EFFECT OF R17 BACTERIOPHAGE-SPECIFIC PROTEINS ON RIBOSOMAL RIBONUCLEIC ACID SYNTHESIS OF *ESCHERICHIA COLI*

by

Virginia L. Waters

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Historical Background

1. Introduction

Viruses have been described as "half-alive:" they can reproduce and are composed of nucleic acid and protein but cannot metabolize, grow, or die. Either as parasites or commensals, they rely on the biochemical machinery of the host cell for their production. Regardless of whether or not viral infection results in an inhibition of the host cell's own macromolecular synthesis, there must be at least a redirection of host cell machinery by the infecting virus in order to propagate itself. Exploring the mechanism of this redirection often results in the development of important models of protein-nucleic acid interactions which contribute to molecular biology.

In 1961 Loeb and Zinder discovered a small male-specific phage of E. coli called f2, with a nucleic acid genome composed of RNA (1). This simgle isolation was soon followed by a series of isolations in Japan and elsewhere of similar RNA-containing coliphages. Such simple RNA phages of E. coli have become appreciated as the best natural messengers available for the study of protein synthesis, have been used in models of RNA synthesis, and have even been used to synthesize the first synthetic antiviral "vaccine" (2). Also, studying the biochemistry of the replication of these RNA phages has revealed them as the first "molecular subunit parasites" and knowledge of the molecular basis of phage transcription and translation, especially in relation to the host cell's biochemistry, continue to elucidate the control of growth in procaryotic cells.

2. RNA Coliphage Grouping

Almost all of the known RNA-containing phages, irrespective of the genus and species of the host, possess similar physical properties (3). Among the RNA coliphages the similarities are even more striking. They all have an RNA genome of similar molecular weight (1.1 X 10^6 daltons) which codes for three proteins: the coat subunit protein, the assembly (A_2) or maturation protein, and the replicase (or "synthetase" or "polymerase") protein (4, 5). A fourth protein, A_1, resulting from "leaky" termination of the coat protein cistron, is translated in Qβ coliphage infections (6, 7). This particular phage also proved to be serologically distinct from the other original isolates, when grouping of these RNA coliphages was attempted in 1967 (8). The serologically related phages have since been shown to possess coat proteins differing from each other by only a few amino acids (9, 10, 11).

The existence of four groups of RNA coliphage based on sensitive serological techniques (8, 12, 13, 14) has been confirmed by various other methods. Based on the fact that the RNA phages attach to millipore filters under conditions of low salt concentration and elute with varying efficiences, Miyake et. al. grouped twenty five different phages which had been isolated in their laboratory or other laboratories in Japan (15). They noted that within the millipore filter groups several other chemical and physical properties were held in common, and that the chemical nature of the coat protein probably accounted for the attachment phenomenon. From the same lab Watanabe et. al. (16) reported on some of the physical and chemical properties, such as UV

sensitivity, density, sedimentation constant, tryptophan content, base ratio, and pH sensitivity. The distinctions of the serological groupings were also confirmed by electrophoresis (15). Groupings according to such varied criteria were in agreement and based on RNA differences as well as on coat protein differences.

Assembly of the protein subunits and of RNA-protein coat subunits into phage-like particles was found to occur with group specificity when phages of two groups were mixed and allowed to reassemble in vitro (17). The specificity of the interactions between viral RNA and coat proteins was also demonstrated when Robertson et. al. (18) investigated the repressor role of coat protein, that is, the mechanism by which phage coat protein specifically inhibits homologous phage RNA translation. By applying a different criterion, the template specificity of an RNA replicase for its homologous RNA, Miyake et. al. (19) used the established serological groupings to suggest subgroups of groups III and IV, and to regroup one phage. The degree of base sequence homology among MS2, f2, and Qβ phages was first approached by hybridization (20), but sequence analysis and the demonstration of structural "loops" of the RNA has begun to show more clearly the nucleic acid relationship between and within the RNA phage groups (21, 22). Similarly, coat protein amino acid sequencing define the differences between phage coat proteins at the molecular level (23).

A sample of the more studied RNA coliphages can be listed according to the aforementioned four groups:*

*compiled from references 11, 19, and 23

I			II		III		IV	
3	f4	R23	EJ	SB	CF	SO	α15	ZG
β	GR	SN	EI	SD	HI	ST	α17	ZL3
FA5	MS2	SJII	GA	SK	NH	VK	FI	ZS3
fc	M12	ZR	KJ	SS	NM		SP	
fr	MY	ZIK/1	MC	SW	Qβ		TW19	
f2	R17		2		SG		TW28	

It is noteworthy that many of the phages which are classified together in a group also have a common source and geographic origin (15).

Of the most extensively studied phages, MS2, f2, and R17 of group I, and Qβ of group III, the differences between Qβ and the others extend beyond the properties of physical and chemical composition. Evidence is accumulating which indicates that group I phages and Qβ use different host proteins for their development. A host factor required for the synthesis of f2 RNA *in vitro* cannot be replaced by the host factors required for Qβ replication (24). The fact that rifampin inhibits group I phage but not Qβ phage RNA replication (25) suggests that either different bacterial proteins are involved in the RNA synthesis of the two phage groups, or that Qβ-specific protein(s) are directly or indirectly responsible for rifampin resistance. Engelberg-Kulka *et. al.* isolated a streptomycin-resistant *E. coli* mutant that is temperature sensitive for Qβ but not for group I RNA phages (26), and the reverse situation, an *E. coli* mutant temperature sensitive for group I phages but not for Qβ, was recently found in their laboratory (27). In the latter case, for example, the inactivation of the

product of the mutant cistron by a temperature shiftup drastically reduced the plaque forming units yielded by MS2 but not by Qβ infection. These kinds of studies may lead to the identification of the host proteins peculiar to each phage group.

3. Coliphage Replication

Coliphage replication is the intracellular event during the infectious process in which the input phage yields its progeny population. The chronological steps in which the infectious process is usually studied are adsorption or attachment, ejection, penetration, translation, transcription, assembly, and cell lysis. Coliphage biosynthesis primarily involves translation and transcription. After phage adsorption onto the E. coli F pilus, which is contingent upon an intact A_2 protein component, the viral particle ejects its RNA genome into the cell as an A_2 protein-RNA complex (28, 29). This single stranded RNA, the "plus" strand, serves as messenger in the translation of viral coat protein and the replicase protein. Once the replicase enzyme becomes available, it uses the plus strand RNA to transcribe complementary minus strands, which in turn serve as templates for the production of progeny plus strand RNA. The assembly protein was first thought to be another "early" protein, the number of A_2 proteins limited by the short time in which the A_2 cistron was available (30). More recently A_2 has been thought of as a late protein, synthesized off the nascent plus strand as the assembly process begins (31).

Continued translation of replicase molecules is prevented by the attachment of six molecules of coat protein per molecule of RNA (32).

The coat protein therby acts as a repressor protein in the control of replicase gene expression (18, 33). Presumably later on in the infectious process, the replicase protein also functions as a repressor: it inhibits the attachment of ribosomes to the coat protein initiation site by binding to an RNA segment which overlaps the coat initiation site (34, 35) without affecting elongation of polypeptide chains already initiated (36). There are several reasons to believe that this event marks the end of "early" phage protein translation and the beginning of viral RNA transcription: 1. translation of the replicase cistron is dependent on the translation of the coat cistron (37) so that inhibiting coat protein initiation precludes replicase synthesis as well; 2. excessive synthesis of replicase is also limited by coat protein repression; 3. the assembly protein is probably only translated on nascent phage RNA (31); 4. RNA template recognition by Qβ replicase is thought to involve an internal site on the RNA molecule, as well as at the 3' end (38), so the binding of replicase to the coat protein initiation site area may have a dual function.

Coat protein synthesis occurs off both the input and progeny plus strand RNA, although more efficiently off the nascent strand (39). The coat protein may regulate, in addition to replicase translation, transcription of plus strand RNA, since amber mutants of coat protein also restrict synthesis of viral plus strand RNA (while allowing minus strand RNA to accumulate), even when the amber mutation does not affect replicase synthesis (39, 40). The details of viral transcription and translation must therefore be considered in the context of their interrelationship, an idea first intimated by the observation that the rep-

licating viral RNA was associated with cellular polysomes (41, 42, 43), and an idea inherent in the coordination of events that must take place in order to economically produce phage particles consisting of one RNA molecule, 180 protein coat subunits, and one assembly protein.

In light of what is known about the functions of phage RNA, coat, and replicase, Robertson presented a model to provide the logistics behind phage replication (39). Called the "butterfly" model, it supposes that the phage replicase molecule remains attached to the 3' end of the template RNA. The replicase enzyme polymerizes the nascent RNA by moving along the template strand, with the 3' end still attached, so that the transcribed portion forms one loop, or "wing" of the "butterfly." The other "wing" is formed by the 5' end of the nascent strand as it is generated. The pair of loops enlarge as the reaction goes to completion, when the enzyme switches over to the newly formed 3' end of the minus strand, which now becomes the template for repeated progeny plus strand synthesis.

Robertson reasoned that the enzyme could easily reinitiate plus strand synthesis by the "permanent" 3' end attachment and butterfly loop formation. The failure of Thach and Thach (44) to find a more rigid and extended double stranded RNA by electron microscopy of undenatured viral RNA extract is consistent with Robertson's model. Removal of the enzyme replicase from the template would, however, result in collapse of the loops into linear double stranded RNA, according to this model. Such double stranded RNA was originally "found" in infected cell cultures, but Weissmann (45) was the first to point out that it probably did not exist <u>in vivo</u> but rather was formed during

extraction procedures. Intermittant, transient helical structures probably do form; however, the limitations in the formation of a helical structure is demonstrated by the accumulation of RNase III-cleaved double stranded RNA in phage infection by certain mutants (39).

Robertson's model does not correlate the messenger activity of parent and progeny plus strands with RNA replication. Since translation proceeds from the 5' to the 3' terminus (46, 47) and replication proceeds from the 3' to the 5' terminus (48, 49), ribosomes must detach from the RNA before or during RNA replication. Kolakofsky and Weissmann (35) suggested that it is the binding of the enzyme replicase which dislodges the ribosomes, before the enzyme begins to advance along the RNA, because they had observed that the binding of molar amounts of Qβ replicase prevents ribosomes from initiating protein synthesis. They presented a simple model for the transition of phage RNA from polysome to replicating complex. First is the initiation of coat protein translation, which alters the RNA secondary structure so as to expose the replicase initiation site. Once replicase is available, it attaches to the coat protein initiation site and blocks ribosome attachment. Replicase then begins minus strand synthesis by moving to the 3' end, when the Robertson butterfly model becomes applicable. As part of the replicating complex, the progeny plus strands may simultaneously be engaged in the translation of coat and assembly proteins. The assembly protein probably remains attached to its own messenger RNA, and it is thought that the completed RNA molecule is catalytic in RNA-capsid protein complex formation by the formation of its quaternary structure (50).

In 1970 Qβ replicase was highly purified and characterized by two independent laboratories (51, 52). Improved purification procedures depended on the use of a Qβ coat amber mutant which overproduces phage replicase in the infected cell. To determine the size and number of individual polypeptides, both laboratory groups used SDS-polyacrylmide gel electrophoreis. Four polypeptides, α, β, γ, and δ, were found to comprise the replicase enzyme. Only one of them, the β subunit, was found to be coded for by the viral genome (51, 52). The combined molecular weights of the coat, assembly, and viral replicase subunits proteins were found to be within the limits of the polypeptide producing potential of the Qβ RNA genome. The other three polypeptides, E. coli gene products, were later characterized and identified according to host cell function in the uninfected state. Subunits γ and δ proved to be identical to elongation factors EF-Tu and EF-Ts, respectively (53). Subunit α was identified as protein synthesis interference factor i (54, 55), which was found to be identical to ribosomal sununit 30S protein S1 (56). In the uninfected cell the 30S ribosomal protein S1 is involved in binding messenger RNA to ribosomes (57, 58), but inhibits translation when present as a free molecule (59, 60). S1, as part of the Qβ replicase molecule, is not required for RNA synthesis directed by templates other than the phage plus strand. S1 is required in the recognition and binding of the plus strand RNA by replicase (54).

S1 may also be required for the enzyme Qβ replicase to act as a translational repressor (61). Elongation factors EF-Tu and EF-Ts, and the elongation factor EF-G, are involved in polypeptide chain elongation on the bacterial ribosome. EF-Tu promotes binding of amino acyl-

tRNA to the 70S ribosome by forming a complex with amino acyl-tRNA and GTP, which binds to the ribosome (62). As GTP is hydrolyzed, EF-Tu complexed to GDP is released from the ribosome. EF-Ts stimulates the exchange between the GDP of the EF-Tu·GDP complex and free GTP, by causing the formation of the EF-Tu·EF-Ts intermediate complex (62).

Separate from the replicase enzyme, at least one host factor is involved in phage RNA replication. Alone, the viral-induced replicase carries out the polymerizing reaction using either plus or minus strand RNA. However, a host specific factor HF (or HFI), is required to initiate synthesis of the minus strand by recognizing the plus strand template (63). The regulation of availability of this factor may be related to the fact that the amount of plus strands synthesized in vivo exceeds that of minus strands by a factor of ten (64). Host factor is a tightly binding hexameric protein with identical subunits, and for maximum synthesizing efficiency, one host factor is required per RNA template (60, 65). Senear and Steitz investigated the interactions of HF and S1 with Qβ and R17 RNA's, and found that HF bound very strongly and specifically to two sites of Qβ RNA (one near the 3' end) and to one site of R17 RNA (located in the replicase cistron) (60). The interactions of S1 were also distinctive for each phage RNA: it bound specifically to only one site on Qβ RNA (near the 3' end) but almost randomly to R17 RNA. They observed that the binding of HF was highly sequence-specific and suggested that, since it bound 16S and 23S ribosomal RNA species on nitrocellulose filters, it may exert control in the metabolism of cellular RNA.

In 1968 Franze de Fernandez, August, and coworkers separated from

their original host factor fraction a second component, designated host factor II or hydroxylapatite factor II (HFII) because it was separated by hydroxylapatite chromatography (66). Omission of either HFI or HFII from their Qβ RNA reaction mixture resulted in the loss of the ability to synthesize Qβ RNA. However, in 1972 Kuo and August found that the requirement for a second factor was equally satisfied by any one of many bacterial basic proteins (67). Kamen developed Qβ replicase purification techniques to exclude the use of hydroxylapatite and Bentonite (68) and subsequent experiments (54, 69-72) did not confirm the requirement for HFII. Kamen, Monstein, and Weissmann (73) compared the components of the replicase systems in their laboratory with those of August and his colleagues (66, 67) and found that the HFII-dependence was a property of the particular preparation used as template and not that of the protein components. They suggested that the role of the basic proteins in supplying HF activity was solely that of counteracting a supposed inhibitor (possibly Bentonite) present in the August preparation.

4. Mutant Coliphage

In 1964 Zinder and Cooper (74) isolated a number of mutants of phage f2 by treating phages with nitrous acid. The mutants were titrated on various permissive (allowing phage growth) and nonpermissive (not allowing phage growth) E. coli strains. They noted that the same bacterial strains that were permissive for some other bacteriophage mutants, such as T4 amber mutants, were also permissive for their f2 RNA phage mutants. The permissive hosts were then thought to contain suppressor

genes that restore function in possibly many different mutated cistrons. Capecchi and Gussin (75) found that the component active in suppression in the permissive strain S26RIE is a serine-accepting tRNA which is not present in the nonpermissive isogenic strain S26. This tRNA suppresses the amber nonsense coden (UAG) with a 63% efficiency (82), that is, 63% of the mutant codons are read as serine codons by the suppressor tRNA, so that polypeptide chains are propagated in sufficient quantity to produce viable phage.

The complementation studies of Gussin (76) on a series of amber mutants of R17 coliphage revealed the existence of the three phage cistrons, one specifying the coat protein, one specifying a protein necessary for infectivity, and one specifying the phage RNA polymerizing enzyme. One of the coat cistron mutants was unable to synthesize either phage coat or replicase. Since the added effect of the mutated cistron was "polarized" in that only the gene thought to be distal (gene order was not then known) to the mutation was affected (77, 78), this mutant was called a polar coat mutant. Lodish observed a similar polar effect with certain amber mutants of the coat cistron of bacteriophage f2 (79). The polar effect was marked when the amber mutation occurred very near the 5' end of the coat protein gene, a situation thought to be analogous to polarity of bacterial operons, in which the degree of polarity on genes beyond the mutation is dependent on the position of the nonsense mutation (80, 81). Tooze and Weber (82) isolated and characterized 55 amber mutants of bacteriophage R17 and found that all of the strongly polar mutants occurred at the sixth amino acid in the coat protein, where a glutamine codon (CAG) was presumably con-

verted to an amber codon (UAG). Other glutamine "hotspots" at positions 50 and 54 in the coat protein produced nonpolar coat mutants. The polar coat mutants, numbered amB23 to amB29, therefore restrict translation of viral replicase; the nonpolar coat mutants, amB17 to amB22, allow large amounts of replicase to be translated. Among the 38 assembly protein mutants, amA30 to amA68, none showed any polar effect. Although Tooze and Weber (82) monitored the strength of the polarity of the various amB coat mutants by the level of uracil incorporation as a reflection of replicase function, they could not detect a gradient of polarity according to position of nucleotide substitution. Such a gradient in polarity was found in the nonsense mutants of the lac and tryptophan operons, which lack messenger RNA beyond the amber mutation (80, 81). In spite of the differences, the then accepted mechanism of polarity for RNA coliphage was the same used to explain polarity in mutant E. coli, i. e., a failure of the ribosome to reattach and initiate the distal gene once translation was terminated by the amber mutation.

Current concepts explain polarity in bacteria in relation to the distance between the nonsense codon and restart codons, ribosome attachment sites, and transcription termination signals which exist beyond the nonsense codon (83). Added features of termination controls are considered in light of the fact that the curve plotting degree of polarity versus position of nonsense mutation within a cistron is a gradient with peaks and valleys (84, 85).

The view that the coliphage coat and replicase cistrons must be translated sequentially and without interruption is no longer a satis-

fying explanation of phage R17 mutation polarity. The gradient observed with E. coli polar mutants has not been demonstrated; free ribosomes are able to initiate synthesis of the replicase cistron if the RNA template structure has been opened (86) and the forty or so nucleotide difference between sites 54 and 6 of the nonpolar and polar coat mutants of R17 is not so great when the total cistron length of 130 nucleotides is considered. The more likely explanation stems from experiments which denature or fragment the native phage RNA and use it to synthesize wild-type or greater amounts of replicase. Translation of the coat cistron past amino acid position 6 probably thus causes a conformation change which exposes the replicase initiation site. Strongly polar mutations at position 6 preclude this necessary conformation change in RNA structure and thereby prevent replicase synthesis.

By relating the locus of Qβ coat protein mutation to the percent of replicase gene expression, Ball and Kaesberg have shown that a gradient of polarity does extend from amino acid position 17 to position 37 to position 86 (87). That their results with Qβ contrast with those of the R17 phage may well reflect the great difference in the intercistronic regions of these two phages. Qβ, with its fourth "read-through" protein, is estimated to have at least 600 nucleotides between its coat and replicase genes (88); R17 contains only 36 nucleotides (89). The RNA conformational changes that occur by Qβ coat and "read-through" protein translation may be more gradual and allow a gradual increase in the initiation of replicase synthesis, resulting in a gradient of polarity, or some of the factors important in bacterial polarity may come into play, such as the location of restart codons,

ribosome attachment sites, and termination signals which exist beyond the nonsense codon.

5. Relation of Viral Infection and Host Cell Metabolism

Host cell metabolism required for coliphage synthesis. Within a year of their report on the existence of RNA-containing bacteriophage f2, Zinder and his colleagues concluded that f2 can replicate when the synthesis of host cell DNA is almost completely blocked (90). Watanabe and August took advantage of the DNA-independence of phage production in developing their methods for isolation of RNA bacteriophages (91). The lack of homology between MS2 RNA and E. coli DNA as tested by hybridization led Doi and Spiegelman (92) to predict the existence of an RNA-dependent RNA polymerase (which Spiegelman later dubbed "replicase"). The evidence implied that the RNA coliphage circumvented the traditional information transfer pathway of DNA-to-RNA-to-protein; however, the treatment of MS2-infected spheroplasts with actinomycin D five minutes post infection reduced phage yield by a factor of 100 (93). Lunt and Sinsheimer (94), and Haywood and Harris (95), studied the kinetics of phage inhibition in actinomycin-treated cells and found that the effect was abruptly manifest at 20 minutes post infection. Lunt and Sinsheimer (94) postulated that phage replication is somehow blocked by actinomycin because some essential component(s) is depleted in 20 minutes. The limiting host factors predicted then in 1966 were most likely the host specific subunits of phage replicase and/or the host factor HF.

At least one other host cell mechanism influences phage RNA synthesis, that regulated by the RC gene. The RC, or "RNA control," or "rel," genetic locus regulates stable cellular RNA synthesis in response to amino acid starvation (96). In its "relaxed" RC^{rel} allelic state, a bacterium allows stable RNA synthesis to continue in the absence of required amino acids; in its "stringent" RC^{str} allelic state, a bacterium restricts stable RNA synthesis when starved for required amino acids (96). To investigate the possible role of the RC gene product on bacteriophage f2 RNA synthesis, Friesen infected various RC^{str} and RC^{rel} E. coli strains (97). He found that phage yield was more reduced by required amino acid starvation in an RC^{str} strain than in an RC^{rel} strain (97, 98). Fukuma and Cohen (99) used agarose acrylamide electrophoresis to analyze the RNA from R17-infected stringent and relaxed strains of E. coli. In R17-infected relaxed cells, the synthesis of both the 16S and 23S species of ribosomal RNA decreased progressively. The same results were obtained using R17-infected stringent cells, as long as the required amino acid was supplied. In the absence of the required amino acid, there was a momentary stimulation of rRNA synthesis relative to the level found at zero time "post infection." The increased level, however, remained a small fraction of the nonstarved level in infected stringent cells. Phage RNA synthesis appeared to be reduced in the amino acid starved stringent cells with respect to the starved relaxed cells (99).

Whatever the degree of influence the rel gene product has on phage RNA synthesis, the cause may again be rooted in the fact that the phage "borrows" host proteins to make up three of the four subunits of the

replicase molecules. The sphere of influence of the rel gene includes, in addition to stable RNA, ribosomal proteins (100-103) and elongation factors EF-Tu, EF-Ts, and EF-G (104, 105), which are also intimately associated with ribosomes. Elongation factor EF-Tu functions in the infected cell as viral replicase subunit γ (53). Rel gene shutdown of EF-Tu in the amino acid starved stringent cell could thus conceivably affect its availability to the viral RNA synthesizing system.

Relationship between host cell metabolism and viral infection in systems other than the RNA coliphage-E. coli system. Lytic bacteriophages, whether DNA- or RNA-containing, hold in common certain features essential to "successful" bacterial infection. Regardless of the particular strategy for genetic regulation, the expression of viral genes occurs in controlled, time-ordered sequences. Among the RNA coliphages, the phage-coded proteins are being characterized as to the quantity of each synthesized, the time sequence in which they are synthesized, the various roles they play and when each role is played, and the relationships they have with the host. The gene products of the other lytic bacteriophages are similarly being characterized, although no other independent phage is as simple as the RNA coliphages, which possess only three (or four) gene products.

What the lytic phages do not hold in common are the regulatory and metabolic interrelationships they have with their hosts. Among the T series are bacteriophages which rapidly and irreversibly inactivate and degrade the host genome, but such a dramatic attack on the host is not the case in other lytic phage infections (106). The synthesis of most host RNA and protein is not altered in the early stages

of infection of E. coli by DNA bacteriophage N4. Curiously, however, N4 rapidly arrests the synthesis of catabolite-sensitive enzymes such as beta galactosidase (107). A similar shutoff occurs during the development of lytic growth in DNA phage λ (107). The same gene that shuts off host RNA synthesis after T7 infection also shuts early phage functions (108), but host protein synthesis in T-even phage infections can be shut off with no viral gene expression at all, as shown by experiments using phage ghosts (109). Specific targets of host gene shutoff in phage T7 infections include the β and β' subunits of E. coli DNA-dependent RNA polymerase, which are phosphorylated (110), and, for T4 and T7 infections, the host cell ribosomes (111).

Among the animal viruses, host RNA and protein synthesis is commonly inhibited and, except among the tumor viruses, the inhibition is usually rapid. The shutoff phenomenon was first described for the picornavirus Mengovirus by Franklin and Baltimore (112), who believed that the rapid inhibition of protein and RNA synthesis was a primary characteristic of virulence among animal viruses. Although the picornavirus-induced inhibition of host macromolecular synthesis is not the direct cause of the typical lesions seen in infected cells, the inhibitions is one of the cell-killing properties of the virus (113). Picornaviruses were the object of most of the ensuing studies, but similar inhibitions were obtained with a variety of animal viruses, such as Poxviruses, Rhabdoviruses, Herpesviruses, and Togaviruses (114-117).

In the polio-HeLa cell system, the host messenger RNA translation must first involve discrimination between viral and host messengers. Such discrimination can occur, however, without a concommitant shutoff

of host protein synthesis, such as in reovirus and SV40 infections (118). The polio mediator of inhibition and discrimination may be the viral capsid protein, because temperature-sensitive poliovirus mutants defective in repression of host protein synthesis are also defective in the polio structural coat protein (119). The effect of the coat protein may be related to alterations in the cell membrane, leading to an influx of sodium ions (120). Somehow the higher salt concentration gives the picornavirus mRNA a competitive advantage over cellular RNA with respect to initiation of protein synthesis, because Koch and his coworkers (121, 122) have shown that polio-infected HeLa cells exposed to high salt concentrations at times early in infection, when both viral and cellular proteins would normally be synthesized, synthesize only virus-specific proteins. However, since poliovirus added to reticulocyte lysates inhibits the initiation of globin synthesis (123), the complex in vivo situation probably involves the coat protein in a multi-component mechanism of host protein shutdown.

Recently, Rose, Baltimore, and coworkers have used a cell-free translation system to study the effect of polio infection on the translation of vesicular stomatitis virus (VSV) by HeLa cell lysates (124). They based their work on the fact that the kinetics of poliovirus inhibition of VSV and host protein synthesis are identical and may therefore occur by the same mechanism. To determine if the inability of extracts from infected cells to translate VSV mRNA was due to the loss of a specific initiation factor, they added various initiation factors and analyzed the in vitro VSV mRNA products. They found that a single purified initiation factor, eIF-4B, can overcome the inhibition of VSV

and host mRNA translation, and that lysates from polio-infected cells can inactivate eIF-4B, presumably by means of a viral protein.

Infection by picornavirus reduces cellular DNA synthesis, an effect which is probably secondary to the inhibition of protein synthesis (125). A virus-specific protein is involved in the inhibition of cellular RNA synthesis, and the analyses of the possible mechanism(s) has been approached by studying the effect of virus infection of the various species of host RNA polymerases present in the cell nucleus (126). In addition to transcription of rRNA, the picornavirus apparently affects the processing of 45S ribosomal precursor RNA (127).

RNA coliphage-induced changes in host cell metabolism. Apart from the remarkable structural and chemical similarity among the RNA coliphages, diverse observations have been made on the quantitative effects that the RNA coliphages exert on their host. The various experimental approaches, and the use of different E. coli strains, in particular strains differing at the rel genetic locus, may have contributed to the diversity in experimental results, but the phages themselves do appear to have distinctions. In light of the multiplicity of criteria that made the serological groupings reflect real differences between the phage groups, it is not surprising that the Qβ phage proved to have a different effect on the host than that of the group I phages. The different effects of the phages within group I, apart from host strain variations, were not so predicatable.

In 1963 Ellis and Paranchych (128) used wild-type E. coli K12 to determine the extent of ribosomal RNA synthesis in R17-infected bacteria. In sucrose gradient analyses of C^{14}-RNA extracted at 20 to 45 minutes

post infection, they saw a rapid inhibition of ribosomal RNA synthesis to about 20% of the uninfected rate. They noted that the inhibition of bacterial RNA synthesis by phage infection was similar to that observed in polio- and Mengovirus-infected mammalian cells, in which the inhibition of ribosomal RNA synthesis had been observed (130). In comparing the overall rate of RNA synthesis, however, the analogy breaks-down: in polio- and Mengovirus-infected cells, overall RNA synthesis is affected relative to control uninfected cells (131), whereas coliphage infection does not appear to significantly affect the overall rate of RNA synthesis in E. coli (132, 133).

By means of in vitro hybridization of purified RNA and E. coli DNA, Hudson and Paranchych (132) confirmed that the level of 16S and 23S ribosomal RNA synthesis was decreased 70 to 80% during the first 20 minutes post infection. Similar results were obtained by Bishop (134) who infected E. coli C3000/L with group I coliphage ZIK/1. Comparing the rates of phage RNA synthesis with total RNA synthesis, and expressing the phage rate as a percent of the total, Watanabe et. al. (133) demonstrated that the group I phage R23 completely dominates RNA synthesis in infected cells, producing 60% of the total RNA. This was in contrast to the other group I phages R17 and f2, which produced about 25% of the newly synthesized RNA. In further studies Watanabe and Watanabe (135) analyzed RNA from R23-infected E. coli K38 and found that the synthesis of 16S and 23S ribosomal RNA rapidly approached 25% that of normal, and that the synthesis of 4S transfer RNA approached 40%. They also examined the DNA synthesis in four different gp. I phages and found that it was about 75% that of uninfected cells at 25 minutes

post infection; group III phage Qβ infection resulted in 80% the normal DNA synthesis. Coliphage-induced inhibition of DNA synthesis had originally been noted by Parachych and Graham (136) and Bishop (137) and had been demonstrated by autoradiographic techniques by Granboulin and Franklin (138).

Central to the question of viral inhibition of rRNA synthesis is the study of Fukuma and Cohen (99) in which R17 was used to infect stringent and relaxed strains of E. coli in the presence and absence of the required amino acid (vide supra). The synthesis of ribosomal RNA species was inhibited in relaxed cells regardless of amino acid supply and in stringent cells in the presence of the required amino acid, but the preexisting low level of synthesis in stringent starved cells was momentarily and slightly stimulated. Under conditions of amino acid starvation of stringent cells, the apparent restriction of virus production may well be due to the limited supply of necessary host proteins, which may temporarily relieve the host of the normal (unstarved) viral-induced inhibition of rRNA synthesis. When the level of essential viral product(s) approaches that of the unstarved infected cells, the normal inhibitory action ensues. The effect may therefore be more complex than the reversal of the stringent response, although it is possible that R17 infection interferes with the action of the rel gene product.

It is generally true that the RNA coliphages inhibit E. coli DNA and rRNA synthesis. No such general statement can be made, however, on their effect of host tRNA synthesis. Although both tRNA and rRNA synthesis are under stringent control, they are probably regulated by slightly different mechanisms, and some tRNA species may be indepen-

dently controlled (139). Accordingly, a more recent approach to viral effect on host tRNA has been directed to individual tRNA species (140). In 1966 Bishop observed an inhibition of 4-5S RNA synthesis on unquantitated sucrose gradients from ZIK/1-infected cells (137). Watanabe obtained similar results using bacteriophage R23 (135). However, little or no decrease in tRNA synthesis occurred in E. coli infected by R17, under conditions in which ribosomal species were inhibited 70 to 80% (141, 142). Hung and Overby (143) noted that infection with Qβ, but not with MS2, results in a codon-accepting alteration in prolyl-tRNA which weakened the tRNA's capacity to bind to ribosomal complexes.

The basic structure of rRNA operons, that is,

promoter-16SrRNA-spacer tRNA-23SrRNA-5S rRNA-distal tRNA,

warrants two comments in regard to phage-induced inhibition of tRNA synthesis. First, sucrose gradient analysis of RNA extracted from infected cells would not, most likely, distinguish the heterogeneous 5S rRNA species from 4S tRNA species. A decrease in the "soluble" RNA peak on a sucrose gradient profile may reflect only a decrease in 5S rRNA synthesis. Second, the existence of tRNA spacer and distal genes on an operon which is transcribed as a unit (144) supports speculation that at least the synthesis of the particular spacer and distal tRNA's, $tRNA^{glu}$, $tRNA^{gly}$, $tRNA^{thr}$, $tRNA^{trp}$, $tRNA^{tyr}$, $tRNA^{asp}$, $tRNA^{ala}$, and $tRNA^{ile}$, are probably inhibited if rRNA synthesis is inhibited.

Among the first indications that E. coli protein synthesis was altered by coliphage infection was the change in sucrose gradient distribution of nascent proteins which occurred concommitant to altered polyribosomal distributions (145). Protein synthesis, as well as DNA

and RNA synthesis, appeared to be reduced in R17-infected cells as visualized by autoradiographic techniques (138). Sugiyama and Stone (146) investigated protein synthesis in MS2-infected cells by several different techniques and found that beta galactosidase synthesis and total protein synthesis were gradually reduced. By infecting E. coli C3000 with a coat protein amber mutant of MS2 (which fails to cause cell lysis), they found that inhibition of protein synthesis was even more apparent. By polyacrylamide gel electrophoresis, they visualized the reduction of host proteins and the appearance of viral proteins. Watanabe and Watanabe (147) used amber mutants of phage R23 to infect E. coli K38 and found that the "late" inhibition of beta galactosidase was absent with mutant phage defective in the RNA replicase cistron, but was present with phage defective in maturation (or assembly) protein. Using polar and nonpolar coat mutants of R17, which produce, respectively, less than and greater than the normal amounts of replicase, Scott demonstrated that the inhibition of beta galactosidase was greater when the abnormally large amount of replicase was present in the infected cell (148). Similarly, the greatest mutant phage-induced effect on E. coli polysome patterns was obtained with nonpolar coat mutant amB22, suggesting again that replicase is a key protein in viral induced host changes (149).

Watanabe and Watanabe (147) noted that an "early" inhibition of beta galactosidase occurred without expression of the viral genome, because UV-inactivated virus were capable of that inhibition. Yamazaki (150) proposed that the phage-induced early inhibition of RNA and protein synthesis was due to the rapid and marked inhibition of cellular amino acid transport. Such an explanation would presumably be in accordance

with the stringent factor response to amino acid starvation, so that a lessor effect would be expected from relaxed cells. Scott (151) however showed that the early inhibition phenomenon was a function of the media in which the infecting phages were suspended: the inhibition occurred if the phages were suspended in tryptone or TCG1 media, but did not occur if the phages were suspended in phosphate buffer. The presence of amino acids appeared to be the critical factor in the inhibiting media, since inoculating a culture of E. coli with a mixture of amino acids caused a 70% inhibition (151).

6. Ribosomal RNA Metabolism in E. coli

That the rare event of redundancy of genes of the E. coli chromosome occurs for the ribosomal RNA genes attests to their importance in fast growing bacteria. Indeed, calculations have shown that for bacteria dividing every 20 minutes, transcription of one rRNA operon at maximum efficiency would not supply the necessary quantity of rRNA (152, 153). The ribosomes account for up to 45% of the mass of rapidly growing E. coli cells, and the controls which determine the fraction of total protein which is ribosomal protein are closely linked to growth rate (154). The indications are, therefore, that the control of ribosome synthesis may be the most important feature in the control of bacterial growth, and at least be critical in the growth of eukaryotic cells.

It appears that ribosomal protein and RNA are coordinately synthesized. Although early studies showed that amino acid starvation affected rRNA and tRNA synthesis but did not affect mRNA synthesis, recent studies have shown that amino acid starvation does affect ribo-

somal protein synthesis (100-104). Since most of the mechanisms which control rates of cellular macromolecular syntheses have been found to operate at the level of transcription, it is not surprising that the E. coli RNA polymerase molecule has become a key enzyme in discussions of the control of ribosome synthesis. The α, β, and β' subunits of RNA polymerase are clustered with ribosomal protein genes, and α at least is probably cotranscribed with ribosomal protein (155). Travers (156) has argued that the two or three conformations of RNA polymerase holoenzyme differ in template specificity and that the stabilization of certain conformation(s) could account for the sparing synthesis of stable RNA in slowly growing cells. In an effort to explain the coupling of transcription and translation in relation to the metabolic state of the cell, Travers (157) devised a model based on the variable initiation specificity of the allosteric enzyme RNA polymerase. According to this model, the particular initiation specificity of RNA polymerase is a function of "effectors" such as ppGpp, fmet-$tRNA_f^{met}$, and EF-TuTs. His model assumes that the number of rRNA operons is in excess of the number of rRNA-transcribing polymerases, so that regulation is effected by increasing the relative concentration of these polymerases (155). The role of ppGpp accumulation in the stringent control of ribosomal RNA synthesis is accounted for by this model, although it does not include the influence that EF-G may have on ppGpp during the stringent response (155). Travers' model does not account for the increased transcription of rRNA operons in cells given a higher dosage of rRNA genes by means of a transducing phage. A model of a more passive, promoter-limiting regulation is suggested by this dosage effect (155),

but such a model is less adaptable to the controls which respond to different nutritional environments.

Once promoted, ribosomal RNA species are cotranscribed (158) and then subjected to processing events which transform the precursor 30S species to 25S and 17S RNA to the mature 23S and 16S species (159). The endonuclease reponsible for the extremely rapid conversion of the primary transcripts from the E. coli operons to the more immediate precursors of the 16S, 23S, and 5S rRNA is RNase III (160, 161). Young and Steitz (161) have shown that the rRNA sequencing data can provide the double stranded character demanded by RNase III in cleaving the 30S precursor to the mature species. (Such complementary interactions between regions of RNA also provide the basis for proposing secondary and teriary structure of the individual rRNA molecules [162], and are becoming important in explaining the role of rRNA's as part of the protein-synthesizing apparatus.) In addition to RNase III, other processing enzymes are needed to generate the mature 16S, 23S, and 5S RNA from their precursors. All three species are cleaved at both ends and methylated (163). Hayes and Vasseur (164) isolated an RNase activity which renders a 16S rRNA product which is identical at the 3' terminus to the mature 16S rRNA. The action of their enzyme appeared to require a system in which protein synthesis could take place. Dahlberg et. al. (165) isolated an endonuclease, called M16, which processes the 5' end of the 16S rRNA. Lowry and Dahlberg (166) had previously shown that methylation of nucleotides accounted for some of the differences between the mature and precursor species.

The connection between structure and function of ribosomal RNA

has been explored by the work of Shine and Dalgarno (167). In 1974 they presented evidence that 3' terminal nucleotides of E. coli 16S RNA could form seven, four, and three base pairs with appropriate regions of the R17 assembly protein, replicase, and coat ribosomal binding sites, respectively. They pointed out that the extent of this base pairing related closely to the ribosome-binding capacities of the three isolated initiator regions of R17 RNA and therefore correlated with Steitz's conclusions that E. coli ribosomes discriminate in favor of the assembly protein initiator fragment 40 times and 11 times over the coat and replicase sites, respectively (168, 169). The Shine and Dalgarno base pairing might also explain why Bacillus stearothermiphilus ribosomes only translated the assembly protein cistron and neither the coat nor replicase cistrons of R17 or f2 RNA (170, 171). Shine and Dalgarno further proposed that the degree of complementarity between the pyrimidine-rich stretch on the 16S rRNA and the purine-rich segment on a bacterial mRNA initiator determines its intrinsic cistron translatability (168). They suggested that the finer controls in initiation be imposed by initiation factors and mRNA secondary structure, so that the 30S subunit does not initiate at the many internal AUG or GUG codons contained within, as well as at the beginning, of a cistron.

In support of the proposals of Shine and Dalgarno, Tanijuchi and Weissmann (172) recently reported that there are great differences in the stabilities calculated for the interactions between the 3' terminal region of 16S rRNA and the Shine-Dalgarno regions of different mRNA's, and that the role of the interaction between initiator site and fMet-

tRNA anticodon is probably complementary to the Shine-Dalgarno interaction. The inhibition of Qβ RNA 70S ribosome initiation complex formation by an oligonuleotide complementary to the 3' terminal region of E. coli 16S rRNA (172) confirms the validity of the Shine-Dalgarno interaction in initiation complex formation.

Statement of Problem

The inhibition of E. coli rRNA synthesis exerted by bacteriophage R17 might be attributed to one or more of the phage-specific proteins, namely, the coat, the replicase, and the assembly proteins. By the use of mutants of the bacteriophage R17 which are defective in one or more of these proteins, the present study investigates:

1. Which, if any, of the phage-specific proteins is involved in the inhibition of host ribosomal RNA synthesis?

2. Does the inhibition occur at the level of transcription, or is the maturation of the transcribed product affected?

Media and Solutions

In experiments in which cells incorporated radioactive precursor, cultures were grown in TCG1 synthetic medium (173). The medium, a modification of the TPG medium of Sinsheimer (174), contains, per liter: 0.6 g NaCl, 8.0 g KCl, 1.1 g NH_4Cl, 0.2 g $MgCl_2 \cdot H_2O$, 0.023 g KH_2PO_4, 0.023 g Na_2SO_4, 1.66 g tris (hydroxymethyl)-aminomethane, 5.72 g tris (hydroxymethyl)-aminomethane hydrochloride (pH 7.4), and 1.5 g casamino acids. After autoclaving, 0.55 g $CaCl_2$, 1.0 ug $FeCl_3 \cdot H_2O$, 0.8 g sodium pyruvate, and 0.2% glycerol were added. Virus stock and E. coli overnight cultures were grown in MS broth (175), which contains per liter: 10.0 g Bacto-tryptone, 8.0 g NaCl, and 1.0 g Bacto-yeast extract. After autoclaving, 10.0 ml of 10% glucose solution, 2.0 ml of 1.0 M $CaCl_2$, and 1.0 ml of 10 mg/ml solution of thiamine hydrochloride were added per liter. Top agar for plating contains the same ingredients as MS broth, plus 0.8% Bacto-agar. Bottom agar contains per liter: 10.0 g Bacto-agar, 10.0 g Bacto-tryptone, 2.5 g KCl and 2.5 g NaCl. After autoclaving 1.0 ml of 1.0 M $CaCl_2$ were added.

RNA extractions were carried out in 0.1 M STE buffer, containing 0.1 M NaCl, 0.05 M tris (hydroxymethyl)-aminomethane(Tris)-hydrochloride, pH 7.4, and 1.0 mM ethylene-diaminetetraacetic acid (EDTA). Electrophoresis solutions were prepared according to Pons and Hirst (176): immediately before use, one volume of a solution containing 9.6% acrylamide and 0.5% N,N'-methylene-bis-acrylamide in 0.486 M Tris-HCl (pH 8.4) containing 0.2% SDS was mixed with one volume of catalyst solution containing 0.16 g ammonium persulfate, 0.15 ml N, N, N', N'-tetramethyl-

enediamine in 50 ml H_2O. After one minute the mixture was mixed with an equal volume of melted 2% agarose in 0.243 M Tris-HCl (pH 8.4) at 45 C. Thus the final concentration of the gels was 2.4% acrylamide containing 1% agarose.

Tritiated uridine (specific activity 20 Ci/mmole) was purchased from Schwartz/Mann, Boston, Mass. Omnifluor scintillation compound was purchased from New England Nuclear Corp., Boston, Mass.

Bacteria and Bacteriophage

E. coli C3000, a nonlysogenic, nonpermissive Hfr strain originally obtained from R. Sinsheimer of the California Institute of Technology, was used in the RNA extraction experiments, and was used to grow wild-type bacteriophage R17. The nonpermissive and permissive indicator strains, E. coli S26 and E. coli S26RIE, respectively, and the R17 amber mutants amA31, amB24, amB22, and amC16, were provided by R. Kamen of Harvard University. Bacteriophage R17 was originally provided by M. Capecchi of Harvard, as were the R17 amber mutants amC8 and amC13.

Standard Agar Layer Plaque Assay of Bacteriophage

Phage titers and reversion rates of phage mutants were obtained using the standard agar layer plaque assay technique. Chloroform treated lysates of infected cultures were diluted serially in tryptone broth. Seed cultures were E. coli inocula grown to 10^8 cells/ml in tryptone

broth in a shaking water bath at 37 C. Phage assay was carried out by mixing 1.0 ml diluted phage and 0.2 ml of a seed culture of E. coli with 2.0 ml of liquid top agar, then pouring the mixture onto the bottom agar plate and allowing the top agar to harden. The plates were incubated at 37 C 12 to 18 hours and scored for plaques in the lawn of bacteria. Reversion rates were calculated from the ratio of plaque-forming units scored on the nonpermissive E. coli S26 to that on the permissive E. coli S26RIE. Only those phage mutant cultures containing less than 1.0% revertants were used in the present study.

Labelling and Preparation of RNA

A fresh overnight MS broth culture of E. coli C3000 was diluted 1:25 in 200 ml of TCG1 medium and grown at 37 C to an early log phase density of 2 X 10^8 cells/ml, as measured by turbidity at 610 mu using a Bausch and Lomb Spectronic 20 Colorimeter. Cells were then harvested by centrifugation at 11,700 g for 5 minutes. The resulting cell pellet was suspended in 3 ml of TCG1 medium containing phage at a multiplicity of 10 plaque-forming units (PFU) per cell. After a three-minute adsorption period, the cells were added to 100 ml of prewarmed medium and incubated in a shaking water bath at 37 C. Uninfected cells were treated identically; the adsorption period was simulated in medium containing no phage. At 45 minutes post infection, each culture was pulse-labelled for two minutes with tritiated uridine at a final concentration of 2.5 uCi/ml, and chased for 10 minutes with a 300-fold excess of unlabelled uridine. At the end of the chase period, 1.0 ml from

each culture was pipetted into a chilled test tube containing 0.2 ml of 60% trichloroacetic acid plus 0.1 ml of chloroform. The rest of the culture was immediately poured over crushed TCG1 ice containing 0.01 M KCN, to stop further incorporation of label. The cells were then harvested by centrifugation at 5 C, and the cell pellets were suspended in cold STE buffer.

RNA was extracted from the cells with phenol in the presence of 2.5% sodium dodecyl sulfate (SDS) by the method of Erikson, Fenwick, and Franklin (177), in which the SDS-lysates are shaken with an equal volume of phenol and the phases separated by centrifugation. Three phenol extractions at room temperature were followed by extraction with ether and removal of ether by the bubbling of nitrogen (173). The nucleic acids were then precipitated with two volumes of cold ethanol, and RNA suspended in 1.0 ml of sample buffer containing 20% sucrose for polyacrylamide gel electrophoresis (176).

Results

It has been found that R17 infection of E. coli does not affect synthesis of transfer RNA (141). To examine the synthesis of 16S and 23S ribosomal RNA in infected and uninfected cultures, stable species of RNA were preferentially labelled with tritiated uridine by chasing with an excess of unlabelled uridine for 10 minutes. Labelled uridine was added to cultures prior to the onset of lysis in R17-infected cells and during the period of maximal inhibition of RNA synthesis (41). Cells were harvested, and total RNA was extracted and subjected to polyacrylamide gel electrophoresis. The resulting RNA profiles from R17-infected and uninfected cells are shown in Fig. 1A. Infection with the R17 phage results in the inhibition of 16S rRNA synthesis. The inhibition of 23S rRNA synthesis could not be determined from this profile because 23S rRNA had not been separated from 27S viral RNA. The identity of the heterogeneous RNA from infected cells migrating in fractions 42 and 72 in unknown.

To resolve 23S from 27S RNA, electrophoresis was carried out for 11.5 hours at 5 ma/gel in 2.4% polyacrylamide gels. Under these conditions tRNA migrated off the gel. The 23S rRNA band was resolved from 27S viral RNA and a new band of labelled RNA appeared between 16S and 23S RNA (Fig. 1B). The pulse-chase experiments shown in Fig. 2 indicated that this new component was probably the precursor to mature 16S rRNA, as originally described by Adesnik and Levinthal (178). The ratio of labelled 17S to 16S rRNA following a 2-, 4-, and 7-minute chase was 1;0.9, 1:2.2, and 1;3.7, respectively. The radioactivity of this com-

Fig. 1. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from R17-infected (---) and uninfected (——) cultures of E. coli C3000. (A) Electrophoresis was carried out for 8.5 hours in 2.2% polyacrylamide gels at 3 ma/gel. (B) Electrophoresis was carried out for 11.5 hours in 2.4% polyacrylamide gels at 5 ma/gel. A standard RNA preparation containing 4S, 16S, and 23S E. coli RNA and 27S viral RNA was used as a migration marker in the polyacrylamide gels. The RNA migrated from right to left and was fractionated by collecting 1.2 mm gel slices.

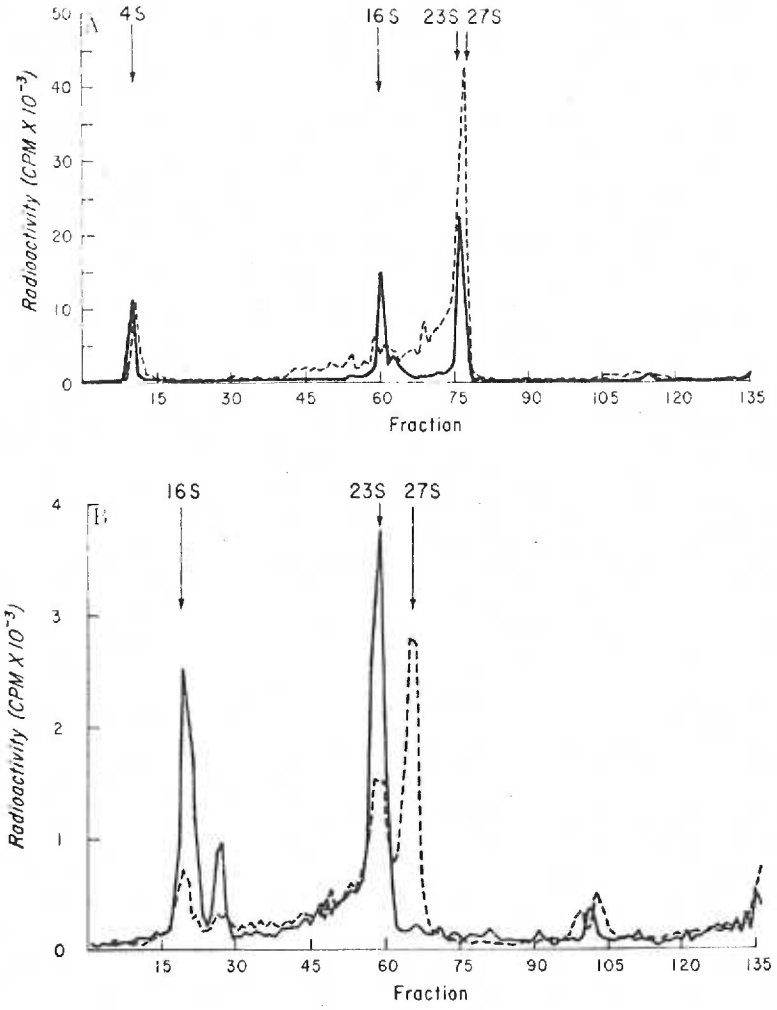
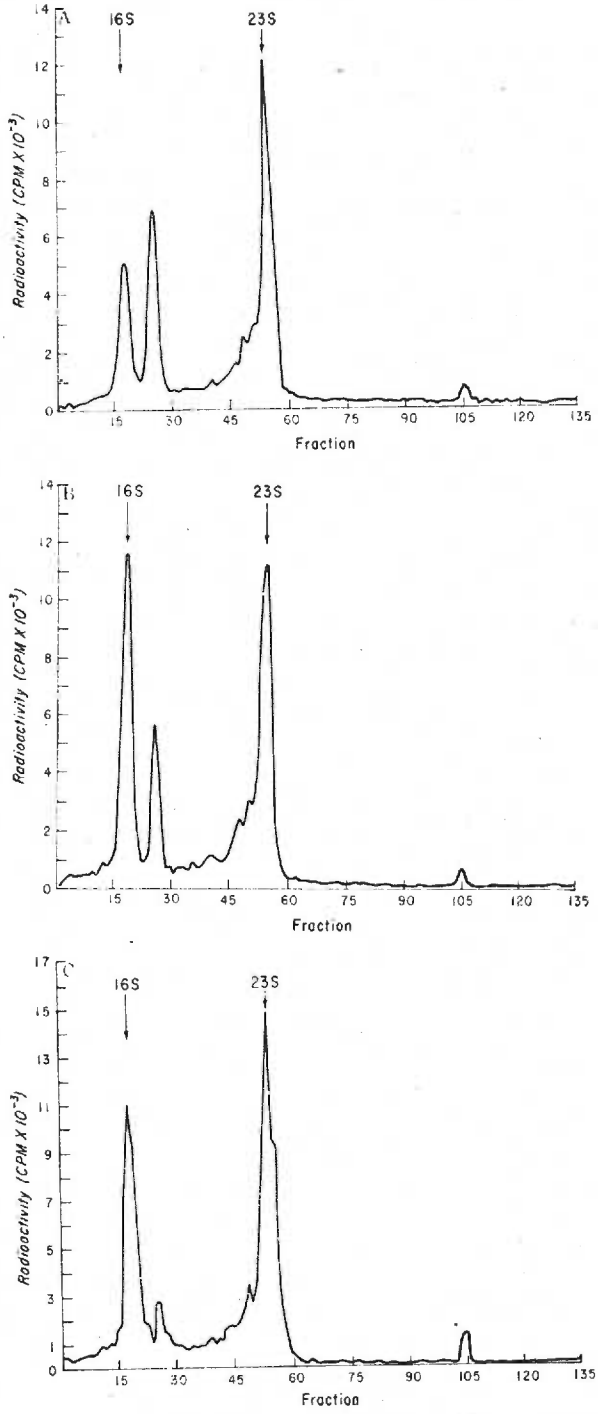

A
4S
16S
23S
27S
Radioactivity (CPM X 10⁻³)
Fraction
B
16S
23S
27S
Radioactivity (CPM X 10⁻³)
Fraction

Fig. 2. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from an uninfected E. coli C3000 culture. Electrophoretic conditions were as in Fig. 1B. The 16S and 23S rRNA species are indicated. This 300 ml culture was pulse-labelled for 2 minutes with ^{3}H-uridine and chased by adding an excess of unlabelled uridine; 100 ml samples were harvested (A) 2 minutes, (B) 4 minutes, and (C) 7 minutes later.


A
16S
23S
Radioactivity (CPM X 10⁻³)
Fraction
B
16S
23S
Radioactivity (CPM X 10⁻³)
Fraction
C
16S
23S
Radioactivity (CPM X 10⁻³)
Fraction

ponent was included with that of 16S rRNA when determining the amount of label incorporated into rRNA during the 2-minute pulse.

Data from electrophoretic profiles resolving viral RNA from rRNA were quantitated. The amount of radioactivity in the 16S and 23S RNA was corrected by dividing by the RNA recovery factor, described in the footnote of Table 1. The recovery factor consisted of the ratio of trichloroacetic acid-precipitable labelled RNA from chloroform-lysed cells to the amount of trichloroacetic acid-precipitable RNA recovered following phenol extraction of the culture. The factor was based on the assumption that the phenol extraction procedure does not preferentially extract a particular species of acid-precipitable RNA and that RNA is equally extractable from infected and control cultures. The recovery factor ranged from 0.67 to 0.85. The major loss of RNA during extraction was due to discarding from 5 to 10% of the aqueous phase at the phenol-buffer interphase during several phenol extractions. However, since the RNA is in solution, the concentration of individual species of RNA should not be affected by the loss of a portion of the solution. Additional extraction did not release additional labelled material. The amount of radioactivity in the 4S RNA served as an internal marker for the extraction procedure. When the amount of radioactivity in the extracted 4S RNA was divided by the recovery factor, incorporation of label into 4S RNA of infected and control cells was similar. This observation would be expected since 4S RNA synthesis is not altered in R17-infected cells (141). In addition, cultures infected with the wild-type R17 incorporated into RNA 28% of the tritiated uridine incorporated by control cultures (Table 1). This value is

Table 1

Ribosomal ribonucleic acid synthesis in infected cultures of E. coli

Culture	Relative amounts of ^{3}H-uridine incorporated[a]	
	% 16S RNA	% 23S RNA
Uninfected control	100	100
R17-infected	28 ± 5	46 ± 7
amA31-infected	36 ± 3	44 ± 9
amB24-infected	80 ± 8	86 ± 10
amB22-infected	46 ± 7	36 ± 5
amC8-infected	100 ± 5	98 ± 7
amC13-infected	100 ± 9	98 ± 7
amC16-infected	97 ± 9	97 ± 9

a. The radioactivity of gel fractions containing 16S and 23S RNA was corrected for the amount of RNA lost during extraction procedures by dividing by a recovery factor (counts per minute of ^{3}H-RNA in final buffer solution per counts per minute of trichloroacetic acid-precipitable RNA in original culture). The corrected radioactivity was expressed as a per cent of that of the uninfected control, and each value in the table is the average per cent obtained from three or more experiments, with standard deviations indicated.

consistent with the corresponding result of 29% obtained from hybridization experments (132). For 23S rRNA, the results indicate that R17-infected cells incorporated 46% of the label incorporated by control cells. During electrophoresis, however, there may have been some slurring of the 27S viral RNA into the 23S rRNA.

The amber mutants of phage R17 used to infect cultures of E. coli are described in Table 2. In the nonpermissive host, the mutant amA31 synthesizes coat protein and viral polymerase but not assembly protein (82). The assembly protein enables the phage to adsorb to the cell (28). Phage amA31 grown in permissive E. coli S26RIE was used to infect nonpermissive E. coli C3000 cells. The electrophoretic profiles of labelled RNA extracted from amA31-infected cells are shown in Fig.3. The amount of inhibition was similar to that observed in R17-infected cells (Table 1). The data indicate that the synthesis of assembly protein is not required for the inhibition of rRNA synthesis. As in experiments with the wild-type phage, viral RNA of amA31 may have been incompletely separated from 23S rRNA, resulting in an apparent increase in the synthesis of 23S relative to 16S RNA.

The amber mutant amB22 does not synthesize coat protein in the nonpermissive host (Table 2). Viral RNA produced during an infection with amB22 is consequently not encapsulated. A second effect of the mutation in the coat protein cistron is the production of an abnormally large amount of viral polymerase (149). Among R17 and related phage, translation of the polymerase cistron is regulated by the binding of the coat protein to the viral mRNA (18, 33). The lack of coat proteins in the amB22-infected cell precludes such regulation of translation, and

Table 2

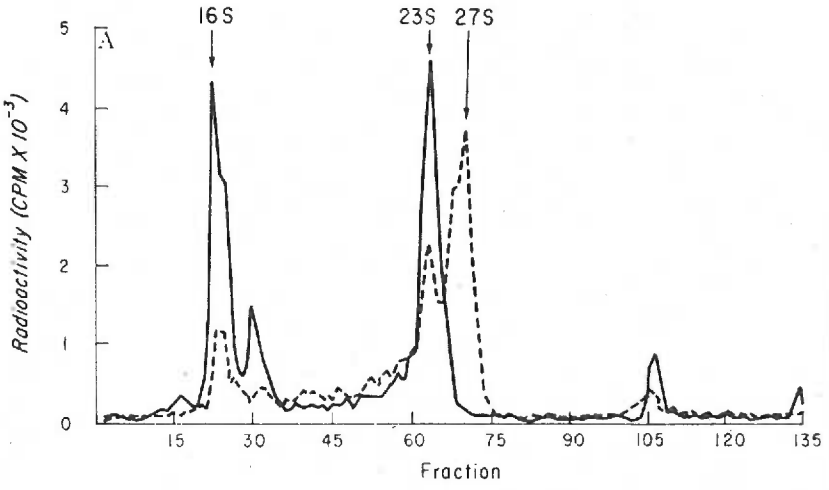
Viral proteins synthesized during phage infections of nonpermissive *E. coli*[a]

Phage	Proteins synthesized		
	Assembly	Coat	Replicase, per cent of wild-type
R17	+	+	100
amA31	-	+	90-100
amB24	+	-	15-30
amB22	+	-	90-105[b]
amC8	-	-	1
amC13	-	-	8
amC16	-	-	3

a. The proteins synthesized by mutants amA31, amB24, amB22, and amC16 were determined by Tooze and Weber (82), and those by amC8 and amC13 by Gussin (76).

b. The percentage of replicase synthesized in the nonpolar mutant amB22-infected cells has probably been underestimated. The *in vivo* replicase activity was measured as a percentage of label incorporated into viral RNA of mutant-infected cells as compared to wild-type-infected cells (82). Polyacrylamide gel electrophoresis of labelled viral replicase from amB22-infected cells indicate a large increase in the amount of replicase synthesized (149). The latter result is in agreement with the 3- to 15-fold increase in viral replicase observed in nonpolar coat f2 mutant-infected cells (40).

Fig. 3. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from assembly protein mutant amA31-infected (---) and uninfected (——) cells. Electrophoretic conditions were as in Fig. 1B.


16S
23S
27S
A
Radioactivity (CPM X 10⁻³)
0
1
2
3
4
5
15
30
45
60
75
90
105
120
135
Fraction

viral polymerase accumulates in excess. The RNA profiles (Fig. 4) indicate that rRNA synthesis was inhibited in cells infected with amB22. The amounts of radioactive precursor incorporated into 16S and 23S RNA were 46 and 36%, respectively, of the control values (Table 1). Since inhibition of rRNA synthesis occurred in cultures infected with amB22, the synthesis of viral coat protein does not appear to be required for the inhibiton.

The amber mutant amB24 is a polar coat mutant (82). It lacks the ability to synthesize coat protein, and the polar effect results in a reduced synthesis of viral polymerase (82, 149) (Table 2). Electrophoretic profiles of RNA from cells infected with amB24 and from uninfected cells are shown in Fig. 5. The incorporation of tritiated uridine into rRNA by infected cells is inhibited, but not to the same extent as in cells infected with amB22 (Table 1). The degree of inhibition of rRNA synthesis in cells infected with mutants amB22 and amB24 appears to be due to the amount of viral polymerase synthesized.

Little single-stranded viral RNA is observed in amB22- and amB24-infected cells under pulse-chase conditions (Figs. 4 and 5). The limited viral RNA synthesis would be expected in amB24-infected cells because of the restricted viral polymerase synthesis (82, 149). However, the lack of single-stranded viral RNA in amB22-infected cells was unexpected since viral polymerase is produced during infection (149). A comparison of 2 minute pulse-labelled viral RNA from cells infected with amB22 and wild-type virus agrees with the expected increase in polymerase synthesis during infection with the mutant (Fig. 6A). The major difference between the mutant and R17-infected cells is the lack of single-stranded

Fig. 4. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from nonpolar coat protein mutant amB22-infected (---) and uninfected (——) cultures. Electrophoretic conditions were as in Fig. 1B.

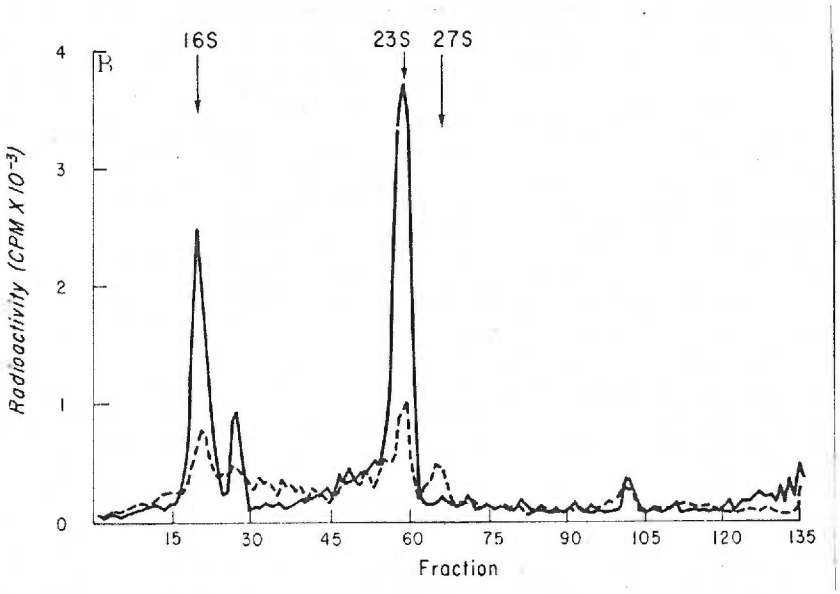

B
16S
23S
27S
Radioactivity (CPM X 10⁻³)
0
1
2
3
4
15
30
45
60
75
90
105
120
135
Fraction

Fig. 5. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from polar coat protein mutant amB24-infected (---) and uninfected (——) cells. Electrophoretic conditions were as in Fig. 1B.

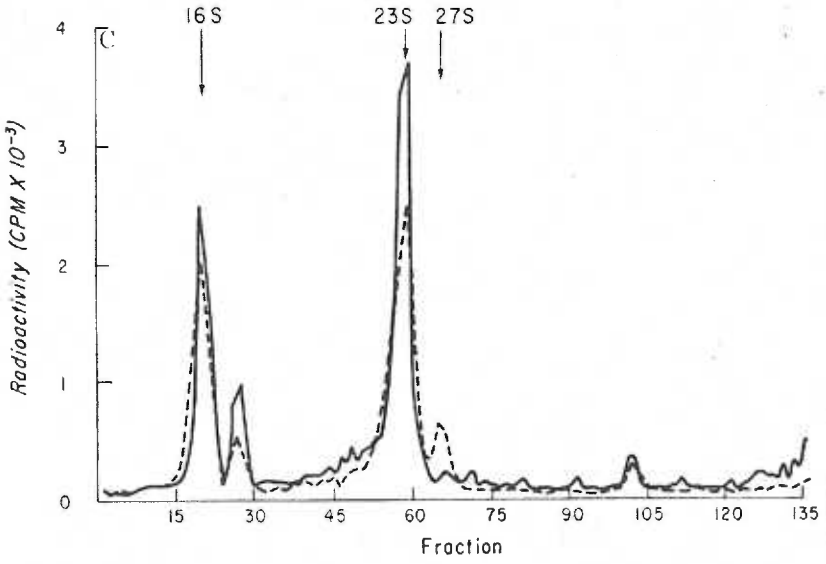
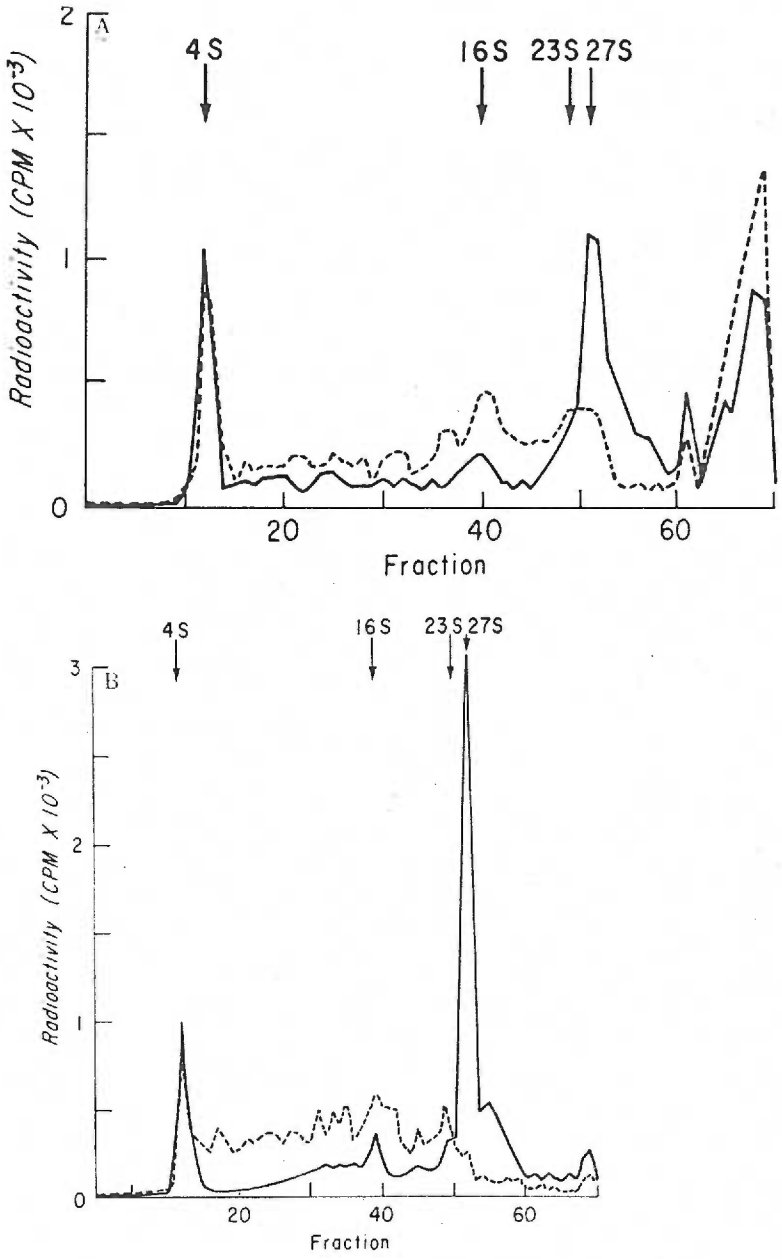

C
16S
23S
27S
Radioactivity (CPM X 10⁻³)
0
1
2
3
4
15
30
45
60
75
90
105
120
135
Fraction

Fig. 6. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from mutant amB22- (---) and R17- (——) infected cultures. Electrophoresis was carried out for 3.5 hours in 2.2% polyacrylamide gels (8.5 by 0.6 cm) at 3 ma/gel. An RNA preparation containing 4S, 16S, 23S E. coli RNA and purified 27S viral RNA was used as a migration marker in the gels. (A) Infected cells pulse-labelled for 2 minutes with ^{3}H-uridine. (B) Infected cells pulse-labelled for 2 minutes with ^{3}H-uridine and then chased for 10 minutes with an excess of unlabelled uridine at 45 minutes post infection.


A
4S
16S
23S
27S
Radioactivity (CPM X 10⁻³)
2
1
0
20
40
60
Fraction
B
4S
16S
23S
27S
Radioactivity (CPM X 10⁻³)
3
2
1
0
20
40
60
Fraction

viral RNA in amB22-infected cells. The amB22-infected culture also contained a slightly larger amount of heterogeneous 4S to 23S RNA. An examination of the labelled RNA from amB22 and R17-infected cells under pulse-chase conditions suggests that the viral RNA in amB22-infected cells may be degraded (Fig. 6B). Most of the labelled viral RNA from R17-infected cells migrates as 27S viral RNA, presumably extracted from mature progeny virus. No distinct peak of 27S viral RNA is observed in the amB22-infected cultures. In addition, a larger amount of the heterogeneous 4S to 23S RNA was consistently observed in the amB22-infected cells. These results are interpreted as being due to the degradation of the viral RNA produced in amB22-infected cells. It seems likely that the viral RNA which is not encapsulated during infection with the coat protein mutant amB22 is probably being degraded. This elevated background of labelled 4S to 23S RNA undoubtedly affected the quantitation of rRNA synthesis in the amB22-infected cells.

Due to amber mutation in the polymerase cistron, phage amC8, amC13, and amC16 do not synthesize viral polymerase in the nonpermissive host. Since these mutants cannot replicate the viral genome, no amplification of viral messenger RNA occurs, and very little of the other viral proteins is synthesized (149). Infection of E. coli C3000 with any of these polymerase mutants had no effect on host synthesis of rRNA (Figs. 7, 8, 9, and Table 1).

Phages that inhibited host rRNA synthesis were the wild-type R17 and the mutants amA31, amB22, and amB24. Electrophoresis of RNA, shown in Figs. 4, 5, 7, and 2B, indicate that there was no accumulation of the precursor form of 16S rRNA in cultures infected with these phage.

Fig. 7. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from replicase protein mutant amC13-infected (---) and uninfected (——) cells. Electrophoretic conditions were as in Fig. 1B.

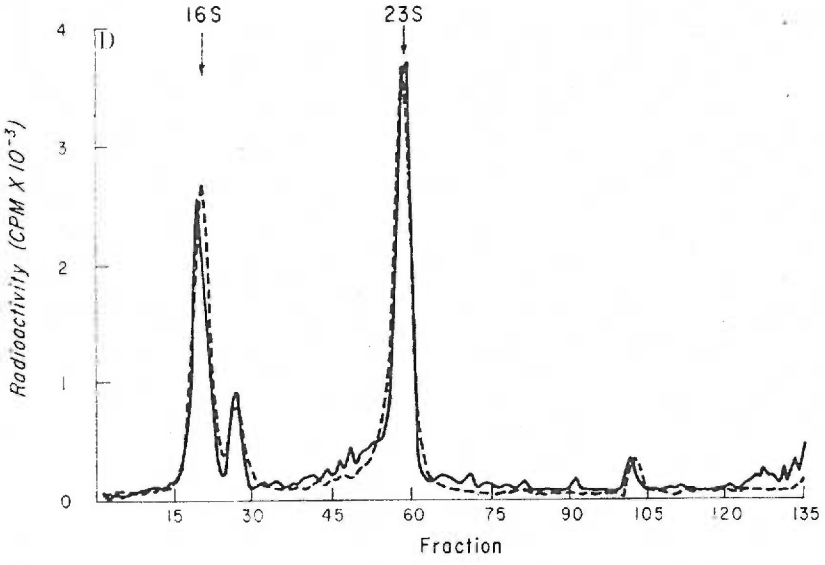

16S
23S
D
4
3
2
1
0
Radioactivity (CPM X 10⁻³)
15
30
45
60
75
90
105
120
135
Fraction

Fig. 8. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from replicase protein mutant amC8-infected (---) and uninfected (——) cells. Electrophoretic conditions were as in Fig. 1B

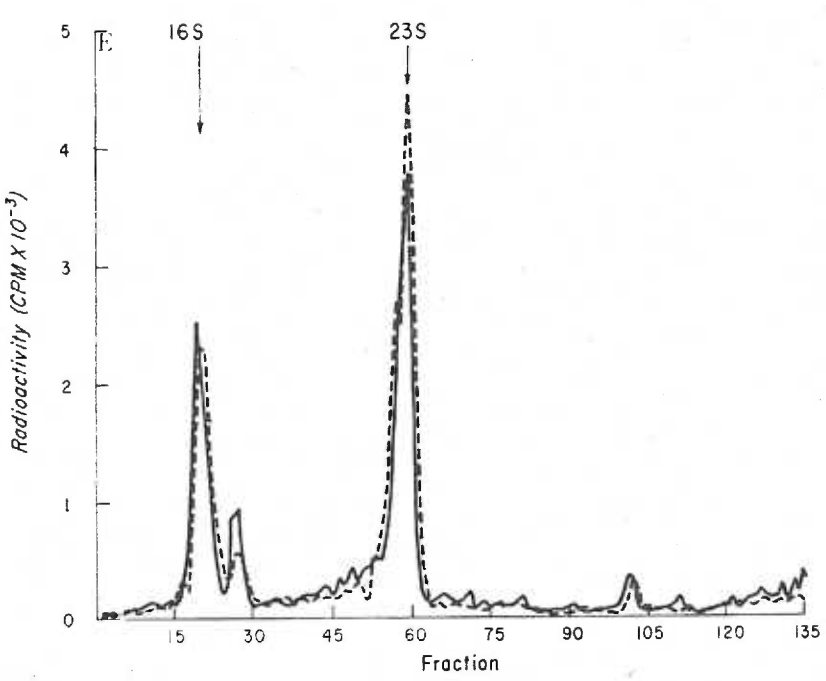
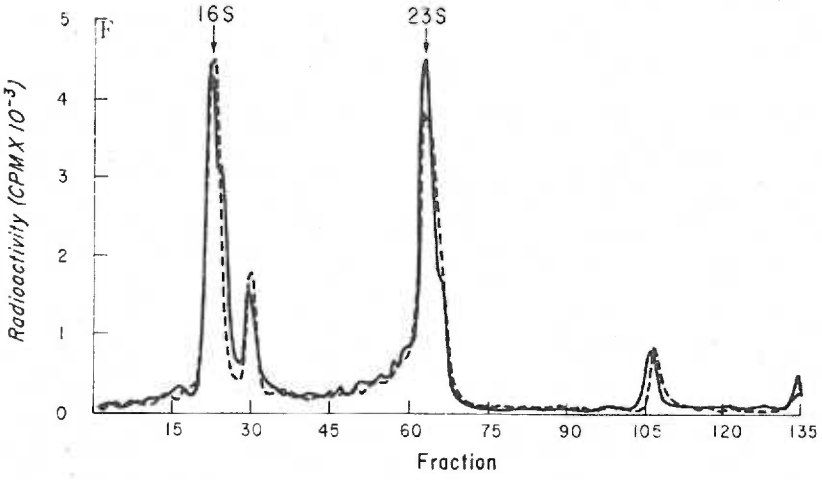

16S
23S
Radioactivity (CPM X 10⁻³)
0
1
2
3
4
5
15
30
45
60
75
90
105
120
135
Fraction

Fig. 9. Polyacrylamide gel electrophoresis of ^{3}H-RNA extracted from replicase protein mutant amC16-infected (---) and uninfected (——) cells. Electrophoretic conditions were as in Fig. 1B.


16S
23S
Radioactivity (CPM X 10⁻³)
0
1
2
3
4
5
15
30
45
60
75
90
105
120
135
Fraction

The amount of precursor synthesized was reduced, and this reduction probably accounts for the decreased amount of mature form observed. The results therefore suggest that the inhibition of 16S rRNA synthesis occurred at the level of transcription rather than at the level of maturation.

Discussion

The early events in the process of R17 infection, adsorption to the cell and penetration of the viral nucleic acid, do not seem to be involved in the inhibition of the host rRNA synthesis. This conclusion is based on results from infections with viral polymerase mutants amC8, amC13, and amC16. Grown in permissive E. coli cultures, these phages were adsorbed to nonpermissive E. coli cells, and the events of infection proceeded normally until the amber mutation in the replicase cistron was expressed. Without functional replicase molecules, viral RNA, and thus viral proteins, were not synthesized in detectable amounts. No inhibition of host rRNA synthesis was observed in infections with the replicase mutants.

The results of experiments with the other amber mutants indicate that synthesis of viral replicase is required for the phage-associated inhibition of rRNA synthesis. Phages that were unable to synthesize assembly protein were able to cause the inhibition, as were phages unable to synthesize coat protein. In all experiments in which the inhibition of host rRNA synthesis was observed, the infecting phage was one which had retained its ability to synthesize viral replicase and viral RNA. However, viral RNA synthesis is not always associated with the inhibition of rRNA, since, at the nonpermissive temperature, a temperature-sensitive replicase mutant of R17 has been shown to inhibit rRNA synthesis in the absence of viral RNA synthesis (142). Presumably this temperature-sensitive protein may be involved in two independent functions: 1. the synthesis of viral RNA, and 2. the

inhibition of rRNA synthesis.

In amB24, amA31, and wild-type R17 infections, the degree of inhibition of rRNA synthesis correlates with the amount of viral replicase synthesized in the infected cell. Restricted replicase production by polar coat mutant amB24 is accompanied by a slight inhibition of rRNA synthesis. Assembly protein mutant amA31 and the wild-type R17 produce comparable quantities of viral replicase, and induce correspondingly similar degrees of inhibition. The nonpolar coat mutant amB22 produces a large excess of viral replicase. However, the inhibition of 16SrRNA synthesis observed in amB22-infected cells was not as great as that observed in cells infected with wild-type phage. The following may explain what was observed in amB22 infections. In amB22 infections, little 27S viral RNA is observed probably because it is not encapsulated and is therefore degraded. The initial nuclease degradation products of R17 RNA, as well as Qβ RNA, are 22S and 15S fragments (179, 180), which would be electrophoretically indistinguishable from rRNA species. What was observed as rRNA synthesis in amB22-infected cells may have been the sum of the syntheses of rRNA and the initial cleavage products of viral RNA into 15S and 22S fragments. The actual synthesis of rRNA, therefore, may have been depressed in amB22-infected cells more than what was observed. Viral RNA degradation may also have slightly affected observations of rRNA synthesis in amB24-infected cells. The effect would be much less pronounced in amB24 experiments because of the restricted synthesis of viral RNA by amB24 relative to that by amB22 (82).

Cells infected with amB22 were reported to synthesize 90 and 105%

of the viral replicase found in R17-infected cells (82). Viral replicase was measured *in vivo* as the percentage of label incorporated into viral RNA of mutant-infected cells as compared to cells infected with wild-type virus. The degradation of unencapsulated viral RNA would have led to an underestimate of the viral polymerase in the amB22-infected cells and would explain the difference in replicase synthesis reported by Phillips *et. al.* (149) and Tooze and Weber (82).

Kondo *et. al.* (51) and Kamen (52) have shown that purified viral replicase from RNA phage Qβ consists of four polypeptide subunits: α β, γ, and δ. Three of these were found to be derived from the host, the β subunit being the only one coded for by the viral genome. The ability of R17 and R17 mutants to synthesize viral replicase therefore refers to the ability to synthesize the β subunit.

The conclusion that viral polymerase synthesis is required for the inhibition of rRNA synthesis is inherent in the hypothesis of Travers *et. al.* (181) on a possible mechanism of inhibition. They suggested that the viral replicase of Qβ and host DNA-dependent RNA polymerase compete for a protein which is necessary for both rRNA synthesis and phage RNA replication. The hypothesis was put forth to explain the relationship between a component of Qβ replicase and a transcription factor they found in *E. coli* extracts. Either the Qβ component or the transcription factor, ψr, could be used in an *in vitro* synthesizing system to specifically stimulate rRNA synthesis (181). The Qβ component ψr consisted of the host-dependent subunits γ and δ.

When Blumenthal *et. al.* (53) later established that the γ and δ subunits of Qβ replicase are identical to elongation factors EF-Tu and

EF-Ts, respectively, they also tested the stimulatory activities of these factors in their own in vitro RNA synthesizing system. They found that the two-fold stimulation observed with γr could be duplicated by the use of elongation factor EF-Ts alone (53). The role of γr, or EF-Ts, as a transcriptional factor has since been subject of controversy (182-184) but Travers has maintained that its activity is one of several among the effectors of E. coli RNA polymerase (157). The effector concept has been supported by the roles that ppGpp (185), EF-G (155), and fMet-tRNA (186) may play in influencing RNA synthesis, but the specificity of the stimulation of EF-Ts has not been confirmed.

Haseltine (182) found that preparations of Qβ replicase subunits γ and δ did stimulate transcription by purified holoenzyme from E. coli DNA, but the two-fold stimulation was not specific for ribosomal RNA. He found that 12% of the total RNA transcript was rRNA, whether or not the Qβ factors were present in the in vitro reaction. It is known, however, that in rapidly growing cells 50% of the RNA synthesized is ribosomal (154), so his in vitro results may arise from only some of the controls that exist in vivo.

One or more of any of the host derived phage replicase subunits could be involved in the phage induced inhibition of ribosomal RNA synthesis, or the inhibition could involve host proteins that are affected by phage infection through the replicase molecule. The limiting quantity of subunit(s) could result in competition between the remaining viral replicase subunits and the E. coli cell transcription system, reducing its availability for host synthesis. The correlation observed between the amount of viral replicase and the degree of inhi-

bition is consistent with, but does not prove, that the inhibition is due to competition for shared factor(s). The sigma factor has been found to be required for preferential in vitro synthesis of rRNA (182), so it too may be affected by coliphage infection. Host factor HF has been observed to bind to 16S and 23S rRNA and may be a control element in RNA metabolism. That HF is required for phage RNA synthesis and therefore may be related to the phage replicase-induced inhibition of rRNA synthesis appears to be contradicted by the observations of Igarashi et. al. (142). They found that a temperature-sensitive replicase mutant of R17 produced no viral RNA in E. coli at 43 C but caused a progressive decrease in rRNA synthesis. Why none of the replicase amber mutants caused the inhibition may however be due to the nature of the mutation (if the same protein is mutated in both systems). The amber mutations of amC8, amC13, and amC16 cause premature terminations of the replicase translation. The resulting incomplete polypeptide may be unable to bind EF-Ts, for example, and thus have neither the ability to polymerize viral RNA nor the ability to inhibit rRNA synthesis. The temperature-sensitive mutations, however, allow translations of the entire replicase molecule. At nonpermissive temperatures this replicase may still be able to bind EF-Ts and thus inhibit rRNA synthesis although it loses enzymatic activity for viral RNA synthesis.

The resulting rRNA synthesis in mutant R17 phage infection of E. coli does not address the question of the various effects that other coliphages exert on E. coli rRNA synthesis. The subtle differences between the coliphages, such as intercistronic distances and the dif-

ferent isoelectric points of the coat proteins, should be considered in the pursuit of the mechanism of phage induced inhibition of rRNA synthesis. The demonstration by Sinear and Steitz (60) that S1 fulfills different roles in the R17 and Qβ RNA synthesizing systems implicates S1 as a factor in the different effects that the two phages exert on the host. In fact, binding experiments (60) indicate that the R17 and Qβ phages and host enzymes may compete for S1 with different efficiences, which could result in different inhibitions of host synthesis.

The role that ribosomal protein S1 plays in the uninfected cell, that of binding mRNA to ribosomes, may implicate it as the indirect inhibitor of rRNA synthesis in infected cells. The infecting phage usurps the S1 protein to use it as a replicase subunit. Limited supply of S1 could limit the initiation of E. coli proteins, including proteins required for rRNA synthesis. The same could be said of EF-Tu and EF-Ts in the elongation of such proteins. The infecting phage usurps EF-Tu and EF-Ts and could thereby disrupt the elongation complex EF-Tu·GTP·amino acyl·tRNA. It may be that GTP (and/or ppGpp) concommitantly accumulate, and inhibit rRNA synthesis by a mechanism that is related to the restricted rRNA synthesis of starved stringent cells. Even as part of a multicomponent system, the demonstration of the activity of EF-Tu, EF-Ts, or S1 in an in vivo inhibition of rRNA synthesis could support the original concept of Travers, that of the competition between viral replicase and the host transcription system.

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