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*RECOGNITION OF SIZE AND SEQUENCE BY AN RNA REPLICASE**

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RNA replicases (RNA-dependent RNA polymerases) induced in *E. coli* by two serologically distinct RNA bacteriophages (MS-2 and Qβ) have been purified[1, 2] to a stage where they are free of detectable levels of RNase, phosphorylase, and DNA-dependent RNA polymerase. The enzymes require homologous RNA and can mediate prolonged and extensive synthesis of polyribonucleotide.

Further experiments with the Qβ-replicase established[3, 4] the following crucial features of the reaction: (1) the enzyme generates a polynucleotide of the same molecular weight (1×10^6) as the viral RNA; (2) when reactions are initiated with template at concentrations below saturation of enzyme, autocatalytic synthesis of RNA is observed; (3) the purified product can, in turn, serve with full effectiveness as a template; (4) the RNA produced by the enzyme is biologically active, being as fully competent as the original RNA to program the synthesis of complete virus particles in protoplasts. These properties demonstrate unambiguously that the enzyme preparation being studied is, in fact, generating identical replicas of the input templates.

We wish here to focus attention on the discriminating selectivity displayed by the two replicases in their response to added RNA. Under optimal ionic conditions, both are virtually inactive with heterologous RNA, including ribosomal and sRNA of the host. Neither replicase can function with the other's RNA. Each recognizes the RNA genome of its origin and requires it as a template for synthetic activity.

The specificity exhibited raises questions of the mechanism employed by the

replicase to distinguish its template from other RNA molecules. An obvious device would invoke the recognition of a beginning sequence, a possibility open to the simple test of challenging the replicase with fragmented preparations of homologous RNA. *If the initial sequence is the sole requirement, RNA fragmented into half and quarter pieces should serve adequately as templates.*

The primary purpose of the present paper is to describe some of the principal findings of the relevant experiments. Fragmented viral RNA was found to be extremely inefficient in stimulating the replicase to synthetic activity, implying that the recognition mechanism involves more than the beginning sequence. It is apparently designed to avoid the replication of fragments of its own genome even if they contain the beginning sequence. In the course of these studies, effects of manganese on the specificity of the reaction have been uncovered and will briefly be noted.

Materials and Methods.—The host and assay organism is *E. coli* Q-13, an Hfr mutant lacking ribonuclease I and phosphorylase activities.[5] The virus is Qβ, isolated by Watanabe.[6] All the methods of preparing infected cells, purification of the replicase, synthesis of radioactive substrates, and assay for enzyme activity have been detailed previously.[1-4]

The RNA of the Qβ virus is apparently more accessible to enzymatic breakage than that of MS-2, and therefore some care and speed has to be exercised in preparing RNA from this virus. The procedures are as follows: *E. coli* (Q-13) cultures were grown to an OD_{660} between 0.2 and 0.3 in the presence of MS-2 antiserum at a dilution of 1 to 10,000. Qβ phage is added at a multiplicity of infection of 10 and the culture is incubated for 30 min at 37°C without shaking. The cultures are then aerated by shaking, lysis occurring after about 2 hr. Shaking is continued for an additional 4 hr and then the cultures are chilled. The lysate is centrifuged for 10 min at 5,000 × *g* and 311 gm of ammonium sulfate are added to each liter of chilled supernatant. After 3–4 hr at 5°C, the particles are collected by centrifugation at 13,000 × *g* for 1 hr. They are then resuspended in TM buffer (10^{-2} *M* Tris, 5 × 10^{-3} *M* $MgCl_2$, pH 7.5) and dialyzed twice for 2 hr against 2,000 ml of TM buffer. The phage suspension is then centrifuged at 10,000 × *g* for 10 min and the supernatant fluid incubated with pronase (1 mg/ml) for 1 hr at 30°C. Sodium dodecyl sulfate is then added to 0.4% and the RNA isolated by phenol[3] at 4°C.

Results.—(a) *Response to fragmented Qβ RNA:* Fragmented RNA molecules are readily obtained from Qβ by prolonging the 4-hr period of the ammonium sulfate precipitation to 10 hr and beyond. The resulting RNA shows evidence of an or-

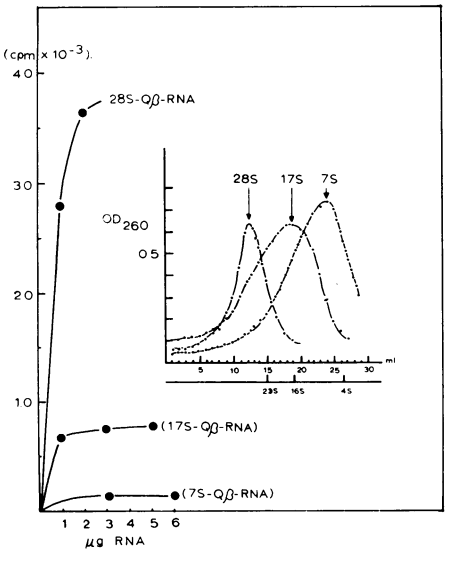

FIG. 1.—Response of replicase to intact and fragmented Qβ-RNA. Inset represents distribution in a linear sucrose gradient (2.5–15%) of RNA prepared and fractionated as described in *Methods*. Each preparation was run in a separate bucket for 12 hr at 25,000 rpm and 4°C in a SW-25 Spinco rotor with markers (ribosomal RNA) to permit *S*-value determinations. The template function of each size class of RNA was determined in the assay described previously.[2] In addition to 40 μg of protein, each standard reaction volume of 0.25 ml contained the following in μmoles: Tris HCl, pH 7.4, 21; $MgCl_2$, 3.2; $MnCl_2$, 0.2; CTP, ATP, UTP, and GTP, 0.2 each. The reaction is run for 20 min at 35°C and terminated in an ice bath followed by precipitation with TCA and washing on a membrane for liquid scintillation counting as described previously.[1, 2] UTP^{32} synthesized as detailed by Haruna *et al.*[1] was used at a level of 1 × 10^6 cpm/0.2 μmole.

dered breakdown revealed by the appearance of reproducible peaks having sedimentation values lower than the 28*S* of the intact viral RNA. These are fractionated for size on a sucrose gradient, collected, and concentrated by alcohol precipitation. The sedimentation profiles of three preparations are shown in the inset of Figure 1. The first is the intact viral RNA (28*S*) prepared as described in *Methods*. The second is a half-piece with a mean of about 17*S*, and the third possesses a sedimentation coefficient of 7*S*. The responses of the replicase to the three RNA preparations are shown in Figure 1. It is obvious that the fragmented material is unable to stimulate the replicase to anywhere near its full activity.

TABLE 1

EFFECTS OF MG AND MN ON TEMPLATE SPECIFICITY OF REPLICASE

Reactions (0.25 ml) contained 1 μg of the indicated RNA and 40 μg of protein in addition to the components detailed in the legend of Fig. 1 with modifications as noted in the following table. Incubations were carried out at 35°C for 20 min; washing and counting was as described in Fig. 1. TYMV refers to intact RNA isolated from Turnip Yellow Mosaic virus. Qβ-28*S* is the intact RNA of the Qβ bacteriophage, and 17*S* is the fragment of half-size.

Template	Mg^{++} (μM)	Mn^{++} (μM)	Incorp. (cpm)
Qβ-28*S*	—	—	146
Qβ-17*S*	—	—	63
TYMV	—	—	0
—	—	—	16
Qβ-28*S*	4	—	4655
Qβ-17*S*	4	—	109
TYMV	4	—	218
—	4	—	1
Qβ-28*S*	—	0.12	474
Qβ-17*S*	—	0.12	222
TYMV	—	0.12	766
—	—	0.12	103
Qβ-28*S*	3	0.2	2804
Qβ-17*S*	3	0.2	240
TYMV	3	0.2	1037
—	3	0.2	103

(b) *The effect of manganese on the size and sequence requirements of the replicase:* In the course of examining template specificity and size requirements of the Qβ-replicase, a striking effect of manganese was uncovered. A typical set of results is summarized in Table 1. In the presence of Mg^{++}, the ability of either the 17*S* fragment or TYMV-RNA to stimulate the enzyme is almost negligible, corresponding to 2 and 4 per cent, respectively, of that observed with intact Qβ-RNA. On the other hand, if Mn^{++} is substituted for Mg^{++}, a striking change is observed. Normal synthesis with 28*S* RNA is strongly inhibited, whereas the abnormal activities observed with 17*S* and TYMV-RNA are stimulated. Indeed, if one were unaware of the optimal conditions for the functioning of this enzyme (Mg^{++} and intact templates), one might be tempted to conclude from the results obtained with Mn^{++} that the Qβ-replicase prefersTYMV-RNA as a template and is comparatively indifferent to the size of the RNA with which it functions.

The abnormalities induced in the reaction by either fragmented RNA or the presence of manganese is even more obvious from the kinetics of the reaction (Fig. 2). As had been observed previously,[2-4] synthesis continues linearly for extensive periods in the presence of saturating amounts of intact template and Mg^{++}. If, however, Mn^{++} is included in the reaction mixture, the reaction stops in about 60 min even with the 28*S* RNA as the template. If we now examine the response of the enzyme to fragmented template (Fig. 2), it is seen that the initial reaction rate is less than 10 per cent of normal and, furthermore, ceases completely in 30 min whether Mn^{++} is present or not.

Discussion and Summary.—Attempts at analyzing replicating mechanisms must recognize the implications of the fact that the enzymes involved are likely to be complicated molecules. High levels of complexity provide the flexibility which permits the occurrence of abnormal activities, a potentiality which can be accentuated by exposure to either strange environments or unusual components. Thus, in

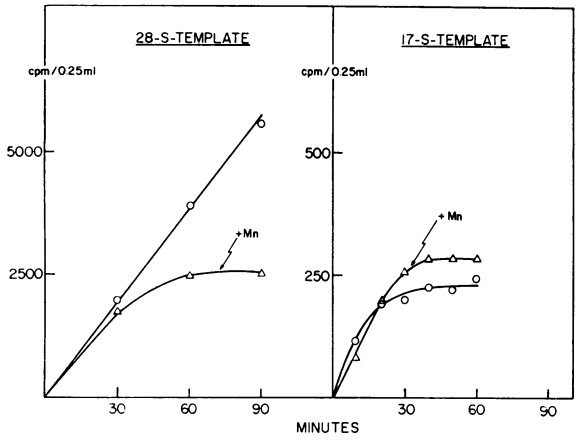

FIG. 2.—Effect of template size and Mn^{++} on kinetics of RNA synthesis. Reactions were set up as detailed in Fig. 1, except that Mn^{++} was omitted or included as indicated. Samples of 0.25 ml were taken and treated for counting as in Fig. 1. Template concentrations were in all cases 1 μg of RNA for each 40 μg of enzyme in 0.25 ml. Note that the ordinate of the right half is 1/10 that of the left half of the figure.

the absence of primer, the DNA-dependent DNA polymerase eventually initiates the synthesis of an A-T copolymer.[7] In the presence of Mn^{++}, the same enzyme will incorporate riboside triphosphates[8] into a mixed polymer. Analogously, the DNA-dependent RNA polymerase (transcriptase) synthesizes poly A if supplied only with ATP, a reaction which is inhibited if the other riboside triphosphates are added.[9, 10] If presented with a single-stranded DNA, the transcriptase synthesizes a DNA-RNA hybrid,[11–14] and if the template is RNA, a duplex RNA results.[15–17] The fact that such artifacts can occur makes it difficult to draw incontestable conclusions from the appearance of a product in a reaction. Evidence, other than simple existence, must be provided before a component is accepted as an obligatory normal intermediate in the polymerization process.

The experiments reported indicate that the response of the replicase to fragmented Qβ-RNA and heterologous RNA is very different from what is observed with intact Qβ-RNA. The nature of the difference in terms of the products produced will be detailed in a subsequent publication. For the present, it must be emphasized that *the study of the normal functioning of the replicase requires intact homologous RNA and the avoidance of* Mn^{++}. The early cessation of the reaction with fragments (Fig. 2) may explain the limited synthesis seen by others[18, 19] with partially purified enzyme preparations from which nucleases have not been completely eliminated.

The inability of the replicase properly to employ fragments of its own genome as templates argues against a recognition mechanism involving only a beginning sequence. The enzyme can apparently sense when it is confronted with an intact RNA molecule, implying that some element of secondary structure is involved. A plausible formal explanation can be proposed in terms of "functional circularity." Thus, a decision on the intactness of a linear heteropolymer can be readily made by examining both ends for the proper sequences. An examination of this sort would be physically aided by forming a circle using terminal sequences of overlapping complementarity. The enzyme could then recognize the resulting region of double strandedness. This particular mechanism is offered only as *an example* of how an enzyme could simultaneously distinguish both sequence and intactness. Whatever the details turn out to be, it appears that the RNA replicases are designed to minimize the futility of replicating either foreign sequences or incomplete copies of their own genome.

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*THE IN VITRO TRANSLATION OF A MONOCISTRONIC MESSAGE**

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Analyses of the translation of RNA into protein are ideally carried out with the aid of defined RNA messages directing the *in vitro* synthesis of proteins.[1] The most convenient sources of homogeneous translatable messages are the RNA viruses. In the cases thus far reported,[2–5] the RNA messages employed have been polycistronic,[5, 6] i.e., they contain information for the coding of several proteins. This feature permits the study of the factors governing the order and frequency of translation of several cistrons linearly arrayed in the same RNA molecule. Multiple cistrons, however, introduce an unwanted complexity if the intent is to relate the detailed chemistry of a message to that of the final polypeptide product produced. For such purposes, one would prefer a monocistronic message containing information for only one protein.

Recent investigations[7] with Satellite Tobacco Necrosis Virus (STNV) suggest that the RNA of this virus is 400,000 in molecular weight and that its coat protein contains approximately 400 amino acid residues. Assuming a nonoverlapping triplet code, it is evident that the genome of STNV contains just enough information to code for its coat protein. The RNA molecule must consequently be a monocistronic message.

The present paper reports the message properties of STNV-RNA. The tryptic