*SELECTIVE INTERFERENCE WITH VIRAL RNA FORMATION IN VITRO BY SPECIFIC INHIBITION WITH SYNTHETIC POLYRIBONUCLEOTIDES**

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Communicated August 29, 1966

In 1963 we reported[1] the isolation of the RNA-dependent RNA polymerase (replicase) induced by the RNA bacteriophage MS-2. The purification was carried to the point of an absolute demand for RNA, permitting therefore an examination of template requirements. The isolated MS-2 replicase responded preferentially to MS-2-RNA; neither the ribosomal nor transfer RNA of the host exhibited significant template function. At the time it was noted that this property ensured *selective synthesis* of viral RNA and thus provided a solution to viral replication in a cellular environment replete with other RNA molecules.

This was the first instance of a nucleic acid polymerase (DNA or RNA) exhibiting a clear-cut requirement for a specific template; the finding was received with some reservation. However, our line of reasoning led to the expectation that other RNA replicases would show a similar preference for homologous template. An opportunity to test the validity of this prediction came with the discovery of a new and serologically unrelated RNA bacteriophage ($Q\beta$) by Watanabe.[2] The $Q\beta$-replicase was purified[3] and the predicted requirement for homologous $Q\beta$-RNA was found.

The unique preferences exhibited by the MS-2 and $Q\beta$-replicases which surprised so many are now accepted. Thus, Weissmann and Feix[4] have confirmed this property with enzyme supplied from this laboratory, and August[5] found that purified $Q\beta$-replicase which he prepared responds also only to $Q\beta$-RNA. Further, the original[1] isolation of MS-2 replicase has been successfully carried out to the stage of complete RNA dependence by Fiers[6] and his colleagues. They confirmed the specific response to MS-2-RNA as well as the autocatalytic kinetics observed[7] when the reaction is initiated at template concentrations below saturation of the enzyme.

The fact that each replicase recognizes its own RNA genome provides an opportunity to examine the basis of the recognition interaction between a protein and a polynucleotide. An obvious device would invoke the initial set of nucleotides, a possibility easily tested by challenging the replicase with fragments of homologous RNA. If the presence of the beginning sequence is the sole requirement, half and quarter RNA fragments should be adequate to initiate synthesis. Haruna and Spiegelman[8] showed that this was *not* the case. Fragments of $Q\beta$-RNA mediate a very slow reaction which soon terminates before 10 per cent of the input has been synthesized. Furthermore, the product is found[9] in a ribonuclease-resistant structure, convertible to sensitivity by heat. This sort of structure is *not* observed[9] when replicase functions with intact $Q\beta$-RNA and is extensively synthesizing biologically active RNA replicas.[9-12]

The inability of the replicase to copy fragments means that the enzyme can sense the difference between an intact and fragmented template, implying that some element of secondary structure of the RNA is involved. We suggested[8] that a

simultaneous decision on sequence and intactness could be made if the two ends were complementary and formed a double-stranded region, sought for and recognized by the enzyme.

This mechanism has some interesting testable consequences in view of the recent demonstration[9] that the first 5–10 per cent of Qβ-RNA synthesized is rich in adenine and poor in uracil. The proposed mechanism would then suggest that the enzyme scans for a secondary structure formed by the pairing of two complementary regions, one predominant in A and the other in U. If this is the case, Qβ-replicase might be specifically inhibited by synthetic polynucleotides composed principally of either A, or U, or both. Conversely, polynucleotides containing mostly C or G should be relatively inert.

The primary purpose of the present paper is to present the relevant experiments. The data obtained confirm the expectations of the model. Qβ-replicase is severely inhibited by poly A, poly U, or copolymers of A and U. It is, however, completely indifferent to the presence of polynucleotides containing principally either C or G. It has also been possible to demonstrate that once the template is complexed to replicase, it is not easily displaced by another polynucleotide.

Methods and Materials.—The virus is Qβ[2] and the host is *E. coli* Q13, an Hfr mutant lacking ribonuclease I and phosphorylase activities. All the methods of preparing infected cells, purifications of the replicase, synthesis of radioactive substrates, and assay for enzyme activity, have been detailed previously.[1, 3, 7–9]

Except for poly G, which was obtained through the kindness of Dr. Marianne Manago-Grunberg, the synthetic polynucleotides used in the present study were obtained from the Miles Laboratory, Inc.

Results.—(a) *Effect of homopolymers:* The effects of the four homopolymers on the activity of Qβ-replicase primed by intact Qβ-RNA is summarized in Table 1. It is evident that both poly A and poly U are extremely effective in inhibiting the reaction, whereas poly C and poly G are virtually without effect.

(b) *Effect of synthetic copolymers:* In view of the striking differences observed, synthetic copolymers containing different combinations of effective and ineffective bases were also examined. The results are summarized in Table 2, from which several facts emerge. No copolymer containing either C or G as a principal com-

TABLE 1

EFFECT OF SYNTHETIC HOMOPOLYMERS ON Qβ-REPLICASE ACTIVITY

Homopolymer	Cpm incorporated	Inhibition, %
Poly A	562	85.5
Poly U	340	91.2
Poly C	3867	0.2
Poly G	4274	0
Control	3878	—

Reactions are run under the standard conditions described previously.[3] Each reaction volume (0.25 ml) contained the following in μmoles: Tris HCl, pH 7.4, 21; $MgCl_2$, 3.2; CTP, ATP, UTP, and GTP, 0.2 each, and 40 μg protein, 1 μg of Qβ-RNA, 1 μg of synthetic polynucleotide. The reaction is run for 20 min at 35°C and terminated by precipitation with 10% TCA in an ice bath, followed by washing on a membrane for liquid scintillation counting all as detailed previously.[1, 3] UTP^{32}, synthesized according to the procedure of Haruna *et al.*,[1] was used at a level of 1×10^6 cpm/0.2 μmole.

TABLE 2

EFFECT OF SYNTHETIC COPOLYMERS ON Qβ-REPLICASE ACTIVITY

Copolymer	Cpm incorporated	Inhibition, %
CA(10:1)	1655	3.2
CA(1:10)	1305	23.7
CA(1:1)	1470	14.0
CU(10:1)	1810	0
CU(1:10)	652	61.9
CU(1:1)	1714	0
UG(1:2:1)	1555	9
UA(3:2)	256	85
Control	1710	—

Details of reactions and subsequent handling are as described in Table 1. Again the Qβ-RNA and synthetic polynucleotide were present at 1 μg each per reaction mixture. The numbers in parentheses of the first column indicate relative composition of the copolymer used. Thus, CA (10:1) indicates a 10:1 ratio of C to A, and CA (1:10) a 1:10 C to A ratio. The enzyme used in these experiments had a specific activity about half that usually encountered.

TABLE 3

PER CENT INHIBITION AT DIFFERENT LEVELS OF TEMPLATE

	Qβ-RNA in μg		
	1	0.6	0.2 γ
Poly A	40.7	37.2	35.5
Poly U	67.0	78.4	91.2

Reactions were run as described in Table 1, except that the Qβ-RNA was varied as indicated and the synthetic polynucleotides were present at 0.1 μg per reaction mixture in all cases. The control reaction incorporated 3790 cpm in 20 min in the absence of poly A and poly U. The numbers give the per cent inhibition observed due to the synthetic polynucleotides.

TABLE 4

INTERACTION WITH FRAGMENTED TEMPLATE

	Polymer, γ	Cpm incorp.	Inhibition, %
Poly A	0.1	1036	10
	1.0	931	19.7
Poly U	0.1	1246	0
	1.0	941	19.0
Control	0	1162	—

Reactions were run as described in Table 1. The Qβ-RNA used was fragmented from 28*S* to 12*S*, each reaction containing 1 μg.

ponent shows any ability to inhibit. Even the two copolymers CA (1 to 10) and CU (1 to 10) are not as effective as the corresponding homopolymers of A and U. The only copolymer examined which approaches the inhibitory capacity of either poly A or poly U is the copolymer containing both of these bases.

(c) *Comparative effectivensss of poly A and poly U:* The syntheses thus far described were carried out at the saturation point of template to enzyme (1 γ to 40 γ). It was of some interest to compare the inhibitory effectiveness of the two polymers by examining the reaction at lower polymer concentrations at varying levels of template. The results (Table 3) reveal that poly U is the more effective of the two. Thus, at 0.2 γ of Qβ-RNA, 0.1 γ of poly A inhibits approximately 36 per cent, whereas an equivalent amount of poly U achieves a 91 per cent inhibition.

(d) *Effects of poly A and poly U on the reaction with fragmented templates:* The abnormality of the reaction mediated by fragments of Qβ-RNA suggests that their interaction with replicase does not involve normal functioning of the recognition mechanism. Consequently, the ability of poly A and poly U to interfere with the limited synthesis observed under these conditions should be lower. That this expectation is realized is shown in Table 4. It will be noted that 0.1 γ of poly A is able to achieve only 10 per cent inhibition in this case, whereas it achieved 40.7 per cent inhibition in the case of the intact template reaction. Similarly, 0.1 γ of poly U has virtually no effect on the fragmentary action, whereas it exerted a 67 per cent inhibition on the intact reaction. Finally, at polymer levels of 1 γ, where over 90 per cent inhibition is achieved with the intact reaction, only a 20 per cent effect is observed with either poly A or poly U.

(e) *Effect of the order of addition on the inhibition:* If the inhibiting polymer and the template are attached to the same enzymatic site, it might be expected that the extent of the inhibition observed would be drastically influenced by the order in which template and polymer are added to the reaction mixture. In the course of these studies, an examination was also made of the effect of the four riboside triphosphates on complex formation between template and replicase to the enzyme. The results are summarized in Table 5 for both poly U and poly A. The preincubation period with the indicated components was carried out for 5 min at 35°. All missing components and UTP^{32} were then added and the incubation was continued for another 5 min at 35°.

A survey of Table 5 reveals that prior addition of the template does have a dramatic effect on the ability of either poly U or poly A to inhibit the reaction. Further, the four riboside triphosphates are not necessary to achieve reversal of the inhibitory effect. There is no significant difference in the response of the enzyme to

TABLE 5

EFFECT OF ORDER OF ADDITION ON INHIBITION BY POLYNUCLEOTIDE

Preincubation Components				Components Added Later			Poly U			Poly A		
							Cpm			Cpm		
RNA	Enzyme	XTP	Polymer	RNA	Enzyme	XTP	Polymer	−	+	Inhib., %	−	+	Inhib., %
+	+	4	−	...	...	...	+	6044	4632	23	5682	5721	1
+	+	3	−	...	...	1	+	2949	3264	0	2705	3617	0
+	+	0	−	...	...	4	+	2559	1820	28	2305	1924	16
−	+	4	+	+	...	...	...	1252	210	84	1962	361	82
−	+	3	+	+	...	1	...	1214	160	87	2129	385	82
−	+	0	+	+	...	4	...	1330	180	86	1662	483	71
−	+	4	+	...	+	...	...	1320	193	85	2002	415	79
+	−	3	+	...	+	1	...	1165	147	87	1417	389	72
+	−	0	+	...	+	4	...	1295	250	81	1295	241	81
+	+	4	+	...	...	...	...	4744	608	87	4905	926	81
+	+	3	+	...	...	1	...	2481	369	85	2722	742	73
+	+	0	+	...	...	4	...	2069	239	88	1695	232	83

Preincubation with the indicated components tested 5 min at 35°C. Missing components and UTP^{32} were then added, and the incubation was continued for another 5 min. XTP refers to the four riboside triphosphates, and the numbers in the corresponding columns indicate whether all (4), 3 (all except UTP), or none were present in the preincubation. All other details as in Table 1.

either poly U or poly A in these experiments. The fact that the order of addition determines the outcome reinforces the conclusion that a specific interaction is being observed between the inhibiting polyribonucleotide and the site on the enzyme which recognizes the template.

(f) *Kinetics of the inhibitory reaction:* It was of interest to examine in greater detail the events following addition of the polymer after the reaction was allowed to proceed for a while with different levels of template. Figure 1 shows the results observed at saturation concentration of template. It will be noted again (see Table 5) that the inhibition is virtually complete if the poly U is added at the same time as the template. On the other hand, addition of poly U or poly A after (2 or 10 min) the reaction has been initiated permits considerable synthesis of RNA. These results suggest that the polymer is unable to displace the template immediately from the enzyme. There is, however, a small but definite probability that displacement will occur and eventually the reaction is inhibited. Note from the zero time addition experiment of Figure 1 that the template is unable to displace the synthetic polynucleotide.

This same question was examined at template inputs below saturation, which leads to autocatalytic synthesis.[7] The autocatalytic kinetics is presumably due in part to the fact that there are unoccupied enzyme molecules which become activated by new strands as they are completed. Presumably, the presence of poly U should make unoccupied replicase molecules unavailable to newly synthesized product. Then it would be expected that the addition of polynucleotide to the reaction mixture should lead to the immediate conversion of autocatalytic to linear synthesis. Figure 2 shows that this expectation is also realized. It will be seen from the control that autocatalytic syn-

thesis extends over a period of about 40 min. The addition of poly U at either 13 or 22 min results in rapid conversion to linear kinetics. It should be noted that the amounts of poly U added corresponded to the amount of Qβ-RNA present in the system at the time of addition and were lower than those used in the experiments of Figure 1. Hence, a more extensive period of linear synthesis is observed in the experiments of Figure 2.

Discussion.—The experiments reported here tested consequences of a specific model of the Qβ-replicase recognition mechanism which stemmed from the following observations: (1) *intact* Qβ-RNA is necessary for the proper activation of the Qβ-replicase;[8] (2) the base composition of the first 100 nucleotides is rich in A and poor in U as determined by a synchronized *in vitro* synthesis.[9] The model then proposes that Qβ-replicase distinguishes one RNA molecule from another, and simultaneously determines intactness, by scanning for a secondary region formed by the pairing of two sequences, one predominantly A and the other correspondingly rich in U.

The fact that poly A and poly U inhibit the reaction, whereas synthetic polynucleotides poor in A and U do not, is consistent with this view. Prior addition of the template to the enzyme markedly reduces the inhibitory effect of both poly A and U, a result which is expected if the interaction is occurring at the enzymatic site used to recognize the homologous template.

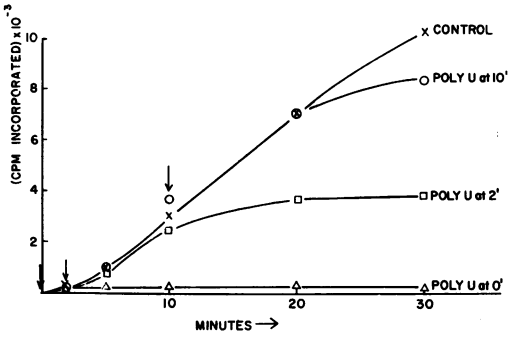

FIG. 1.—Effect of adding poly U after initiation of synthesis. Conditions are as specified in Table 1 with the following exceptions: 0.5-ml reaction volume from which 50 λ samples were removed. Qβ-RNA was 1.2 γ, and the poly U added was 1 γ. The UTP^{32} specific activity was such that the incorporation of 6470 cpm represents the synthesis of 1 μg of RNA.

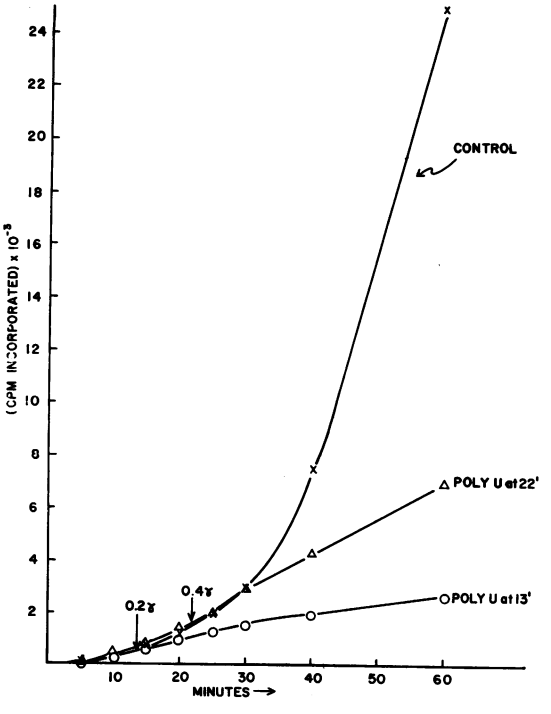

FIG. 2.—Effect of adding poly U during autocatalytic synthesis. Conditions are as specified in Fig. 1 with the following modifications: 0.2 γ of Qβ-RNA added initially and the amounts of poly U added at the indicated times corresponded to the concentration of Qβ-RNA present at the time. The UTP^{32} specific activity was such that the incorporation of 12,900 cpm represents the synthesis of 1 γ of RNA.

The inability of Qβ-RNA to reverse the inhibition with time and the failure of either poly A or poly U to effect immediate displacement of Qβ-RNA from the enzyme-template complex suggest that the union between replicase and nucleic acid is comparatively irreversible. This may explain the unusually rapid approach to plateaus generally observed[6, 7, 13] in curves examining saturation of enzyme with template.

It is of immediate interest to examine unrelated replicases in a similar manner, and for this purpose the MS-2 replicase is being investigated.[14] It is already evident that its reponse to synthetic polynucleotides is different. Thus, unlike the Qβ-replicase, the MS-enzyme is indifferent to poly U but strongly inhibited by poly G.

It should be obvious that the observations recorded open up a new approach to achieve highly selective interference with viral multiplication.

Summary.—Previous experiments have established[1, 3] that RNA replicases induced by RNA viruses require *homologous* and *intact* RNA for proper synthetic activity. Studies[8, 11] of the Qβ-replicase suggested that this enzyme recognizes a secondary structure formed by the pairing of two complementary sequences, initial and terminal, one containing predominantly A and the other U. In conformity with this model, it has been found that Qβ-replicase is specifically inhibited by synthetic polynucleotides composed principally of either A or U. Other polynucleotides, containing mainly or solely C or G, are inert. It was further shown that prior attachment of homologous template to enzyme eliminates the immediate inhibition by either poly A or U.

The discovery of specific template requirements and the studies reported here suggest a new approach in the search for a novel and highly selective interference with viral replication via compounds which can neutralize the recognition mechanism between a replicase and its homologous template.

The authors would like to express their appreciation for the assistance of Dr. B. H. Leichtling in the performance of the kinetic experiments described.

* This investigation was supported by U.S. Public Health Service research grant CA-01094 from the National Cancer Institute and grant GB-2169 from the National Science Foundation.

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