Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 50, No. 5, pp. 905–911. November, 1963.

*AN RNA "REPLICASE" INDUCED BY AND SELECTIVE FOR A VIRAL RNA: ISOLATION AND PROPERTIES**

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Communicated by M. M. Rhoades, August 26, 1963

The existence of the RNA viruses raises obvious questions concerning information transfer in organisms with an RNA genome. Doi and Spiegelman[1] have shown that neither before nor after infection can sequences be detected in the DNA of the host cell which are complementary to the viral RNA. These results suggest that RNA viruses do not employ DNA as an informed intermediary in any synthetic step required for components unique to the formation of virus particles. On this ground and others one is led to predict[2, 3] a mechanism of RNA replication involving an RNA-dependent RNA polymerase, which we will hereafter refer to as a "*replicase.*" (We have elsewhere pointed out[2] that the repetitive use of such phrases as "DNA-dependent RNA polymerase," "RNA-dependent RNA polymerase," etc., can be avoided by introducing three simple terms. As noted, the RNA-dependent RNA polymerase which functions to turn out RNA replicas is termed a "*replicase.*" The corresponding enzyme which synthesizes DNA duplicates can be called a "duplicase." Finally, the transcribing enzyme which employs DNA as a template to synthesize complementary RNA can be referred to as a "transcriptase." These terms have the advantage of brevity, precision, and a useful alliterative allusion.)

Available evidence makes it unlikely that an enzyme using an RNA template functions in uninfected cells. All recognized cellular RNA components, including the "message" fraction,[4–6] ribosomal,[7, 8] and amino acid transfer RNA[9, 10] have been shown to be complementary to some sequences in homologous DNA. Consequently, the pathway of their formation can be adequately explained by the DNA-dependent RNA synthesizing mechanism. This conclusion is further strengthened by the observations that actinomycin D inhibits[11] normal cellular RNA synthesis without interfering with the appearance of viral RNA.[12]

The following two predictions are generated by the considerations just summarized: (1) the infecting RNA strand of the virus must serve as a message and be conserved during its translation into protein; (2) an enzyme should be obtainable from cells infected with RNA viruses which is uniquely dependent on RNA to exhibit RNA polymerizing activity.

The first prediction was confirmed[13] through the use of double labeling (N^{15} and P^{32}) and the demonstration that the two isotopes could be recovered in the same RNA strands at the end of a complete lytic cycle. It is the purpose of the present paper to offer evidence which confirms the expectation that a new type of polymerase is induced by an RNA virus.

The experiments to be described were performed with the RNA bacteriophage MSϕ2 used in the earlier investigations noted,[1, 13] and is similar to the f2 of Loeb and Zinder.[14]

A search for a unique RNA-dependent polymerase is complicated by the presence of a variety of enzymes[15] which can incorporate ribonucleotides either terminally or subterminally into pre-existent RNA chains. In addition, there are others (e.g.,

RNA phosphorylase,[16] DNA-dependent RNA polymerase,[17] polyadenylate synthetase[18]) which can effect extensive synthesis of polynucleotide chains. Many of these sources of confusion can be avoided by suitable adjustment of the assay conditions and supplementary tests for a requirement of all four triphosphates, etc.

The most serious difficulty is introduced by the DNA transcriptase, since it appears that, when isolated, it can employ certain types of RNA as substitutes for DNA as templates for polyribonucleotide synthesis.[19, 20] Under these circumstances, the use of actinomycin D or DNAase does not ensure against observing its activity. The only certain way to avoid interference with the DNA-dependent enzyme is to eliminate it from the fraction of interest. It is obvious that a claim for a new type of RNA polymerase must be accompanied by evidence for RNA dependence and a demonstration that the enzyme is distinguishable in one or more of its properties from previously known enzymes with which it can be confused.

Several recent reports offer suggestive evidence of polyribonucleotide synthesis activity induced by RNA viruses of animal[21] and bacterial hosts.[22] However, in no case was an enzyme isolated in a sufficiently purified state to permit a definitive demonstration of RNA dependence. Under these circumstances, investigation of template specificity was obviously impossible.

It is the purpose of the present paper to show that an RNA-dependent polymerase can be isolated from *E. coli* cells infected with an RNA bacteriophage (MSϕ2). Further, the enzyme shows a selective preference for its own RNA as a template.

Materials and Methods.—1. *Bacteria and virus:* The bacterial virus, MSϕ2, was provided by Dr. Alvin J. Clark; it was grown and assayed according to the procedures of Loeb and Zinder.[14] Preparation of virus stocks and purified viral RNA followed the methods of Doi and Spiegelman.[13]

2. *Preparation of infected cells:* The medium used contained the following in gm/liter: Bactotryptone, 10; NaCl, 10; Difco yeast extract, 5; glucose, 1; to each liter was added 1.3 ml of 2 *M* $CaCl_2$. The procedure was as follows: (1) one liter of a log phase culture ($O.D._{660}$ of 0.150) is inoculated with phage at multiplicity of 0.1–0.2 and allowed to go into complete lysis and then used as a source of virus inoculation for larger batches. Thirty-five liter quantities of cells are grown up in cyclone pumps to an $O.D._{660}$ of 0.3. They are then infected with a multiplicity of 10 and allowed to aerate for several min. The aeration is interrupted 10 min for absorption, reinstituted, and the infection allowed to proceed 30–40 min. The process is stopped by chilling with crushed ice, the cells are harvested, and then stored frozen at −20°C. Such cells retain their ability to yield active enzyme for periods exceeding 4 months. Uninfected cells are prepared by the same procedure and stored in the same manner.

3. *Labeled substrates:* C^{14}-labeled riboside triphosphates were all purchased from Schwarz BioResearch, Inc. They were used at the following specific activities: ATP-C^{14}, 2×6 cpm/μM; GTP-C^{14}, 0.72×10^6 cpm/μM; UTP-C^{14}, 1.8×10^6 cpm/μM; CTP-C^{14}, 1.7×10^6 cmp/μM. P^{32}-labeled nucleotide was prepared by a modification of Tener's[23] procedure suggested to us by A. M. Michelson. The method is illustrated by the synthesis of UMP^{32}: a mixture of 100 μM of inorganic phosphate and 200 μM of isopropylidine is dissolved in 3 ml of dry pyridine, and the solution evaporated to dryness. More pyridine is added, and the procedure is repeated several times. The residue is finally dissolved in 0.1 ml of dimethylformamide and 1 ml of pyridine, to which is added 200 μM of β-cyanoethanol and then 1,000 μM of dicyclohexylcarbodiimide, and the mixture is left at 37°C overnight under anhydrous conditions. Pyridine is removed under reduced pressure, and to the residue is added 2 ml of 2 *N* NaOH; the mixture is kept at 100°C for 30 min. To remove the protecting isopropylidene residue, the mixture is adjusted to pH 1.0 with 2 *N* HCl, kept at 100°C for 45 min, and then neutralized to pH 8.0. (If applied to purine nucleosides, the acidic hydrolysis should be at pH 2.0 at 100°C for 30 min.) Usually 80–90% conversion of inorganic phosphate into nucleoside-5′-phosphate is achieved. Conversion to the triphosphate is accomplished by the baker's yeast kinase as described by Weiss.[24] The UTP^{32} was employed at 2×10^6 cmp/μM.

4. *Reagents:* Unlabeled riboside triphosphates were from Pabst Laboratories, Milwaukee, Wisconsin. DNAase was 2 × recrystallized from Worthington Biochemical Company, Freehold, New Jersey. It was further purified on DEAE columns to remove contaminating ribonuclease.[25] Phosphoenolpyruvate (PEP) and the corresponding kinase (PEP-kinase) were from C. F. Boehringer and Soehne, Mannheim, Germany. Lysozyme was purchased from Armour and Company, Kankakee, Illinois. Poly A, Poly U, and Poly C were obtained from the Miles Chemical Company, Clifton, New Jersey. Poly G was a gift from Dr. J. Fresco, and turnip yellow mosaic virus (TYMV) RNA was kindly provided by Dr. R. Haselkorn. Tobacco mosaic virus RNA was isolated and purified from infected plants supplied by Dr. L. M. Black.

5. *Assay of enzyme activity by incorporation of radioactive nucleotides:* The standard reaction of 0.25 ml contained the following in μM: Tris-HCl pH 7.5, 21; $MgCl_2$, 1.4; $MnCl_2$, 1.0; KCl, 3.75; mercaptoethanol, 0.65; spermine, 2.5; phosphoenolpyruvate, 1.0; $(NH_4)_2SO_4$, 70; CTP, ATP, GTP, and UTP, 0.5 each. In addition, it contained pyruvate kinase, 5 μg, DNAase, 2.5 μg, and, where indicated, 10 μg of the polynucleotide being tested as template. Enzyme was assayed at levels of 50–300 μg protein per sample. DNAase was always omitted in assaying for DNA-dependent polymerase activity. Incubations were carried out at 35°C for 10 min and terminated by placing the reaction mixture in an ice bath and by the addition of 0.15 ml of neutralized saturated pyrophosphate, 0.15 ml of neutralized orthophosphate, and 0.1 ml of 80% trichloracetic acid (TCA). The precipitate was washed onto a millipore filter and washed five times with 10 ml of cold 10% TCA containing 0.9% of Na pyrophosphate. The millipore membrane was then dried and counted in a liquid scintillation counter, as described previously.[4] The pyrophosphate was included in the wash, since we found in agreement with Kammen *et al.*[26] that its presence lowered zero time backgrounds to acceptable levels (40–70 cpm per sample containing input counts of 1×10^6 cmp).

6. *Preparation of enzyme:* A suitable aliquot of the frozen infected cells is removed and put through the following procedure which is specified for 2 liters of infected cells at 1×10^9 cells per ml. (*a*) The frozen cells are suspended in "standard buffer" (0.01 *M* Tris, pH 7.2; 0.005 *M* $MgCl_2$; 0.0005 *M* mercaptoethanol) and allowed to thaw. To this, 5 μg/ml of DNAase and 1 mg/ml lysozyme are added, and the mixture is frozen and thawed twice, as described by Hayashi and Spiegelman.[4] The resulting mixture is allowed to incubate for 10 min at 20°C after increasing the level of DNAase to 10 μg/ml. (*b*) The extract is centrifuged at 15,000 *g* for 20 min, and the supernate and pellet are separated. The pellet is frozen with dry-ice acetone and ground in prechilled (−15°C) mortar for 10 min. It is then resuspended in 5 ml of "standard buffer" and again centrifuged at 15,000 *g* for 20 min. The two supernates are combined and possess an $O.D._{260}$ of about 300. (*c*) The crude supernate is made up of 25 ml with "standard buffer" and adjusted to 0.01 *M* EDTA and incubated at 0°C for 5–15 min. The appearance of a light, white precipitate indicates the onset of ribosomal destruction. The extract is then centrifuged at 15,000 *g* for 20 min and the precipitate discarded. (*d*) The extract is now subjected to a protamine fractionation which is designed to remove the DNA-dependent RNA polymerase, while leaving the RNA-dependent enzyme in the supernatant. The amount of protamine required to remove virtually all of the DNA-dependent activity varies from 11–13 mg per ml and must be titrated by assay for DNA-dependent activity. After appearance of the precipitate, the extract is centrifuged at 10,000 *g* for 10 min. (*e*) The supernate of the protamine fraction is kept, and to 40 ml is added 23.6 ml of a saturated ammonium sulfate (pH adjusted to 7). After 10 min the material is centrifuged at 10,000 *g* for 10 min. (*f*) The activity is found in the supernate, and to it is added a further 12 ml of saturated ammonium sulfate followed by centrifugation at 10,000 *g* for 10 min. (*g*) The supernate is discarded; the precipitate is dissolved in 6 ml of "standard buffer" and dialyzed against 1 liter of the same buffer for 2 hr. (*h*) The dialysate is then put on a DEAE cellulose column (1.2 × 7 cm) and washed with 40–100 ml of the "standard buffer" made 0.1 *M* with respect to sodium chloride. This effectively removes remaining protamine sulfate and contaminating nucleases. Following the 0.1 *M* wash, the enzyme is eluted with 20 ml of the "standard buffer" made 0.3 *M* with respect to sodium chloride. (*i*) To 20 ml of the eluted enzyme is added 20 ml of a saturated ammonium sulfate, and the enzyme is centrifuged down at 10,000 *g* for 10 min. (*j*) The precipitate is dissolved in 4 ml of the "standar l buffer."

The usual preparation obtained from the above procedure contains 7.4 mg protein per ml

with an O.D.^{280}/O.D.^{260} ratio of 1.2. The enzyme activity is unstable unless the ionic strength is restored. The addition of $(NH_4)_2SO_4$ to 10% of saturation was found to prevent detectable decay of activity for several weeks providing the enzyme is stored in a 0°C ice bath.

Results.—Reasons for including certain components in our "standard assay mixture" (see *Methods*, § 5) may be specifically noted. The riboside triphosphate generating system (PEP and PEP-kinase) was routinely included to avoid contributions by RNA-phosphorylase to the observed incorporation. DNAase was added to eliminate DNA-dependent synthesis. Spermine had previously been found[27] to be an effective neutralizer of ribonuclease commonly found in crude extracts.

From our first examinations of extracts from infected cells, evidence was obtained for DNAase-resistant incorporating activity associated with the pellet fraction which was obtained at 100,000 *g* in 2 hr (100G120P). This feature is illustrated in the data of Table 1 in which the pellet contains a fair amount of DNA-dependent activity of which 16 per cent continues in the presence of DNAase. If the pellet fraction is treated with EDTA as in *Methods*, § 6(*c*) and the supernatant then fractionated with protamine as in *Methods*, § 6(*d*), activities exhibited are as shown in Table 2. Prior to the protamine fractionation there is considerable DNA-dependent activity. The protamine step effectively removes this, the ratio of DNA- to RNA-dependent activity changing from 23.3 to 0.09.

It was found with experience that separation of the extract into the 100G120P pellet and supernatant was unnecessary, and the procedure described in *Methods* § 6 was adopted for routine purposes. Table 3 shows some of the general characteristics of the enzyme fraction thus obtained. It has a clear requirement for viral RNA, and synthesis is only observed when all four triphosphates are present. The omission of any one of them completely abolished incorporation of UMP^{32}. It should also be noted (from the last line of Table 3) that this preparation does not contain detectable amounts of the polyadenylate synthetase.

TABLE 1

LOCATION OF ENZYME ACTIVITY IN INFECTED CELL EXTRACTS

Fraction	Template	NT incorporated in mμM/10 min/mg protein
Pellet	MSϕ2-RNA	0.34
	0	0.28
	CT-DNA*	1.94
Supernatant	MSϕ2-RNA	<0.02
	0	<0.02

* DNAase omitted from assay mixture.
Assay made with UTP^{32} under the "standard conditions" as described in *Methods*, § 5. All template polynucleotides added to a level of 10 μg per reaction mixture.

TABLE 2

SEPARATION OF RNA-DEPENDENT FROM DNA-DEPENDENT POLYMERASE

Fractions	Template	NT incorporated in mμM/10 min/mg protein
(A) Supernatant of EDTA treated 100G120P	0	0.14
	MSϕ2-RNA	0.54
	CT-DNA*	12.6
(B) Prot. sulfate (12 mg/ml) supernatant of A	0	0.18
	ϕ2-RNA	3.5
	CT-DNA*	0.31

* DNAase was omitted from assay mixture.
Assays were carried out with UTP^{32} under conditions of *Methods*, §5. See text for details on fractions.

TABLE 3

TEMPLATE AND TRIPHOSPHATE DEPENDENCE OF ENZYME

Assay mixture	Template	NT incorporated in mμM/10 min/mg protein
(1) Complete	0	0.12
(2) "	MSϕ2-RNA	4.73
(3) −ATP	" "	<0.02
(4) −GTP	" "	<0.02
(5) −CTP	" "	<0.02
(6) *−GTP, CTP, UTP, i.e., C^{14}-ATP only	" "	0.08

* C^{14}-ATP was present as the only triphosphate.
Enzyme was carried through all the steps described in *Methods*, §5. It had an O.D.^{280}/O.D.^{260} ratio of 1.21. UTP^{32} incorporation assayed according to *Methods*, §6.

TABLE 4

NEAREST NEIGHBOR ANALYSIS OF PRODUCT SYNTHESIZED BY RNA POLYMERASE UNDER INFLUENCE OF VIRAL RNA

Per cent mole fractions

Cp^{32}U	Ap^{32}U	Up^{32}U	Gp^{32}U
25.5	25.1	16.3	33.1

0.8 ml of the "standard reaction mixture" (*Methods*, §5) containing UTP^{32} as the labeled riboside triphosphate was incubated for 15 min. The reaction was stopped with 0.3 ml of neutralized saturated Na pyrophosphates and 7 ml of cold 3.5% perchloric acid (PCA). The precipitate was washed 5 times with 7 ml of 3.5% PCA. At each washing 0.8 mg of *E. coli* ribosomal RNA was added as carrier. The final precipitate was dissolved in H_2O and the perchloric acid removed by addition of 0.5 ml *N* KOH and centrifugation. The supernate was incubated at 37°C for 18 hr, and the nucleotides were separated and analyzed on Dowex-1 formate.[3]

It was necessary to demonstrate that the UMP^{32} was incorporated into the internucleotide linkages of a heteropolymer. A larger-scale reaction was run, the product hydrolyzed with alkali, and a nearest neighbor to U determined. The results (Table 4) provide clear evidence that a proper heteropolymer is being synthesized.

A very interesting feature was revealed when the template specificity of the enzyme preparation was examined (Table 5). It will be noted that there is a striking preference for its own RNA. There is little detectable activity with either the host s-RNA or ribosomal RNA. Further, ribosomal RNA does not interfere with the template activity of the MSϕ2-RNA. Of the two plant viruses tested, TMV exhibits low but probably significant activity. TYMV-RNA definitely serves as a template with an efficiency which routinely corresponds to 25 per cent of that observed with MSϕ2-RNA.

TABLE 5

TEMPLATE SPECIFICITY OF PURIFIED RNA-DEPENDENT POLYMERASE*

Template (all at 10 γ/0.25 ml)	NT incorporated in mμM/10 min/mg protein
0	0.08
MSϕ2-RNA	8.5
s-RNA	0.09
Ribosomal RNA	0.06
Ribosomal RNA + MSϕ2-RNA	8.0
TMV-RNA	0.3
TYMV-RNA	2.2
CT-DNA†	0.11

* UTP^{32} incorporation assayed according to *Methods*, §5.
† DNAase omitted from assay mixture.

The enzyme preparations used in the experiments of Tables 4 and 5 contained very little of the DNA-dependent RNA polymerase activity. Consequently, the properties shown cannot be ascribed to contamination by the transcriptase. Nevertheless, as a final check a purified DNA transcriptase was prepared from *E. coli*

according to the procedure of Chamberlin and Berg,[28] and it had a specific activity with DNA of 6,000/mg protein in their units. When presented with MSϕ2-RNA as a template, it showed virtually no activity. Finally, it should be noted that many preparations were made from noninfected cells according to the procedure described in *Methods* (§ 6). None of these showed the specific stimulation by MSϕ2-RNA nor the requirement of all four riboside triphosphates shown in Tables 3 and 5 for the same preparations derived from virus-infected cells.

Discussion.—The experiments described demonstrate that cells infected with an RNA virus contain an RNA polymerase possessing a number of characteristics which serve to identify it as a unique entity. When sufficiently pure, it needs an RNA template for activity. The fact that DNA will not substitute in this requirement clearly distinguishes it from the DNA-primed polymerase. When presented with a heteropolymer containing all four bases, the enzyme has an absolute requirement for all four riboside triphosphates. This characteristic, coupled with the direct demonstration of inactivity with ATP alone (line 6, Table 3), clearly eliminates the polyadenylate synthetase of August *et al.*[18] These same characteristics also serve to distinguish this enzyme from RNA-phosphorylase.

An outstanding feature of this enzyme is its preference for its homologous RNA as a template for activity. The fact that the ribosomal RNA and s-RNA molecules of the host cell are virtually completely inactive as templates is perhaps not too surprising. The production of an enzyme which ignores the mass of pre-existent cellular RNA represents an obvious advantage to the virus. Replica production can thus be focused on the single strand of viral RNA which is the ultimate origin of the final yield of progeny.

It is to be noted that the polymerase induced by MSϕ2 can readily distinguish its own RNA from that of TMV, and even to some extent that of TYMV. This selectivity differentiates this enzyme from other sorts of RNA synthetases. It also may serve as a unique label to permit identification among the class of polymerases induced by the RNA viruses. One is inclined to predict, for example, that the RNA polymerase induced by TMV-RNA will show an equally one-sided preference for its own RNA as a template. It seems likely that the basis of this sort of specificity will be found in the sequences of the RNA, but the details remain to be unraveled.

It will be recalled that with only one possible exception[29] all RNA viruses are single-stranded. Further, no evidence for an RNA-replicating duplex, analogous to that of the single-stranded DNA virus, ϕX174,[30,31] has thus far appeared, despite the fact that RNA-RNA duplexes are known to be very stable structures and resistant to ribonuclease.[32] These observations raise the obvious possibility[13] that RNA replication may not mimic in all details the mechanism of DNA duplication. However, now that the RNA "replicase" has been obtained free of RNA it should be possible to perform the experiments necessary for an understanding of its mode of action.

Summary.—An RNA polymerase has been isolated and purified from cells infected with an RNA virus. It shows a dependence on RNA for polymerizing activity and a mandatory requirement for all four riboside triphosphates when employing a template which contains all four bases. The enzyme exhibits a selective preference for its homologous RNA, being completely inactive with host s-RNA and ribosomal RNA. It is partially functional with certain other viral RNA's.

We would like to express our deep appreciation to Dr. A. M. Michelson who during a stay at Illinois introduced us to the intricacies of nucleotide chemistry. The synthesis of labeled intermediates was thereby converted from a terrifying adventure in uncertainty to a pleasant chore.

* This investigation was aided by grants-in-aid from the U.S. Public Health Service and the National Science Foundation.

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