Volume 6 Number 5 1979 Nucleic Acids Research

The regulatory region of MS2 phage RNA replicase cistron. III. Characterization of fragments resulting from S_1 nuclease digestion

I.Jansone, V.Berzin, V.Gribanov and E.J.Gren

Laboratory of Nucleic Acid Chemistry, Institute of Organic Synthesis, Latvian Academy of Sciences, Riga 226006, USSR

Received 15 March 1979

ABSTRACT

The functionally active fragments MS2 R(-53→6) and MS2 R(-53→3) comprising the regulatory region for the replicase cistron have been isolated from MS2 RNA-coat protein complex following T_1 RNase digestion. In order to obtain shorter fragments, active in coat protein binding and initiation of translation, MS2 R(-53→6) was cleaved with S_1 nuclease. The results indicate that S_1 nuclease attacks the most susceptible loop regions of the two hairpin helices of MS2 R(-53→6). Among the three fragments which have been isolated, only MS2 R(-35/33→6) containing the intact hairpin (b) region with initiation codon AUG is active in the coat protein binding. Functional activity exerted by another polynucleotide MS2 R(-17→6) supports the assumption that specific binding with the coat protein is determined by the hairpin (b) region prior to the replicase cistron.

INTRODUCTION

The region preceding the RNA phage replicase cistron provides true initiation of replicase cistron translation and is the site of coat protein binding, which leads to the repression of replicase synthesis /1,2/. The short regulatory region (R-region)-containing fragments isolated from phage R17 /3/ and MS2 /4,5/ RNAs retain their functional activity and offer a useful tool for the investigation of molecular mechanisms involved in initiation of translation and protein-RNA recognition.

In order to locate in these fragments the ribosome and coat protein recognition sites, we have attempted to cleave further one of the polynucleotides MS2 R(-53→6) /5/ to obtain shorter active fragments. This was achieved by using S_1 nuclease, specifically attacking single-stranded structural-

ly unmasked regions of RNA /6,7/. Owing to the well pronounced secondary structure of MS2 R(-53→6) /8/, the polynucleotide has only a few sites susceptible to digestion by S_1 nuclease, resulting in the production of fairly long fragments.

The present study is mainly concerned with optimization of conditions for cleavage of MS2 R(-53→6) at the exposed single-stranded regions by S_1 nuclease and characterization of the products of digestion. The results presented here show that S_1 nuclease attacks the most susceptible loop regions of the two hairpin helices of MS2 R(-53→6), the single-stranded region connecting them being completely protected.

The coat protein binding activity of the fragments originating from limited S_1 digestion and some other MS2 R-region polynucleotides is also described. Our findings confirm the conclusions previously reported by Gralla et al. /10/ that only the hairpin (b) of phage R17 RNA R-region is involved in the binding of the coat protein to produce repressor complex.

The data concerning the functional activity of MS2 R-region polynucleotides in formation of the initiation complex of protein biosynthesis are presented in the accompanying paper /9/.

MATERIALS AND METHODS

Preparation of MS2 R-region polynucleotides.

^{32}P-labeled MS2 RNA(27.0 nmol, 5.9×10^8 cpm) was incubated with 91.0 nmol of coat protein in 5 ml 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 80 mM KCl (TMK buffer) for 10 min at 30°C, and the resultant complex was treated with T_1 RNase for 30 min at 25°C. Further isolation and purification of the coat protein-protected RNA fragments was performed as described previously /5/.

Limited S_1 nuclease digestion of MS2 R(-53→6).

S_1 nuclease isolated from Aspergillus oryzae according to the conventional method described in /14/ was the kind gift of

M.Ya.Karpeisky and L.L.Kisselev (Institute of Molecular Biology, Moscow). The standard reaction mixture (200 µl) contained 10 mM sodium acetate, pH 5.0, 10 mM $MgCl_2$, 45 mM NaCl, 5 mM $ZnSO_4$, 5% glycerol (buffer A), 1.8×10^6 cpm (0.8 A_{260} units) of MS2 /^{32}P/ R(-53→6) and 144 units of S_1 nuclease. Incubation was carried out at 2°C. Samples of reaction mixture were withdrawn at various times - 1 µl for coat protein binding assay and 3 µl for electrophoretic analysis; 2 µl 50 mM EDTA-Tris, pH 8.3, 2 µl 50% sucrose containing xylene cyanol and bromphenol blue were added and the solution was loaded onto 1-cm identations of a 22 × 30 × 0.04 cm slab of 16% polyacrylamide gel (pH 8.0) containing 6 M urea /15/. The gel was electrophoresed at 400 V for 14 h. Bands, visualised by autoradiography, were excised and measured by Cerenkov counting. For the isolation of preparative amounts of fragments of MS2 R(-53→6) produced by S_1 nuclease, the reaction mixture was incubated for 75 min and digestion was stopped by 0.03 volumes of 0.1 M EDTA-Tris (pH 8.3) followed by 80% phenol extraction. The aqueous phase containing 10% sucrose and dye markers was layered onto a slab (21 × 30 × 0.2 cm) of 16% polyacrylamide (pH 8.0) / 6 M urea gel and run at 400 V for 20 h. The desired fragments were eluted as described previously /5/.

Nucleotide sequence analysis of ^{32}P-labeled MS2 R(-53→6) fragments.

Pancreatic and T_1 RNase digests were fractionated by two-dimensional separation method, involving high-voltage electrophoresis on cellulose acetate (pH 3.5) and electrophoresis on DE81 paper (pH 1.9) /12/ or homochromatography with mixture "ß" on PEI-cellulose thin layer plates /13/ in the second dimension. 30% triethylamine bicarbonate was used for oligonucleotide elution and further analysis was subsequently performed by RNase digestion and identification of the products by electrophoresis at pH 3.5 on DE81 or Whatman 3MM paper /12/.

Assay for binding of MS2 coat protein to MS2 R-region polynucleotides.

10-20 pmol ^{32}P-labeled polynucleotides in 100 µl TMK

buffer were incubated with saturating amounts of the fresh MS2 coat protein for 10 min at 2°C. The reaction mixture was diluted to 1.0 ml with ice-cold TMK buffer and the solution was immediately filtered through a duplicate 24-mm Selectron BA85 nitrocellulose filters. The filters were washed twice with 1 ml TMK buffer, dried, and their radioactivity was measured in a toluene-based scintillator.

RESULTS

Isolation of MS2 R(-53→3).

The coat protein, which forms repressor complex with MS2 RNA, protects from cleavage by T_1 RNase an RNA region around the beginning of the replicase cistron, thus permitting isolation of MS2 RNA fragments comprising a region responsible for the regulation of replicase synthesis (MS2 R-region) /4,5/. A high molar ratio of coat protein to RNA (9:1) in the reaction mixture results in a fraction of predominantly long fragments, MS2 R(-53→50) and MS2 R(-53→6). At low protein : RNA molar ratios (3:1) a fragment MS2 R(-53→6) is mainly formed. Both these fragments have been isolated and their structure and functional properties have been studied /5/.

In several cases isolation of MS2 R(-53→6) by electrophoresis in 12% polyacrylamide gel (Fig.1) revealed the occurrence of band 1a, corresponding to a somewhat shorter polynucleotide, as compared to MS2 R(-53→6) of band 1. Intensity of this band varied considerably in separate experiments. As a rule, the amount of material in band 1a did not exceed 10% of MS2 R(-53→6) in band 1. The material of band 1a was further re-electrophoresed in 16% polyacrylamide gel in the presence of 6 M urea, with subsequent purification using the standard procedure /5/ in order to remove contamination of the material with the closely located band 1. Two-dimensional separation of pancreatic or T_1 RNase oligonucleotides derived from band 1a polynucleotide and comparison of the relative molar yields of products with that obtained for MS2 R(-53→6) (Tables 1 and 2) allowed to identify the sequence of band 1a as MS2 R(-53→6) (Fig.3).

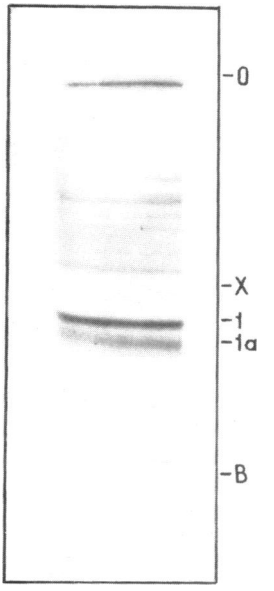

Fig. 1 12% polyacrylamide (pH 8.0) gel electrophoresis of MS2 R(-53→6) (Band 1) and MS2 R(-53→3) (Band 1a) obtained by digestion of MS2 /^{32}P/RNA-coat protein complex with T_1 RNase. B, position of bromphenol blue; X, xylene cyanol FF dye marker; O, origin.

Cleavage of MS2 R(-53→6) by S_1 nuclease.

Since S_1 nuclease preparations purfied from Aspergillus oryzae by the procedure of Vogt /14/ may be contaminated by traces of RNases T_1 and T_2 /6/, a study of the specificity of enzyme action was undertaken. It is known that activity of S_1 nuclease at pH above 6.0 must not exceed 2.5% of the maximal value at pH 4.0-4.3 /14/. On the other hand, RNase T_1 at pH 6.2 amounts to about 75% of its activity optimum at pH 7.5 /18/. The radioactivity of acid-insoluble fraction and polyacrylamide gel electrophoresis pattern (not shown) revealed that ^{32}P-labeled MS2 R(-53→6) during 150 min at pH 6.2 (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $ZnSO_4$) retains complete resistance to the S_1 nuclease preparation used (S_1/RNA=90 units/1A_{260} unit). The same amount of enzyme was sufficient to degrade MS2 R(-53→6) in 10 mM sodium acetate, pH 4.3, 5 mM $ZnSO_4$

during 30 min at 2°C. Complete inhibition of digestion was obtained by the addition of 50 mM EDTA. Since RNase T_2 is known to be resistant to EDTA treatment, it is concluded that the S_1 nuclease used is free from RNase T_2 contamination.

To obtain the set of partial products of MS2 R(-53→6), digestion with S_1 nuclease was carried out in buffer A (10 mM sodium acetate, pH 5.0, 10 mM $MgCl_2$, 45 mM NaCl, 5 mM $ZnSO_4$ and 5% glycerol) at 2°C. The above conditions (Mg^{2+}, salt concentration, low temperature) are known to increase the stability of helical structure of polynucleotide. At the same time, pH 5.0 of the digestion mixture is not pH optimum of S_1 nuclease. The latter fact may be also related to the factors stabilizing the secondary structure of MS2 R(-53→6), since deprotonation of RNA bases at pH above 5.0 has been observed /19/. In buffer A S_1 nuclease cleaves MS2 R(-53→6) into fairly long fragments. Electrophoretic separation of digestion products in 16% polyacrylamide gel containing 6 M urea revealed 5 major bands and several less distinct ones (Fig.2-a). We have studied distribution of radioactivity among bands (see Materials and Methods) in the time course of S_1 hydrolysis of ^{32}P-labeled MS2 R(-53→6). As seen in Fig. 2-b, the rate of appearance of bands is subject to considerable variation. S_1 nuclease produce bands 2, 3 and 4 almost simultaneously, but the yield of band 2, unlike other bands, reaches maximum within 60 min of incubation and drops abruptly after 90 min. On the contrary, the material in bands 3 and 4 continues to accumulate during 120 min. Kinetics of band 3 polynucleotide appearance and its sequence analysis (see below) lead us to conclude that this fragment results mainly from further cleavage of the previously formed polynucleotide of band 2.The proportion of polynucleotides active in the coat protein binding also diminished during the hydrolysis (Fig.2-c). However, the binding activity was still retained even after the reaction mixture had been completely depleted of MS2 R(-53→6). This indicates the presence of functionally active fragments among the products of S_1 nuclease digestion of MS2 R(-53→6).

To carry out preparative isolation of the products

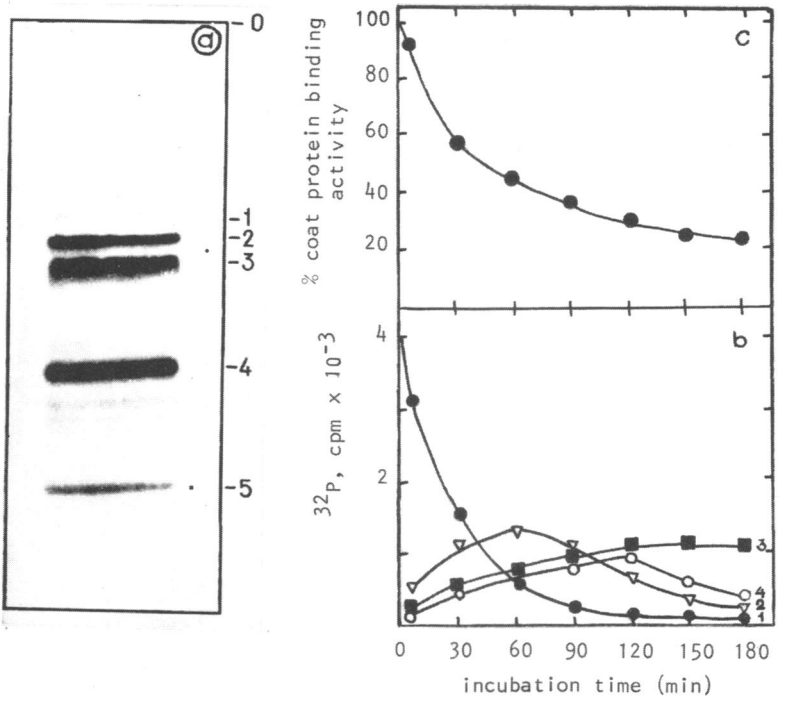

Fig. 2 Limited S_1 nuclease digestion of ^{32}P-labeled MS2 R(-53→6).
a - autoradiogram of 16% polyacrylamide (pH 8.0)/6M urea gel electrophoresis of preparative amounts of S_1 fragments. 1 - position of uncleaved MS2 R(-53→6).
b - distribution of radioactivity among bands in the time course of S_1 nuclease hydrolysis (see Materials and Methods). Radioactivity was measured by Cerenkov counting.
c - effect of S_1 nuclease digestion on coat protein binding activity of MS2 R(-53→6).

derived form S_1 nuclease cleavage of MS2 R(-53→6), conditions were selected permitting accumulation of the longest polynucleotides migrating as bands 2 and 3. Fig. 2-a shows an autoradiogram of S_1 nuclease digests of MS2 /^{32}P/R(-53→6) subjected to gel electrophoresis after 75 min of incubation. It is noteworthy that polynucleotides in bands 2,3, and 4 are characterized by rather low yields, amounting to 20% of theoretically estimated values, which is probably

due to the further digestion of originally formed long fragments by S_1 nuclease. Following separation and purification of polynucleotides corresponding to bands 2,3, and 4 they were identified by complete pancreatic RNase digestion (polynucleotide of band 2 were also exposed to T_1 RNase) with subsequent two-dimensional separation of oligonucleotides. The results of oligonucleotide analysis are presented in Tables 1 and 2. The material of band 5 was found to be heterogeneous and unaccessible to structural analysis.

Among T_1-oligonucleotides of band 2 polynucleotides two of them, T1 and T2, were represented in small amounts, whereas pancreatic RNase digests (P-oligonucleotides) have lower content of A-A-Up. The presence of all the other T_1-oligonucleotides, therein included 3'-terminal U-C-Gp, as well as

Table 1. Catalogue of T_1-oligonucleotides

Sequence	Molar yield in polynucleotides[a]			
	R(-53→6) (Band 1)[b]	R(-53→3) (Band 1a)[b]	R(-53→6) (Band 1)[c]	R(-35/33→6) (Band 2)[c]
Gp	4.13 (3)	3.45 (3)	4.31 (3)	2.57 (2)
A-Gp	1.06 (1)	1.11 (1)	1.23 (1)	1.38 (1)
A-C-Gp	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
C-C-Gp	1.37 (1)	1.26 (1)	1.11 (1)	1.00 (1)
U-C-Gp	0.90 (1)	0.12 (0)	1.21 (1)	0.78 (1)
C-A-A-A-C-U--C-C-Gp(T1)	1.22 (1)	0.90 (1)	0.64 (1)	0.11 (0)
C-A-U-C-U-A--C-U-A-A-U-A-Gp(T2)	0.73 (1)	0.81 (1)	0.56 (1)	0.17 (0)
C-C-A-U-U-C--A-A-A-C-A-U-Gp(T3)	0.90 (1)	1.00 (1)	0.66 (1)	0.42 (1)
A-U-U-A-C-C--C-A-U-Gp(T4)	1.00 (1)	0.89 (1)	0.72 (1)	0.52 (1)

[a] Expected values are given in parentheses.
[b] Separation of T_1-oligonucleotides by a standard fingerprinting method /12/.
[c] Separation of T_1-oligonucleotides by a mini-fingerprinting procedure /13/.

Table 2. Catalogue of P-oligonucleotides

Sequence	Molar yield in polynucleotides[a]				
	R(-53→6) (Band 1)	R(-53→3) (Band 1a)	R(-35/33→6) (Band 2)	R(-35/33→-7/6) (Band 3)	R(-53→-36/34) (Band 4)
Cp	11.70(10)	8.96(9)	6.20(6)	3.58(3)	3.69(4)
Up	6.46(5)	4.70(5)	1.98(2)	1.25(1)	2.00(2-3)
Gp	1.28(1)	0.88(1)	0.46(1)	0(0)	0(0)
A-Cp	2.56(2)	1.91(2)	0.92(1)	0.28(0)	0.93(1)
G-Cp	1.38(1)	0.99(1)	0.95(1)	0.86(1)	0(0)
A-Up	4.25(4)	4.06(4)	2.65(3)	2.00(2)	1.10(1)
G-Up	1.12(1)	0.40(0)	1.05(1)	0(0)	0(0)
A-A-Up	1.11(1)	1.03(1)	0.28(0)	0.14(0)	0(0)
G-G-Cp	2.00(2)	2.00(2)	1.00(1)	1.00(1)	1.00(1)
A-A-A-Cp	1.90(2)	1.73(2)	0.69(1)	1.36(1)	0.91(1)
A-G-A-Cp	0.99(1)	0.96(1)	0.81(1)	0.83(1)	0(0)
G-A-G-G-A-Up	1.04(1)	0.95(1)	0.69(1)	0(0)	0(0)
G-A-G-G-A-(U_{OH})	0(0)	0(0)	0.13(0)	0.27	0(0)
G-A-G-G-(A_{OH})	0(0)	0(0)	0(0)	0.31 (1)	0(0)

[a]Expected values are given in parentheses.

G-A-G-G-A-Up and G-Up among P-oligonucleotides indicates that the polynucleotide chain of MS2 R(-53→6) is primarily attacked by S_1 nuclease in the hairpin (a) region producing two fragments, a long 3'-terminal fragment moving as band 2 and a shorter 5'-terminal fragment located in band 4. P-oligonucleotide composition of band 4 polynucleotides supports this conclusion. The precise site of the cleavage was difficult to localise because we failed to detect any new 3'- and 5'-terminal oligonucleotides in appreciable amounts, which was probably due to heterogeneous cleavage. However, the presence of theoretically expected amounts of A-Cp and A-G-A-Cp in polynucleotides of bands 4 and 2, respectively, locates the attack site of S_1 nuclease within the loop of

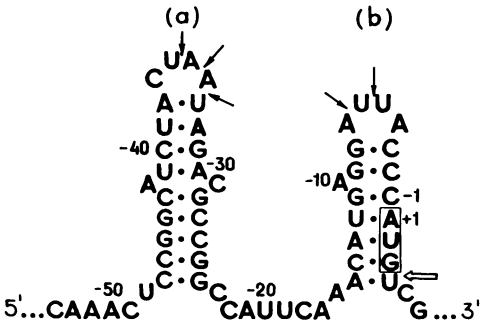

Fig. 3 Secondary structure model of the MS2 R(-53→6). Thin arrows indicate the possible splitting points by S_1 nuclease. The boxed arrow denote the partial removal of U-C-Gp by T_1 RNase during isolation of MS2 R(-53→6).

hairpin (a) involving internucleotide bonds U_{-36}-A_{-35}-A_{-34}-U_{-33}.

Further cleavage of polynucleotides constituting band 2 occurs within the loop of hairpin (b) and it produces polynucleotides of band 3. The latter lacks P-oligonucleotides A-Cp and G-Up characteristic of hairpin (b), and instead of G-A-G-G-A-Up two shorter derivatives of it were detected, which were present in equal amounts and whose tentative structure is G-A-G-G-A-(U_{OH}) and G-A-G-G-(A_{OH}) (unphosphorylated 3'-terminal nucleotides are not detected directly). Therefore the polynucleotides of band 3 may be formed as a result of cleavage between A_{-7}-U_{-6} or U_{-6}-U_{-5}.

On the basis of considerations stated above the following structures may be deducted for the fragments obtained after S_1 nuclease treatment of MS2 R(-53→6): band 4 - MS2 R(-53→-36/34); band 3 - MS2 R(-35/33→-7/6); band 2 - MS2 R(-35/33→6).

It should be noted that the material of band 2 was found to be slightly heterogeneous and was contaminated with variable amounts of other polynucleotides which were derived from MS2 R(-53→6) after the cleavage only in the loop of hairpin (b). This is evidenced by the presence of small but detectable amounts of T_1-oligonucleotides T1, T2 and P-oligonucleotides A-A-Up, G-A-G-G-A-(U_{OH}).

Binding of the coat protein to MS2 R-region polynucleotides.

MS2 R(-53→6) and MS2 R(-53→50) described in the previous study have been shown to be active in binding the coat protein; their affinity for the coat protein being close to that of intact MS2 RNA /5/.

To determine activity of the newly-isolated polynucleotides originating from MS2 R-region, we used a conventional technique of coat protein-polynucleotide complex binding on nitrocellulose membrane filters (see Materials and Methods). To achieve complete binding of polynucleotides, the coat protein was used in 10-20-fold molar excess and the binding procedure was performed in TMK buffer at 2°C. The results are shown in Table 3.

Removal of 3'-terminal U-C-Gp from MS2 R(-53→6) appears to have no appreciable effect on the binding of the resulting fragment to the coat protein, for MS2 R(-53→3) exerts similar activity. Among the fragments derived from S_1 nuclease digestion of MS2 R(-53→6) only MS2 R(-35/33→6) (band 2) is active in binding the coat protein. Some decrease in binding was apparently due to contamination with a functionally inacti-

Table 3. Binding of MS2 R-region polynucleotides to coat protein

MS2 R-region polynucleotides	/^{32}P/polynucleotides		Coat protein	/^{32}P/polynucleotides bound	
	cpm	pmol	pmol	cpm	%
R(-53→6)	3882	14.4	211.3	3172	81.7
R(-53→3)	4809	16.8	298.9	3465	72.1
R(-35/33→6)	2965	26.3	422.5	1540	51.9
R(-17→6)	1310	3.2	42.3	757	57.8
R(-35/33→-7/6)	2422	21.0	253.5	64	2.6
R(-53→-11)	4028	11.8	122.0	138	3.4
R(-53→-36/34)	1649	47.7	422.5	26	1.6
Polynucleotides of Band 5	2300	106.3	196.0	119	5.2

ve fragments lacking the complete hairpin (b) region (see above). MS2 R(-53→-11) structurally related to the contaminating polynucleotide, containing only a part of hairpin (b), is completely inactive in binding the coat protein /5/. For this very reason the other two fragments isolated from S_1 nuclease digests of MS2 R(-53→6) - MS2 R(-35/33→-7/6) (band 3) and MS2 R(-53→-36/34) (band 4), respectively, as well as the heterogeneous material containing the short fragments of band 5 fail to bind coat protein (Table 3).

The shortest polynucleotide derived from MS2 R-region which retains coat protein binding activity appeared to be MS2 R(-17→6) possessing the complete hairpin (b) region (Table 3). Isolation of this polynucleotide from the ribosomal initiation complex on MS2 R(-53→6) template has been described in an accompanying paper /9/.

DISCUSSION

The coat protein binds to the MS2 bacteriophage RNA and protects from cleavage by T_1 RNase a region of 59 nucleotides - MS2 R(-53→6) /4,5/. A similar fragment carrying one or two base substitutions can be isolated from R17 RNA/1,10/. A two hairpin secondary structure model has been proposed for R17 R(-53→6) on the basis of temperature-jump melting experiments and high resolution proton magnetic resonance data /10,11/. The same model may be applicable also for MS2 R(-53→6) (Fig.3). Kethoxal modification of MS2 R(-53→6) and its partial digestion with T_1 RNase have demonstrated that in contrast to the strong helix (a), helix (b) is less stable and under physiological conditions it may exist in equilibrium with the open form /8/.

S_1 nuclease preferentially degrades single-stranded regions in both RNA and DNA /6,16,17/, and, therefore, tRNA molecules which have compact structure are cleaved by the enzyme only at the anticodon loop /7/. The cleavage of MS2 R(-53→6) by S_1 nuclease in the loops of both hairpins suggests the presence of the typical double-hairpin structure of the polynucleotide also at pH 5.0, 2°C in the Mg^{2+} containing buffer of high ionic strength. However, somewhat surpris-

ing is the observed low susceptibility to digestion not only terminal single-stranded regions, but even of a rather long single-stranded region of MS2 R(-53→6) connecting the two hairpins. Presumably, these parts of the molecule are somehow burried in the tertiary structure and are thus made inaccessible to S_1 nuclease action. This is confirmed by the failure to perform polynucleotide cleavage selectivelly between hairpins (a) and (b) and by retaining the latter when using some other nucleases as RNase T_1 and T_2 (not shown).

As to the boundaries of the active region of MS2 RNA, determining the interaction with the coat protein in repressor complex formation, our findings are in full accord with the conclusions drawn initially by J.Steitz implying that only hairpin (b) in R17 RNA is involved in the recognition of the coat protein /10/. Activity is only inherent in those MS2 R(-53→6) fragments with the complete hairpin (b) region: MS2 R(-53→3), MS2 R(-35/33→6) and MS2 R(-17→6). Other fragments - MS2 R(-53→-11), MS2 R(-35/33→-7/6) and MS2 R(-53→-36/34) are entirely inactive. Hence, the coat protein interacting with the R-region of MS2 RNA recognizes a comparatively short nucleotide sequence prior to the replicase cistron. The active site does not extent out the region limited by the initiation codon AUG on the 3'-terminus and the nucleotides of the intercistronic region A_{-17} on the 5'-terminus.

ACKNOWLEDGEMENTS

We thank Drs. M.Ya.Karpeisky and L.L.Kisselev for providing S_1 nuclease preparations and Dr. G.P.Borisova for her assistance in isolating fragments obtained from MS2 R(-53→6) enzymatic hydrolysis.

REFERENCES

1 Bernardi, A. and Spahr, P.F. (1972) Proc.Nat.Acad.Sci. USA 69, 3033-3037.
2 Weber, H. (1976) Biochim.Biophys.Acta 418, 175-183.
3 Steitz, J.A. (1974) Nature 248, 223-225.
4 Berzin, V.M., Borisova, G.P., Gribanov, V.A., Rosenthal, G.F., Cielens, I.E., Jansone, I.V. and Gren E.J. (1976) Dokl.Akad.Nauk S.S.S.R. 229, 741-744.

5 Berzin, V., Borisova, G.P., Cielens, I., Gribanov, V.A., Jansone, I., Rosenthal, G. and Gren, E.J. (1978) J.Mol.Biol. 119, 101-131.
6 Rushizky, G.W., Shaternikov, V.A., Mozejko, J.H. and Sober, H.A. (1975) Biochemistry 14, 4221-4226.
7 Harada, F. and Dahlberg, J.E. (1975) Nucl.Acid.Res. 2, 865-871.
8 Berzin, V.M., Jansone, I.V., Gribanov, V.A., Tsimanis, A.J. and Gren, E.J. (1978) Molecular Biology (USSR) 12, 1288-1298.
9 Borisova, G.P., Volkova, T.M., Berzin, V., Rosenthal, G. and Gren, E.J. (1979) Nucl.Acid.Res., accompanying paper.
10 Gralla, J., Steitz, J.A. and Crothers, D.M. (1974) Nature 248, 204-208.
11 Hilbers, C.W., Shulman, R.G., Yamane, T. and Steitz, J.A. (1974) Nature 248, 225-226.
12 Brownlee, G.G. (1972) Determination of Sequences in RNA. pp. 67-99, North-Holland, Amsterdam.
13 Volckaert, G., Min You, W. and Fiers, W. (1976) Anal. Biochem. 72, 433-446.
14 Vogt, V.M. (1973) Eur.J.Biochem. 33, 192-200.
15 De Wachter, R. and Fiers, W. (1972) Anal.Biochem. 49, 184-197.
16 Flashner, M.S. and Vournakis, J.N. (1977) Nucl.Acid.Res. 4, 2307-2319.
17 Beard, P., Morrow, J.F. and Berg, P. (1973) J.Virol. 12, 1303-1313.
18 Warrington, R.C. (1974) Biochim.Biophys.Acta, 353, 63-68.
19 Rushizky, G.W. and Mozejko, J.H. (1977) Anal.Biochem. 77, 562-566.