Preparation of Large Oligonucleotides from High Molecular Weight Ribonucleic Acid*

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ABSTRACT: High molecular weight ribonucleic acid (RNA) of the *Escherichia coli* phage MS 2 was adsorbed on DEAE-cellulose and hydrolyzed *in situ* with one of four different ribonucleases. After nuclease removal or inactivation, stepwise elution with solutions of increasing salt concentrations yielded predominantly large oligomers as ascertained by end-group chain-length determination and column chromatography on polyacrylamide gels. With *Bacillus subtilis* ribonuclease, significant differences were obtained between the base ratios of the oligomers and that of MS 2 ribonucleic acid.

Penswick and Holley (1965) reported that alanine soluble ribonucleic acid was selectively hydrolyzed at only one site with small amounts of RNAase T_1 at 0° in the presence of 0.01 M Mg. With high molecular weight RNA from bacteriophage MS 2, the same conditions of hydrolysis yield a variety of oligomers as well as undegraded RNA. The larger oligomers (n = 10–100) so produced have base ratios identical with those of the parent molecule and require further fractionation in order to remove native MS 2 RNA (G. W. Rushizky, unpublished data, 1966).

As described here, enzymatic hydrolysis of RNA previously adsorbed to DEAE-cellulose (rather than in the presence of Mg) followed by stepwise removal with eluents of increasing salt concentrations yields predominantly oligomers of n = 10–100 which are free of native RNA. When *Bacillus subtilis* RNAase is used, the base ratios of these oligomers differ significantly from that of MS 2 RNA. No such differences are obtained with micrococcal, *Bacillus cereus*, or pancreatic RNAase.

Experimental Procedure

Spectrophotometric measurements were made in cells of 1-cm light path and are expressed as absorbance at 260 mμ (A_{260}).

Enzymes. Micrococcal nuclease was a gift of Drs. H. Taniuchi and C. B. Anfinsen. *B. subtilis* RNAase (Nishimura and Nomura, 1958) was prepared as described (Rushizky *et al.*, 1963). Two samples of alkaline phosphatase from *Escherichia coli* were used. One was a gift from Drs. R. W. Brockman and L. A. Heppel, and the other was isolated from *E. coli* strain C-90 according to Neu and Heppel (1965). *B. cereus* RNAase was isolated as described (Rushizky *et al.*, 1964); pancreatic RNAase A (twice recrystallized) was obtained from Sigma.

RNA. The high molecular weight RNA of the *E. coli* phage MS 2 was prepared essentially as described by Strauss and Sinsheimer (1963). MS 2 phage was obtained as reported (Rushizky *et al.*, 1965). Hepta- and octanucleotides from RNAase T_1 digests of MS 2 RNA were prepared by column chromatography on DEAE-Sephadex A-25 in 7 M urea (Tomlinson and Tener, 1962).

Adsorbents. DEAE-cellulose (Schleicher and Schuell Selectacel Lot No. 1710, 0.8 mequiv/g, 100–200 mesh) was washed (Peterson and Sober, 1956) and preequilibrated in appropriate buffer (Table I). Bio-Gel P-300, 50–150 mesh, was obtained from Bio-Rad Laboratories, Richmond, Calif., and was allowed to hydrate for several hours prior to use.

Enzymatic Hydrolysis of RNA. DEAE-cellulose (17 g dry weight) was equilibrated in 0.1 M NH_4HCO_3, pH 8.6 (digestion buffer) to the proper pH and conductivity values. The adsorbent was filtered off and suspended in a polyethylene flask in 500 ml of digestion buffer. RNA of the *E. coli* phage MS 2, 100 mg in 25 ml of digestion buffer (total A_{260} = 2480), was added to the adsorbent with stirring and the suspension was shaken at 37° for 1 hr. An aliquot of the suspension was centrifuged and the A_{260}/ml of the supernatant found to be 0.042, indicating that all the added RNA had been adsorbed. *B. subtilis* RNAase, 270,000 units in 100 ml of digestion buffer, was then added. This amount of nuclease, designated as E would suffice to hydrolyze unprotected RNA to completion, *i.e.*, to mono- and dinucleotides (Rushizky *et al.*, 1963). After shaking at 37° for 17 hr, the suspension was diluted to 2500 ml with H_2O and stirred for 15 min. The DEAE-cellulose was filtered off, and after washing on a funnel with several portions to a total of 8 l. of 0.02 M NH_4HCO_3, pH 8.6, the adsorbent was packed in the same buffer in a 4 i.d. × 20 cm high column. Stepwise elution was begun with 0.1 M NH_4HCO_3, pH 8.6, and continued with 0.3, 0.5, 0.7, and

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TABLE I: Fractionation of Partial Enzymatic Digests of MS 2 RNA by Stepwise Elution with NH_4HCO_3, pH 8.6.

Expt	Length of Digestion at 37° (hr)	Amt of Enzyme (E)[a]	Digestion Buffer (M)	Amt of RNA (% of total A_{260} eluted at the indicated molarity of NH_4HCO_3) 0.1	0.3	0.5	0.7	1	Recov (in %) of Total A_{260} Adsorbed in DEAE-Cellulose
MN 1[b]	17	0.5	Tris–Cl (0.5)[c]	3	5	15	34	11	68
MN 2	17	1	Tris–Cl (0.5)[c]	4	17	23	28	12	84
MN 3	17	1	Tris–Cl (0.5)[c]	4	2	19	46	12	83
MN 4	1	1	Tris–Cl (0.5)[c]	3	2	6	24	16	51
MN 5	17	1	Tris–Cl (0.1)[c]	2	4	4	3	8	21
B 1	17	1	NH_4HCO_3 (0.1)[c]	8	18	14	18	3	61
B 2	17	2	NH_4HCO_3 (0.1)[c]	40	28	15	19	1	103
B 3	17	1	NH_4HCO_3 (0.1)[c]	17	24	24	14	1	80
P 1	17	0.4	Tris–Cl (0.1)[d]	22	9	19	15	1	66
P 2	17	1	Tris–Cl (0.1)[d]	54	7	7	4	2	74
C 1	17	1	Tris–Cl (0.1)[e]	18	1	1	6	15	41
C 2	17	2	Tris–Cl (0.5)[e]	16	1	1	5	20	43

[a] E = amount of enzyme to digest the RNA to completion in the absence of DEAE-cellulose (see text). [b] MN = micrococcal nuclease; B = *B. subtilis* RNAase; P = pancreatic RNAase; C = *B. cereus* RNAase. [c] At pH 8.5. [d] At pH 7.5. [e] At pH 8.0.

1 M NH_4HCO_3, pH 8.6, at a flow rate of 60–100 ml/hr. Eluents were changed after the A_{260}/ml fell below 0.05. Effluents obtained at the same salt concentration were pooled, the total A_{260} was determined, and the solutions were lyophilized. As an alternative, the effluent was diluted 1:5 with H_2O and concentrated by passage through DEAE-cellulose (Rushizky and Sober, 1962). Oligomer fractions were stored at 23° as lyophilized powders over $CaCl_2$.

Digestion of MS 2 RNA by micrococcal nuclease was carried out as above but in digestion buffer of 0.001 M $CaCl_2$ in either 0.1 or 0.5 M Tris–Cl, pH 8.5. To inhibit the enzyme after digestion, dilution to 2500 ml was with 0.001 M EDTA. Enough enzyme was used (E = 0.04 mg of 90% pure nuclease) to obtain complete hydrolysis of 100 mg of RNA (Roberts *et al.*, 1962). RNAase from *B. cereus* (E = 15 units of enzyme/100 mg of RNA) was employed in digestion buffer of 0.001 M $CaCl_2$ and either 0.1 or 0.5 M in Tris–Cl, pH 8.0, and washed out of the DEAE-cellulose with 0.001 M EDTA as described for micrococcal nuclease digests. Pancreatic RNAase (E = 5 mg of enzyme/100 mg of RNA) was used in digestion buffer of 0.1 Tris–Cl, pH 7.5, and removed from DEAE-cellulose in the same manner as *B. subtilis* RNAase.

Determination of base ratio and of chain length are described in the legends of Tables II and III. Conditions for exclusion chromatography on Bio Gel P-300 are given in Figure 1.

TABLE II: Average Chain Length of Oligomers Derived from Partial Enzymatic Digests of MS 2 RNA.

Expt	Chain Length[a] of Oligomers Eluted at the Indicated Molarity NH_4HCO_3, pH 8.6 0.3	0.5	0.7	1.0
MN 1		24	67	>150
MN 2		29	58	177
MN 3		25	81	138
MN 4		28	256	>1000
B 2	14	65	95	
B 3	20	86	114	
P 1		90	143	
P 2		72	91	
C 1			>1000	>1000
C 2			>1000	>1000

[a] Determined as ratio of total to terminal phosphate released by alkaline phosphatase. Aliquots of about 0.5 and 1.0 mg of oligomer material were hydrolyzed with 400 units of phosphatase (Neu and Heppel, 1965) in 0.25 M Tris–Cl, pH 8.5, for 17 hr at 39°. There was no significant increase in inorganic phosphate between 2 and 17 hr of digestion.

Results

RNA from bacteriophage MS 2 was adsorbed on DEAE-cellulose in buffers at the pH optimum of micrococcal, *B. subtilis*, *B. cereus*, and pancreatic RNAase. Sufficient enzyme (E) to hydrolyze an equal amount of free RNA was then added to the suspension,

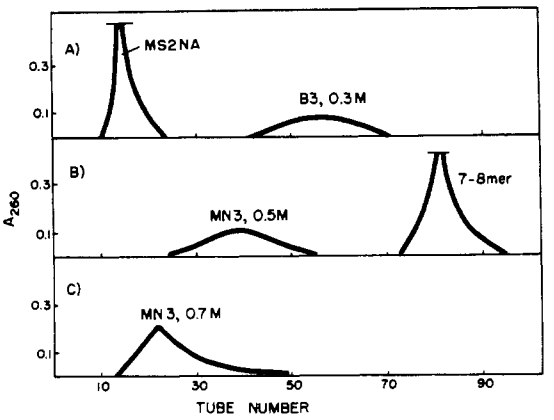

FIGURE 1: Column chromatography of oligomer fractions (see Table I) on two 1.2 i.d. × 182 cm high columns in series containing Bio-Gel P-300, 50–150 mesh, in 0.1 M NaCl, 0.01 M Tris–Cl, pH 7.5, and 0.001 M EDTA. The flow rate was 4.0–4.2 ml/hr tube. (A) Original MS 2 RNA, and oligomer fraction from B 3 (see Table I), eluted with 0.3 M NH_4HCO_3. (B) Oligomer fraction from MN 3, eluted with 0.5 M NH_4HCO_3, and (as marker) oligomers of chain length seven to eight derived from RNAase T_1 digests of MS 2 RNA. (C) Oligomer fraction from MN 3, eluted with 0.7 M NH_4HCO_3.

and the mixture was shaken for 1 or 17 hr at 37°. The adsorbent was filtered off, washed extensively to remove the added nucleases, and repacked in a column which was then developed stepwise with buffers of increasing concentration from 0.1 to 1.0 M NH_4HCO_3, pH 8.6. Under these conditions, unhydrolyzed MS 2 RNA is not eluted from DEAE-cellulose.

The recovery of A_{260} material in the fractions so obtained is shown in Table I. The recoveries ranged from 21 to 103% of the total A_{260} of RNA adsorbed on DEAE-cellulose, depending on the extent of enzymatic digestion. When elution with 1 M NH_4HCO_3, pH 8.6, was followed by elution with 0.1 M NaOH, the recovery was better than 97% of the A_{260} of adsorbed RNA (corrected for changes in ultraviolet absorption with pH). Recoveries of material after micrococcal nuclease digestion in 0.1 M Tris–Cl buffer were lower than those obtained in 0.5 M Tris–Cl (MN 5 *vs.* MN 3, Table I). Changes in salt concentration had no such effect with *B. cereus* RNAase digestion.

Groups of oligomers so prepared were further characterized by chain-length determination with alkaline phosphatase (Table II). The micrococcal enzyme, unlike the other three enzymes, does not produce oligomers with 2′,3′-cyclic-terminal phosphate groups. While unstable at alkaline pH, such cyclic phosphate groups are resistant to alkaline phosphatase. This may explain why corresponding oligomer fractions appear to have higher chain lengths with *B. subtilis* RNAase and with *B. cereus* RNAase. Since alkaline phosphatase functions best with small oligomers, the chain-length values are considered as approximate rather than absolute values. The size range and number of kinds of oligomers in any one of the fractions may depend not only on chain length, but also on nucleotide sequence and/or shape of the oligomer.

As expected from the chain-length determinations, none of the 0.5, 0.7, and 1 M NH_4HCO_3-eluted oligomer fractions moved in the centrifugal field in a 5–20% sucrose gradient, 0.05 N in NaCl and 0.01 M Tris–Cl, pH 7.5, during 26 hr at 0° and 24,000 rpm. However, these fractions were found to be free of MS 2 RNA. On Bio-Gel P-300, oligomers obtained with buffers of the same salt concentration were found to be poly-

TABLE III: Base Ratios of Fractions Derived from Partial Enzymatic Digests of MS 2 RNA.[a]

MS 2 RNA		C 26	A 23	G 27	U 24
Expt	Fractions Eluted with NH_4HCO_3 as Described in Table I (M)				
MN 1	0.5	25	22	26	27
	0.7	27	22	28	24
	1	29	21	26	24
MN 2	0.5	27	22	27	24
	0.7	27	21	28	24
	1	25	24	26	25
MN 3	0.5	28	20	27	25
	0.7	28	21	27	24
	1	26	23	27	24
B 1	0.3	28	23	22	27
	0.5	34	24	15	27
	0.7	35	23	17	25
B 2	0.3	31	21	20	29
	0.5	37	23	13	26
	0.7	41	19	12	29
B 3	0.3	25	23	24	28
	0.5	33	23	16	28
	0.7	33	24	15	28
P 1	0.3	18	30	29	23
	0.5	25	20	30	26
	0.7	26	21	30	23
P 2	0.3	16	30	29	25
	0.5	25	20	26	29
	0.7	26	23	28	23
C 1	0.7	27	21	27	25
	1	24	23	30	24
C 2	0.7	27	21	27	24
	1	26	23	27	24

[a] Base ratios were determined by hydrolysis with RNAase T_2 followed by paper electrophoresis in 0.03 M Tris–acetate, pH 3.6, at 6 v/cm for 17 hr.

disperse. Fractions eluted with different buffers from DEAE-cellulose were also retained on Bio-Gel P-300 to a different degree.

The base composition of oligomers from micrococcal and *B. cereus* RNAase digests closely resembled that of the original MS 2 RNA. With pancreatic RNAase, the smaller oligomers (Table II, P 1–P 2, 0.3 M NH_4HCO_3 fraction) were low in Cp and Up and high in Ap and Gp, as expected from the specificity of the enzyme. Oligomer fractions eluted with 0.5 and 0.7 M NH_4HCO_3 had base ratios closer to that of MS 2 RNA. *B. subtilis* RNAase gave different results. Here the smaller oligomers resembled MS 2 RNA while the larger fractions (0.5 and 0.7 M NH_4HCO_3 fractions, B 1–B 3, Table III) had Cp:Gp ratios of 2:1 as compared to Cp:Gp ratios of 1:1 in MS 2 RNA.

Discussion

Degradation of high molecular weight RNA to molecules of s-RNA size might be a useful step toward determination of the nucleotide sequence of such polymers. Ideally, such a degradation should not be random but favor specific sites in the RNA. In the case of s-RNA, magnesium stabilizes the molecule (and/or inhibits attacking nucleases) so that only certain specific sites are preferentially hydrolyzed (Nishimura and Novelli, 1963; Litt and Ingram, 1964; Penswick and Holley, 1965). By contrast, no such selective hydrolysis is obtained under identical conditions with MS 2 RNA.

MS 2 RNA adsorbed to DEAE-cellulose was found to be more resistant to enzymatic hydrolysis than free RNA. Enzymatic hydrolysis of "bound" RNA was therefore investigated with enzymes having a different preference of specificity. Micrococcal nuclease first attacks d-Ap and d-Tp or Ap and Up sequences (Roberts *et al.*, 1962) while *B. subtilis* RNAase preferentially hydrolyzes Gp-Ap and Gp-Gp sequences (Rushizky *et al.*, 1963) and is virtually inactive toward poly-C (polycytidylic acid) (Whitfield and Witzel, 1963). However, at completion of digestion, both enzymes degrade RNA to mono- and dinucleotides. No preferential specificity has been reported for *B. cereus* RNAase, an enzyme which hydrolyzes RNA completely to mononucleotides (Rushizky *et al.*, 1964). By contrast, these three RNAases are not specific at all compared to the pyrimidine specificity of pancreatic RNAase (Schmidt and Levene, 1938).

The enzymes employed were also selected because they are readily inactivated and/or eluted from DEAE-cellulose without concomitant elution of partially hydrolyzed RNA. Thus, micrococcal and *B. cereus* RNAase are inactive in the presence of EDTA, and, like *B. subtilis* and pancreatic RNAase, eluted from DEAE-cellulose with 0.1 M NH_4HCO_3, pH 8.6.

Conditions for enzymatic hydrolysis of adsorbed RNA were chosen so as to achieve "completion" of digestion, *i.e.*, a high yield of large oligomers. Rather than slowing down the attack on RNA by nucleases by using low temperatures at digestion and/or small amounts of enzyme, all RNA was adsorbed to DEAE-cellulose as evidenced by the absence of A_{260} in the supernatant, enough enzyme added to achieve complete digestion of the RNA were the adsorbent not present, and the suspension was shaken at 37° for 1–17 hr.

With three of the four enzymes studied, the larger oligomers ($n = >10$) did not differ significantly from the parent molecule with respect to their base ratios. However, with *B. subtilis* RNAase, the large oligomers had Cp:Gp ratios of 2:1 as compared to the corresponding ratio of 1:1 in MS 2 RNA. This is in agreement with the preference of the enzyme for Gp-Gp and Gp-Ap linkages (Rushizky *et al.*, 1963) and its inability to hydrolyze poly-C (Whitfield and Witzel, 1963). This effect obtained by attachment of RNA to DEAE-cellulose, a polyamine, may be similar in nature to the effect of polylysine which has been found to protect RNA segments with high Gp-Cp content against nuclease hydrolysis (Sober *et al.*, 1965).

Enzymatic hydrolysis of high molecular weight RNA adsorbed on DEAE-cellulose should be useful for nucleotide sequence determinations since large oligomers may be obtained in good yields. Hydrolysis with *B. subtilis* RNAase offers promise with respect to the isolation of specific portions of the RNA molecule as evidence by the unusual base ratios of the isolated oligonucleotides. Other nucleases, other hydrolysis conditions with the enzymes employed here, and further fractionation of the groups of oligomers obtained by stepwise elution may also yield large oligonucleotides with base ratios other than the original RNA.

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Fidelity in the Translation of Messenger Ribonucleic Acids in Mammalian Subcellular Systems*

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ABSTRACT: Magnesium, low temperature, aminoglycoside antibiotics, and polyamines are known to produce mistakes in translation of the genetic code in subcellular bacterial systems. In the present study, the effects of these same environmental factors on fidelity in the translation of native messenger ribonucleic acids (m-RNA's) and of polyuridylic acid (poly-U) have been examined in a subcellular rat liver and a rabbit reticulocyte system. When protein synthesis was directed by native m-RNA's, high concentrations of magnesium, low temperature, or the addition of streptomycin, spermine, and spermidine inhibited protein synthesis and decreased the ratio of leucine to phenylalanine present in the product. When protein synthesis was directed by polyuridylic acid, these same factors did not appreciably enhance the ability of poly-U to code for leucine or isoleucine. In both mammalian systems, poly-U-directed leucine incorporation seldom exceeded 6% of that obtained with phenylalanine. There was no stimulation of isoleucine incorporation. The results suggest that the translation mechanism of mammalian cells functions with higher fidelity *in vitro* than that of bacteria.

Previous studies from this laboratory have indicated that specificity in the translation of synthetic messenger RNA's (m-RNA's) in a subcellular system derived from *Bacillus stearothermophilus* is influenced to a considerable extent by temperature, magnesium concentration, polyamines, and dihydrostreptomycin (Friedman and Weinstein, 1964, 1965a,b). It has also been demonstrated that with extracts of *Escherichia coli* a variety of factors can modify specificity in the translation of a synthetic m-RNA. These factors include: temperature (Szer and Ochoa, 1964), magnesium concentration (Szer and Ochoa, 1964; So and Davie, 1964; Grunberg-Manago and Dondon, 1965), streptomycin and related aminoglycoside antibiotics (Davies *et al.*, 1964, 1965; Pestka *et al.*, 1965), organic solvents (So and Davie, 1964, 1965), pH (Grunberg-Manago and Dondon, 1965), and the concentration of amino acids, or s-RNA (soluble ribonucleic acid) (So and Davie, 1965; Grunberg-Manago and Dondon, 1965; Pestka *et al.*, 1965; Davies *et al.*, 1965). We have chosen the term "fidelity" to refer to specificity in the translation of nucleotide sequences in m-RNA into amino acid sequences in protein (Friedman and Weinstein, 1964).

Previous studies with extracts of *Chlamydomonas* (Sager *et al.*, 1963) and mammalain cells (Weinstein and Schechter, 1962; Weinstein, 1963; Ochoa and Weinstein, 1964a) indicated that, in addition to directing the synthesis of polyphenylalanine, polyuridylic acid (poly-U) coded to a small extent for leucine. This suggested the existence of ambiguity for the UUU codon. It was of interest, therefore, to determine whether the factors which alter translation of RNA in bacterial systems have a similar effect in mammalian subcellular extracts.

In the present study we have examined the fidelity of protein synthesis in mammalian systems when directed by native m-RNA's ("endogenous reaction") and also when directed by poly-U. Preliminary reports of portions of this work have appeared in abstract form (Friedman *et al.*, 1966; Ochoa and Weinstein, 1966).

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