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Symposium on Replication of Viral Nucleic Acids

II. Ribonucleic Acid Virus Replication[1]

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INTRODUCTION

A cell infected with a ribonucleic acid (RNA)-containing virus has the unique capability of producing viral progeny from information encoded in the RNA strand of the infecting particle. The role of viral RNA in initiating its own replication is of major interest because of the insight this special process may provide into mechanisms for transfer of genetic information. It is believed that RNA of the infecting virus serves both as messenger, to direct the synthesis of proteins required for phage multiplication, and as template, for replication of viral RNA. At present, two viral-specific synthetic functions are recognized. The RNA genome must provide information for synthesis of coat protein, a function demonstrated in vitro (40), and it has been shown that a virus-specific enzyme, or series of enzymes, is involved in the synthesis of progeny RNA (3, 4, 8, 25, 26, 58). Whereas the de novo synthesis of both protein and RNA are vital for these processes (12–18, 46), deoxyribonucleic acid (DNA) synthesis does not appear necessary. Replication of RNA viruses was not inhibited by mitomycin C, or fluorinated pyrimidines (11, 49). Furthermore, transcription of bacterial DNA may not be required for the replicative process, because a normal yield of the bacteriophage MS2 was obtained from protoplasts treated with actinomycin (27). In these experiments, however, protoplasts were prepared 5 min after infection, and actinomycin was added 10 min later. Under these circumstances, an early function requiring bacterial DNA could have escaped detection. There remains the possibility that some function involving bacterial DNA may influence viral RNA synthesis. Evidence from several sources indicates that regulatory mechanisms similar to those exerted on bacterial RNA synthesis affect RNA virus replication. The synthesis of bacteriophage RNA appears to be controlled by amino acids in a manner similar to that of cellular RNA and to be influenced by the RC locus, "RNA control gene," of the bacterial chromosome (21). Phage RNA synthesis is also subject to control by energy source "shift-down" (21). Another mechanism for the control of cellular RNA is the poorly understood process whereby infection with T-even bacteriophage inhibits bacterial RNA synthesis (43). It was recently found that infection by T-even phage inhibits RNA phage replication as well (41; Leventhal, *unpublished data*). It is not known, however, whether this control operates at the level of host DNA. Another observation was that infection of ultraviolet-treated bacteria by RNA phage results in the accumulation of double-stranded viral RNA (41, 44). It was suggested that a host-cell function which normally prevents the accumulation of this double-stranded form was damaged by radiation (41). RNA phage replication may not exhibit the autonomy of the T-even phages which are capable of multiplying in bacteria treated with ultraviolet light (2). From these accumulated data, it seems possible that the ultimate expression of the message contained in the entering viral RNA strand is not completely independent of some function involving bacterial DNA.

Replication of viral RNA requires that the parental strand be used to specify the unique sequence of ribonucleotides in progeny RNA.

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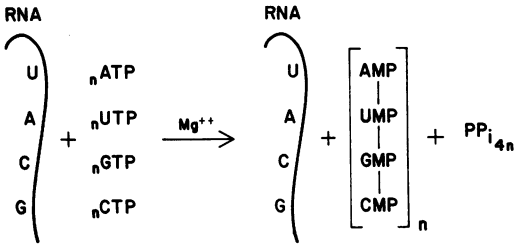

FIG. 1. *Predicted activity of RNA-dependent RNA polymerase.*

One molecular mechanism for template-directed nucleic acid synthesis involves specific hydrogen bonding between purine and pyrimidine bases as set forth in the Watson and Crick (54) model of DNA structure. If the replication of single-stranded RNA viruses is also to involve hydrogen-bonded base pairing, a nucleic acid of complementary base composition must be synthesized. A possible alternative mechanism is the direct synthesis of RNA identical to parental RNA, eliminating the intervention of a complementary polynucleotide (51). There have been many reports, however, of virus-specific RNA with characteristics of a double-stranded structure (7, 19, 30, 33, 39, 44, 56, 57). Parental virus RNA has been found in this form (18, 19, 32, 53, 56), and double-stranded RNA was reported to be infectious (1, 39, 45). There is also evidence that replication of RNA in a reaction catalyzed by an RNA virus RNA polymerase occurs by a template mechanism utilizing specific hydrogen bonds between complementary base pairs (47, 48). By analogy to the double-stranded DNA replicative form of the bacteriophage ϕX-174 (50), it has been postulated that single-stranded RNA viruses require the formation of an intermediate for the formation of progeny RNA. Conclusive identification of the RNA directly involved in replication of progeny viruses, as well as a detailed description of secondary structure, has not been presented, however.

The series of events following RNA virus infection logically suggest a requirement for RNA-mediated RNA replication and the synthesis of at least one virus-specific RNA polymerase. A reaction utilizing RNA as template and ribonucleoside triphosphates as substrates is not known to occur in vitro in normal uninfected cells, whether animal, plant, or bacterial. Several reports have indicated the presence of new RNA polymerase activity after RNA virus infection (3, 4, 8, 25, 26, 58). It is the purpose of this review to discuss the characteristics of this new enzyme(s) and to present data elucidating the biochemical mechanism whereby parental single-stranded RNA leads to the synthesis of many identical copies of RNA for progeny virus.

INDUCTION OF AN RNA VIRUS RNA POLYMERASE

An RNA-dependent RNA polymerase could be predicted to act as outlined in Fig. 1. The *in vitro* assay of such an enzymatic activity utilizes ribonucleoside triphosphate substrates and is carried out in the presence of RNA, Mg^{++}, deoxyribonuclease, and phosphate buffer. The deoxyribonuclease is included to inhibit DNA-dependent RNA polymerase activity, and phosphate buffer excludes polynucleotide phosphorylase activity. Under these conditions, in vitro ribonucleotide incorporation was found to increase after infection with RNA viruses (Fig. 2). The inhibition of this RNA polymerase activity by chloramphenicol suggests that synthesis of a new protein is required for the expression of this activity (36). New RNA polymerase activity has been found after mengovirus infection of L cells (8); bacteriophage MS2 (25, 58), f2 (4), and Qβ (26) infection of *Escherichia coli*; and turnip yellow mosaic virus infection of Chinese cabbage leaves (3). It is generally assumed that this new activity is that of an RNA-dependent RNA polymerase induced by virus infection. It is also possible, however, that the activity present in crude extracts, in whole or in part, is that of the host RNA polymerase utilizing a new type of RNA as template.

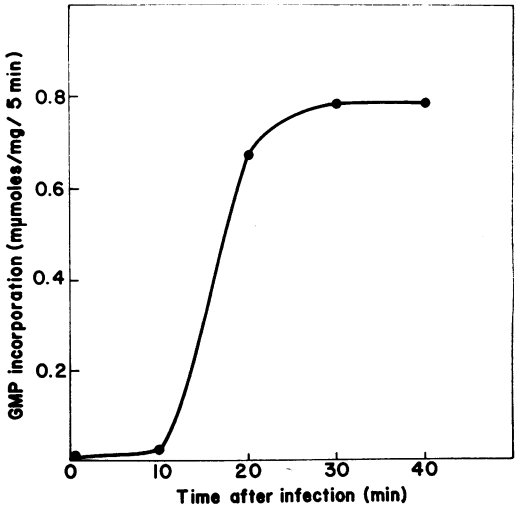

FIG. 2. *Effect of RNA phage infection on ribonucleotide incorporation. Alumina extracts were prepared from Escherichia coli collected before and after infection with phage f2 at intervals as shown on the abscissa. The incorporation of guanosine monophosphate (GMP) was measured as described in Table 1.*

TABLE 1. *Requirements for guanosine monophosphate (GMP) incorporation**

System†	GMP incorporation ($\mu\mu$moles/5 min)
Complete	72.5
Minus $MgCl_2$	3.2
Minus ribonucleic acid	6.6
Minus CTP, UTP, and ATP	2.5
Minus GTP, CTP, UTP, and ATP: plus [C^{14}] dGTP, dCTP, dTTP, and dATP	1.0
Minus GTP, CTP, UTP, and ATP: plus [C^{14}] GDP, CDP, UDP, and ADP	1.9
Minus ribonucleic acid and deoxyribonuclease; plus thymus deoxyribonucleic acid	10.1
Minus deoxyribonuclease; plus ribonuclease (1.0 μg)	0.8
Minus deoxyribonuclease	71.6
Plus actinomycin D (25 μg)	74.1

* The ribonucleic acid (RNA) virus RNA polymerase assay measured the rate of incorporation of radioactivity from a labeled ribonucleoside triphosphate into an acid-insoluble form (6). The standard reaction mixture (0.2 ml) contained 25 μmoles of potassium phosphate buffer, *p*H 7.0; 5 μmoles of β-mercaptoethanol; 2 μmoles of $MgCl_2$; 20 mμmoles of [C^{14}]-GTP (8.0×10^6 counts per min per μmole); 50 mμmoles each of ATP, CTP, and UTP; 300 mμmoles of *Escherichia coli* R-RNA; 5 μg of pancreatic deoxyribonuclease; and 3 μg of protein of the enzyme fraction.

† Abbreviations: CTP, cytidine triphosphate; UTP, uridine triphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; dGTP, deoxyguanosine triphosphate; CDP, cytidine diphosphate; dTTP, deoxythymidine triphosphate; dATP, deoxyadenosine triphosphate; GDP, guanosine diphosphate; UDP, uridine diphosphate; ADP, adenosine diphosphate.

It is not known whether synthesis of progeny RNA is entirely the function of a new virus-induced enzyme(s). It is possible that, once a new enzyme catalyzes the synthesis of RNA complementary to the parental strand, a replicative form serves as template for the host RNA polymerase. It has been reported that double-stranded RNA of reovirus serves as primer for the normally DNA-dependent RNA polymerase, in vitro (24, 35). Precise identification of the RNA-dependent polymerase activity in crude extracts of virus-infected cells must await clarification of the biochemical details of viral RNA synthesis.

PURIFICATION AND CHARACTERIZATION OF AN RNA VIRUS RNA POLYMERASE

One approach to understanding the biochemical mechanism of RNA virus multiplication is to purify and characterize the virus-induced polymerase enzyme(s). Purification of one RNA virus RNA polymerase was facilitated by use of a mutant phage, f2 su-11. Bacteriophage su-11 produces viable progeny only in bacteria containing a suppressor gene. In *E. coli* K38, a strain lacking a suppressor gene, su-11 does not produce progeny, either viable or defective (36). However, the RNA virus RNA polymerase activity in extracts of K38 infected with su-11 is about five times greater than normal, and an excess of phage-specific RNA is produced.

Purification and Properties of the Enzyme

The RNA virus RNA polymerase, isolated from *E. coli* K38 infected with f2 su-11, has been purified approximately 100-fold (6). RNA synthesis catalyzed by the partially purified enzyme was dependent on the presence of Mg^{++}, RNA, and the four ribonucleoside triphosphates (Table 1). The reaction did not occur when manganous ions replaced magnesium ions or when DNA replaced RNA; nor did deoxyribonucleotides or ribonucleoside diphosphates replace the ribonucleoside triphosphates. Ribonucleoside triphosphate dependence was demonstrated even when adenosine triphosphate (ATP) was the labeled substrate, indicating the absence of polyriboadenylate polymerase activity (5). Enzymatic synthesis of homopolymers with any of the ribonucleoside triphosphates as substrate was never observed. The reaction was completely insensitive to deoxyribonuclease, but was inhibited by 0.1 μg/ml of ribonuclease. Actinomycin D, in concentrations as high as 100 μg/ml, did not inhibit the reaction; by comparison, DNA-primed synthesis of RNA was inhibited by 1 to 4 μg/ml of actinomycin D (23, 28).

The purified enzyme was dependent upon the addition of RNA for activity. RNA from many different sources satisfied this requirement. Preparations of bacterial soluble and ribosomal RNA and viral RNA were equally effective in stimulating the reaction catalyzed by the RNA virus RNA polymerase isolated from cells infected with f2 su-11 (Table 2). Synthetic polyribonucleotides, either homopolymers or copolymers, did not satisfy the RNA requirement for either complementary or noncomplementary incorporation of ribonucleotides. Changes in the secondary structure of the synthetic polymers were without effect; polyadenylic acid plus polyuridylic acid and polyinosinic acid plus polycytidylic acid were ineffective as primers.

In the presence of a high concentration of inorganic pyrophosphate, the RNA virus RNA polymerase catalyzed the incorporation of pyrophosphate into ribonucleoside triphosphates

TABLE 2. *RNA requirement for guanosine monophosphate (GMP) incorporation**

RNA added	GMP incorporation ($\mu\mu$moles/5 min)
None	8
f2	103
TMV	106
Escherichia coli B ribosomal	104
Streptococcus faecalis ribosomal	101
Micrococcus lysodeikticus ribosomal	108
Clostridium pasteurianum ribosomal	107
E. coli B soluble	101
E. coli W soluble	105
S. faecalis soluble	105

* The standard assay procedure was used with C^{14}-guanosine triphosphate (8.2×10^6 counts per min per μmole) and 5 μg of enzyme protein in each tube, except that *E. coli* R-RNA was omitted. Different RNA preparations were added as indicated, by use of 200 to 300 mμmoles of each preparation.

(6). This is in accord with an enzymatic mechanism utilizing nucleoside triphosphates as substrate (10, 22). Inorganic pyrophosphate (PP_i) exchange required the presence of ribonucleoside triphosphates, Mg^{++} and RNA.

Comparison Between RNA Virus RNA Polymerase and Host RNA Polymerase

Both the RNA virus RNA polymerase and the DNA-dependent RNA polymerase catalyze the synthesis of RNA. There are several obvious similarities in the details of the reactions, but there are also significant differences (Table 3). The outstanding difference is in the nature of the template utilized in the reaction. The RNA virus RNA polymerase will utilize RNA but not DNA as template, whereas single-stranded RNA is a poor template in the normally DNA-dependent RNA polymerase reaction (52). However, the DNA-dependent enzyme will utilize ribohomopolymer templates very efficiently and will also catalyze ribohomopolymer synthesis (20, 34, 42). In contrast, the RNA virus RNA polymerase appears to be more specific, for it will utilize only natural RNA as template and does not catalyze homopolymer synthesis (6). Furthermore, the viral polymerase purified from cells infected with f2 su-11 is insensitive to antiserum which specifically inactivates the host RNA polymerase (Shapiro and August, *unpublished data*). This characterization of the partially purified RNA virus RNA polymerase appears to establish the fact that at least one new enzyme is involved in the replication of the RNA bacteriophage f2.

RNA Product of the Reaction

The accumulated evidence supports the conclusion that an intermediate or "replicative form" of RNA may be involved in the multiplication of single-stranded RNA viruses (7, 19, 30, 33, 37, 39, 44, 56). Thus, the enzyme(s) associated with the replication of viral RNA may be involved either in the formation of a replicative form or in the use of the replicative form during synthesis of progeny RNA, or both. With the assumption that the formation of hydrogen-bonded base pairs occurs in these processes, both steps would require the synthesis of a complementary copy of the RNA directing the reaction. Further elucidation of the role, in these processes, of the viral RNA polymerase induced by f2 su-11 infection has been obtained (i) by analysis of the base composition of the RNA product, (ii) by studies of base analogue substitution, and (iii) by characterization of some of the physical properties of the RNA product of the reaction.

The base composition of the RNA product was determined by the technique of nearest neighbor

TABLE 3. *Comparison of RNA polymerase enzymes*

Characteristic of the reaction	Viral RNA-dependent RNA polymerase	DNA-dependent RNA polymerase
Substrates	ATP, UTP, GTP, CTP*	ATP, UTP, GTP, CTP*
Metal ion	Mg^{++}	Mn^{++} (Mg^{++})
DNA requirement	No	Yes
RNA requirement	Yes	No
Ribohomopolymer as template	No	Yes
Ribohomopolymer synthesis	No	Yes
PP_i exchange	Yes	Yes
Actinomycin inhibition	No	Yes
Sensitive to RNA polymerase antiserum	No	Yes

* See second footnote to Table 1.

TABLE 4. *Base composition of enzymatically synthesized RNA and primer RNA**

RNA	Base			
	Cytidine	Guanosine	Adenosine	Uridine
TMV				
RNA primer....	18.5†	25.3	29.8	26.3
RNA product...	25.8	19.6	23.6	31.7
f2				
RNA primer....	26.4	27.2	22.7	23.7
RNA product...	26.0	28.2	23.7	22.3

* The base compositions of the RNA products were determined from nucleotide base pair frequencies, and the base compositions of TMV RNA and f2 RNA were obtained from the literature as described elsewhere (47).

† Results expressed as moles per cent.

TABLE 5. *Replacement of natural bases by analogues**

Analogue†	Per cent incorporation of natural base			
	Uridine monophosphate	Guanosine monophosphate	Cytidine monophosphate	Adenosine monophosphate
BrUTP.....	*85.0*	0.2	1.8	3.6
ψ Uridine triphosphate.....	*78.0*	0.2	2.4	0.2
rTTP.....	*44.0*	0.2	0.2	0.2
AzaUTP.....	2.5	0.2	1.0	0.2
Inosine triphosphate..	0.2	*23.0*	—	0.2
XTP.....	0.2	0.2	0.2	0.2
6MeATP.....	0.2	0.2	0.2	*32.0*
BrCTP.....	0.2	0.2	*41.0*	0.2

* The standard assay procedure was used with C^{14}-guanosine triphosphate (3.6×10^7 counts per min per μmole), C^{14}-adenosine triphosphate (1.5×10^7 counts per min per μmole), or C^{14}-cytidine triphosphate (5×10^7 counts per min per μmole); and 50 mμmoles of the appropriate natural or analogue nucleoside triphosphate, as indicated.

† Abbreviations: BrUTP, 5-bromouridine triphosphate; rTTP, ribothymidine 5′-triphosphate; AzaUTP, 4-azauridine 5′-triphosphate; XTP, xanthosine 5′-triphosphate; 6MeATP, 6-methylaminopurine ribonucleoside triphosphate; BrCTP, 5-bromocytidine triphosphate.

analysis (29). The product of the reaction resembled a complementary copy of the primer (Table 4). That is, the molar proportion of cytidylate, guanylate, adenylate, and uridylate of the primer was similar to that of guanylate, cytidylate, uridylate, and adenylate of the product, respectively. This is particularly evident with tobacco mosaic virus (TMV) RNA because of large differences in the molar proportion of each base. Synthesis of RNA in a reaction catalyzed by the purified RNA virus RNA polymerase is thus consistent with a mechanism based on complementary base pairing.

To elucidate further the mechanism of synthesis of RNA from an RNA template, the enzyme-catalyzed incorporation of purine and pyrimidine analogues of ATP, uridine triphosphate (UTP), guanosine triphosphate (GTP), and cytidine triphosphate (CTP) was also studied (48). Base analogues replaced the natural substrates in reactions catalyzed by DNA polymerase or RNA polymerase, utilizing DNA as template (9, 31), provided that the specific functional groups involved in hydrogen bonding were not affected. The results of experiments with the RNA virus RNA polymerase indicated that RNA-directed RNA synthesis also adheres to a mechanism utilizing hydrogen-bonded base pairing (Table 5). Natural analogues and chemically modified nucleotides specifically substituted for the four natural ribonucleoside triphosphates when the 6-keto or 6-amino group remained unchanged. BrUTP, ψUTP and rTTP substituted only for UTP, whereas azaUTP was not incorporated in place of any substrate. Inosine triphosphate (ITP) would substitute for GTP, but XTP would not. 6-CH_3 ATP and BrCTP substituted for their respective natural nucleotides. These results support the observations that the base composition of the RNA product is complementary to that of the RNA added as template.

Further studies of the RNA product showed that it possessed a high degree of secondary structure, as indicated by resistance to ribonuclease (6, 47). When heated and quickly cooled, a greater proportion of the product was sensitive to ribonuclease. Changes in ribonuclease sensitivity exhibited a sharp thermal transition with a temperature of 85 C in 0.015 M NaCl (Fig. 3). The loss of ribonuclease resistance as a result of heat denaturation was reversible in a manner very similar to that shown to occur with double-stranded DNA by Marmur and Doty (38) (Fig. 4). The results indicated that in the RNA virus RNA polymerase reaction nucleotides were incorporated into a product with DNA-like secondary structure, possibly double-stranded RNA.

The accumulated data suggest that the RNA virus RNA polymerase induced after *E. coli* infection with bacteriophage f2 su-11 functions in vivo to catalyze the synthesis of a complementary copy of the parental single-stranded RNA, pos-

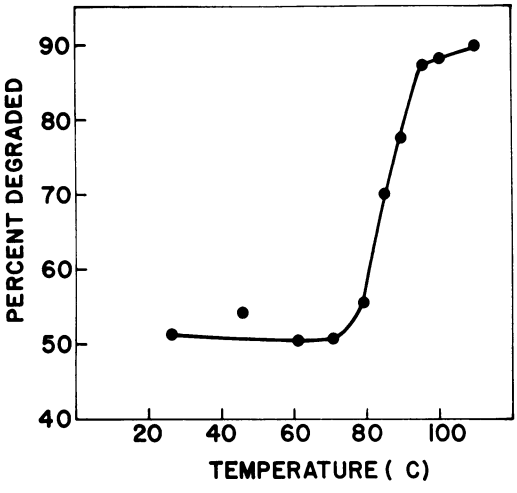

FIG. 3. *Ribonuclease sensitivity of the RNA product as a function of temperature. The C^{14}-labeled RNA product was synthesized in the reaction mixture described in Table 1, and isolated by phenol extraction. The ribonuclease sensitivity of samples heated and quickly cooled was determined (47).*

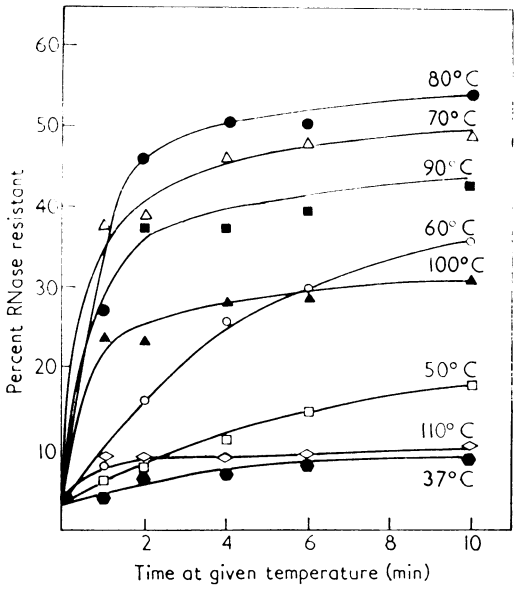

FIG. 4. *Rate of increase in resistance to ribonuclease of heat-denatured RNA product during incubation at different temperatures. The C^{14}-labeled RNA product was heated and quckly cooled. Equal portions of this solution were then incubated at the indicated temperatures and at different times, and fractions were tested for the sensitivity of the RNA product to ribonuclease (47).*

sibly yielding a double-stranded replicative form (the first reaction in Fig. 5). This conclusion is based on (i) the observation that single-stranded RNA was utilized as template in the synthesis of RNA with an average chain length of at least 300 to 400 nucleotides; (ii) the results of nearest neighbor analysis, indicating complementary copying of a single-stranded RNA; (iii) base analogue incorporation consistent with the hydrogen-bonded base pairing mechanism of replication; and (iv) evidence that the product had a high degree of secondary structure.

POLYMERASE ACTIVITIES ASSOCIATED WITH OTHER RNA VIRUSES

Other viral RNA polymerase activities (3, 4, 8, 25, 26, 58) have much in common with the f2 su-11 enzyme. All utilize ribonucleoside triphosphates as substrates, require magnesium ions, and are sensitive to ribonuclease but not to deoxyribonuclease. Haruna et al. (25, 26) reported the purification of viral RNA polymerase enzymes extracted from *E. coli* infected with MS2 as well as Qβ. These enzymes were dependent on the presence of homologous RNA in the reaction mixture. The enzyme isolated from MS2-infected cells required MS2 RNA, and that from Qβ-infected cells required Qβ RNA (26). TYMV RNA supported incorporation of only 6 to 25% that found with homologous RNA. More recently, this group of workers has presented evidence that an enzyme fraction isolated from *E.*

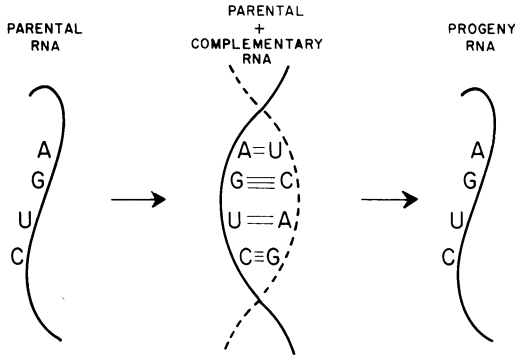

FIG. 5. *Schematic representation of a possible mechanism for the replication of RNA virus RNA based on the principle of hydrogen-bonded base pairing. In the first reaction, a complementary copy of the single-stranded parental RNA is synthesized. In a second reaction, the complementary strand is used as template for synthesis of progeny RNA.*

coli infected with Qβ will utilize the homologous phage nucleic acid to catalyze the synthesis of infectious RNA. Questions emerging from these studies are whether one or two enzymes are

present in the purified enzyme fraction and whether a polynucleotide complementary to the viral RNA exists in the reaction mixture. Further elucidation of the enzymatic mechanism and the RNA products of the MS2 and $Q\beta$ reactions will permit a clearer analysis of the RNA requirement exhibited by these enzymes and of the viral polymerase isolated from *E. coli* infected by f2 su-11.

Activity from another enzyme isolated from MS2-infected *E. coli* has been studied by Weissmann and his co-workers (51, 55, 58). This enzyme was purified in association with endogenous RNA and in this state appeared to catalyze the synthesis of progeny RNA (57). It was proposed that the enzyme, as isolated, utilized the replicative form as template and yielded progeny RNA via a semiconservative mechanism of replication (55). It is as yet unknown whether this enzyme has properties in common with the other enzymes isolated from cells infected with f2 su-11, MS2, or $Q\beta$.

CONCLUSIONS

The evidence presented favors the concept that replication of single-stranded viral RNA proceeds by the known mechanism of base pairing, requiring the synthesis of RNA with a base composition complementary to that of parental RNA (Fig. 5). Once formed, the complement of the entering viral strand would act as template for the synthesis of progeny RNA by means, perhaps, of asymmetric semiconservative replication. Whether a single virus-induced polymerase or two different enzymes catalyze the synthesis of viral complement and progeny is a matter of speculation at this time. Although much has been learned about the replication of the small RNA viruses, it is apparent that there are many details of this mechanism that remain to be elucidated.

ACKNOWLEDGMENTS

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