denaturation of the αA chain and the subsequent resistance to denaturation of the β Rainier chain.