Patterns of Ribonucleic Acid Synthesis in T5-infected Escherichia coli

IV. EXAMINATION OF THE ROLE OF DEOXYRIBONUCLEIC ACID REPLICATION*

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SUMMARY

The role of DNA replication in the synthesis of RNA and proteins during the "late" period of the infective cycle of T5-infected Escherichia coli F has been examined. Inhibition of DNA synthesis was achieved by addition of chloramphenicol to T5-infected cultures or by use of a mutant of T5 (T5 am I), which is unable to cause the synthesis of an active DNA polymerase upon infection of the nonpermissive host, E. coli F. The synthesis of late or Class III RNA and lysozyme, a presumed late protein, was measured. In the absence of DNA synthesis a small amount of late or Class III RNA species is formed by 9 to 12 min after infection, but much greater quantities are produced under conditions allowing concurrent DNA replication. The synthesis of T5 lysozyme occurs normally in both the permissive and nonpermissive hosts.

After the infection of host cells by bacteriophage there occurs a series of sequential, highly controlled reactions starting with the entrance of phage nucleic acid into the host cell and ending with the production of infective phage particles. The transcription of the viral genome has been divided into at least two periods during the infective cycle, the "early" or prereplicative and the "late" or replicative (2-9). Based on the time of their appearance after T5 infection, three major classes of proteins can be observed by polyacrylamide gel electrophoresis (10). At 37°, Class I proteins are formed between 0 and 6 min by transcription of a small segment of the phage DNA, approximately 8%, that is first injected into the host cell (10-12). Class II protein synthesis commences at 5 min and continues until 24 min, whereas Class III protein synthesis starts at 14 min and continues until lysis. Class I proteins function in the shutoff of transcription and translation of the host genome and the injection of the remaining 92% of the phage genome into the host cell. During the period of synthesis of Class II components, the proteins necessary for replication are made. The replication of the phage genome, the synthesis of coat proteins, and the buildup of infective phage occur during the third period. As found by Sirbasku and Buchanan (1, 13) and Moyer and Buchanan (9), at least three classes of RNA are sequentially formed during transcription of the phage genome. Each period of RNA synthesis is followed closely by a period of protein synthesis. Furthermore, proteins formed in one period may be needed for synthesis of RNA in a succeeding period (1, 9, 13).

The relationship between the synthesis of DNA and the late components has been studied most extensively with T-even phages. Snyder and Geiduschek (14) and Lembach, Kuninaka, and Buchanan (15), with T4, and Sekiguchi and Cohen (16), with T6, found that newly synthesized DNA or the process of DNA synthesis is a requirement for the transcription of late genes. It also has been reported that continuous synthesis of DNA is, under certain conditions, a necessity for extended late RNA synthesis (14, 15). Since T5 and T4 DNA differ markedly in composition of nucleotide bases and in physical structure (17), we have determined whether DNA replication is a requirement for formation of Class III RNA and the late proteins of the T5 system.

EXPERIMENTAL PROCEDURE

Bacteria, Bacteriophage, and Media—Escherichia coli F and bacteriophage T5st(0), a temperature-resistant wild type T5, were those used by Sirbasku and Buchanan (13). E. coli F sup+ was kindly supplied by Dr. D. J. McCorquodale and bacteriophage T5 am I (DNA polymerase-) by Dr. Y. T. Lanni. The phages were grown and purified by a slight modification of the procedure described by Adams (18). The growth medium used was the Tris-maleate medium prepared as described by Oleson, Pispa and Buchanan (19), with the exception that Casamino acids were omitted and the concentration of glucose was reduced to 0.5%. Cells were suspended in a modified glycerol medium of Fraser and Jerrel (20) from which all carbon sources were omitted. Prior to infection the phages were suspended in Tris-maleate buffer containing 0.11 M Tris-0.05 M maleate-0.01% gelatin-0.002 M CaCl2, pH 7.2.

Growth and Infection of Cells—The growth of cells and the phage infection were performed as described by Sirbasku and...
Buchanan (13). The cells at a concentration of 5 x 10⁹ per ml were infected at a multiplicity of 10 by addition of an equal volume of phage. The phage were adsorbed to starved host cells first at 0° for 2 to 3 min and then at 37° for 10 min (13). Starvation of the cells prevents protein synthesis and hence full transfer of DNA. RNA synthesis was initiated in infected cells at 37° as previously described (13).

**Pulse Labeling of RNA**—The preparation of ¹⁴C pulse-labeled RNA for gel electrophoresis and ³²P pulse-labeled RNA for DNA-RNA hybridization analysis was described by Sirbasku and Buchanan (13). Both ²⁻¹⁴C-uracil and ⁸⁻¹⁴C-adenine (50 μCi per μmole each) were used for ¹⁴C-RNA. ⁸⁻³²P-Adenine (1.2 mCi per μmole) was used for ³²P-RNA.

**Preparation of RNA**—RNA to be analyzed by disc electrophoresis was prepared by the phenol-cresol procedure (21), and that used for DNA-RNA hybridization by phenol extraction at 60° (22).

**Other Methods**—Lysozyme activity in crude extracts of infected cells was assayed by the method of Sekiguchi and Cohen (16). The kinetics of synthesis of both RNA and DNA were followed by incorporation of ⁸⁻¹⁴C-adenine as described by Sirbasku and Buchanan (13). The initial concentration of labeled adenine in the incubation mixture was 0.20 μm of specific activity 0.4 μCi per μmole. Labeled nucleic acids were precipitated from acid solution, subjected to a washing procedure to remove radioactive precursors, and treated with alkali. The radioactivity in the acid-insoluble, alkali-stable fraction and in the alkali-labile fraction is a measure of the synthesis of DNA and RNA, respectively. DNA-RNA hybridization was performed by the method of Gillespie and Spiegelman (23). The DNA from T5 was prepared by the method of Kaiser and Hogness (24). The hybridization buffer contained 0.03 M sodium citrate-0.3 M sodium chloride-0.1% sodium dodecyl sulfate, pH 7.1. Electrophoresis of RNA was performed as described by Sirbasku and Buchanan (13). The Packard Tri-Carb liquid scintillation counter was used for the determination of radioactivity.

**Chemicals**—⁸⁻³²P-Adenine (22 Ci per mmole), ⁸⁻¹⁴C-adenine (50 mCi per mmole), and ²⁻¹⁴C-uracil (50 mCi per mmole) were purchased from Schwarz BioResearch. Phenol and cresol were redistilled and stored at -20° before use. Sodium dodecyl sulfate was purchased from Fisher, acrylamide and N,N-methylacrylamide from Eastman, and egg white lysozyme and deoxyribonuclease from Worthington. Chloramphenicol was a gift of Parke-Davis. Nitrocellulose filters (grade B6, 24 mm) were purchased from Carl Schleicher and Schuell Company.

**RESULTS**

The experiments designed to study the relationship between DNA replication and synthesis of late (Class III) RNA in E. coli F cells infected with bacteriophage T5st(0) were carried out in two parts. First, chloramphenicol was used to prevent DNA synthesis, and RNA synthesis was followed at different stages of infection. Secondly, a comparison has been made of RNA and DNA synthesized during infection of E. coli F with T5st(0) and during infection of both the permissive and nonpermissive host cells with a DNA polymerase negative amber mutant of T5. In addition, we have studied the appearance of lysozyme, an enzyme known to be a late protein in other bacteriophage systems (25).

**Effect of Chloramphenicol on Synthesis of DNA in Bacteriophage T5 Infection**—Several investigators have shown that in some cases, in particular with phage T5, the continuous synthesis of phage DNA requires sustained protein synthesis in the phage-infected cell (26-30). We have confirmed the experiments of Pfefferkorn and Amos (26) and of Crawford (28) showing that the addition of chloramphenicol to T5-infected cells either prevents or stops DNA synthesis within a short time. As shown in Fig. 1 we have followed the incorporation of ¹⁴C-adenine (added at zero time) into DNA in a T5-infected culture to which chloramphenicol had been added at the indicated times. There is a complete block in adenine incorporation into DNA when chloramphenicol in a concentration of 100 μg per ml was added at 8 min after infection. When chloramphenicol was added later than 8 min, there was a gradual decrease in incorporation of ¹⁴C-adenine into DNA depending upon the time that chloramphenicol was added. The time between the addition of chloramphenicol and the cessation of incorporation of ³²P-adenine into DNA probably reflects completion of replication, since it has been shown that protein synthesis is required for the initiation of DNA replication (31, 32).

**Analysis of RNA Pulse-labeled in Presence of Chloramphenicol by DNA-RNA Hybridization Technique**—Since it has been shown that RNA synthesis in contrast to DNA synthesis is not affected by addition of chloramphenicol to T5-infected cells (1, 9, 13), we have studied the type of RNA synthesized after addition of chloramphenicol by the DNA-RNA hybridization-competition technique.

The main purpose of these experiments was to correlate, if possible, the time of appearance of Class III RNA and the beginning of phage DNA synthesis. As has been previously noted,
three classes of T5-specific RNA may be defined by the time of their appearance. Starting from the time that phage-cell complexes are diluted into aerated medium, Class I RNA is synthesized from 0 to 4 min after which its synthesis declines rapidly. The syntheses of Class II and Class III RNA start approximately at 4 min and 9 min, respectively, and continue up to lysis. DNA synthesis begins at about 8 min.

A comparison was made of the type of T5-specific RNA synthesized between 24 to 28 min after infection with T5st(0) when chloramphenicol was added at 3, 8, and 15 min after infection. The unlabeled, competitor RNA prepared from cells infected for 6 min without chloramphenicol addition has been reported to contain Class I and Class II RNA (13). The 6-min RNA was completely competitive with labeled RNA formed between 24 and 28 min in the presence of chloramphenicol added at 3 min. RNA pulse-labeled at 24 to 28 min after addition of chloramphenicol at 8 min contained a small amount of sequences not found in the 6-min competitor RNA. With RNA synthesized between 24 to 28 min after addition of chloramphenicol at 15 min, the competition was clearly incomplete, an indication that this labeled RNA contains new species of RNA, in all probability Class III RNA.

In order to establish the presence of Class III RNA in this sample, the labeled RNA was placed in competition with either 6-min unlabeled RNA (Class I and Class II) or with RNA obtained 36 min after a normal T5st(0) infection (36-min RNA), which contains all three classes (Fig. 2B). In confirmation of the experiment reported in Fig. 2A, 6-min RNA is only partially competitive whereas 36-min RNA is completely competitive with the labeled RNA. Hence RNA labeled between 24 to 28 min in the presence of chloramphenicol added at 15 min contains at least Class II and Class III RNA. Thus, when DNA synthesis is allowed to start at 8 min and chloramphenicol is added at 15 min, Class III RNA is synthesized approximately in normal quantities.

To return to the question of whether RNA labeled between 24 and 28 min in the presence of chloramphenicol added at 8 min contains Class III RNA, a mixed competition experiment was performed with 6-min unlabeled RNA and unlabeled competitor RNA extracted from T5st(0)-infected cells at 36 min. One can observe that the addition of unlabeled 36-min RNA to 6-min unlabeled RNA results in loss of further 3H-RNA remaining in hybrid form (Fig. 3). However, since the final points of both curves fall closely together and since the competition is so nearly complete in either case, one cannot state definitively that this labeled RNA contains sequences of Class III RNA. The increased competition seen upon addition of 36-min unlabeled RNA could be explained by the relatively larger accumulation of the more infrequent species of Class II RNA at the late stage of infection.

In order to study more rigorously the possibility that Class III RNA is made without DNA synthesis, i.e. when chloramphenicol is added at 8 min, the following experiment was performed. The RNA pulse labeled between 28 and 32 min after normal T5st(0) infection was hybridized with T5 DNA. Competition was performed with unlabeled RNA extracted from T5st(0)-infected cells after 28 min of infection when chloramphenicol was added at 3, 6, and 8 min. Normal 36-min unlabeled RNA served as a competitor in a control vessel (Fig. 4). The normal 36-min unlabeled RNA containing Class I, II, and III RNA exhibits full competition with this labeled RNA. The unlabeled RNA extracted at 28 min when chloramphenicol is added at 8 min shows slower competition, i.e. higher ratios of unlabeled to labeled RNA are required to reduce the base-line of radioactivity retained in hybrid form. However, with excess of unlabeled RNA full competition can be achieved. When unlabeled competitor RNA was prepared from cells treated with chloramphenicol at either 6 or 3 min, a great part of labeled RNA (30 to 50%) still remained in hybrid form at the highest ratio of unlabeled to labeled RNA. These results indicate that the synthesis of Class III RNA starts between 6 and 8 min after infection. They suggest, furthermore, that DNA synthesis is not required at least for a small amount of Class III RNA synthesis. However, as will
be shown in a later section, the quantity of Class III RNA synthesis is affected by DNA replication.

Polyacrylamide Gel Electrophoresis of T5-specific RNA Labeled in Presence of Chloramphenicol—In previous experiments we have shown that, in spite of the lack of DNA synthesis, Class III RNA synthesis can be detected in T5st(0)-infected cells by the hybridization-competition technique. In the following experiment we have analyzed by polyacrylamide electrophoresis the RNA obtained when chloramphenicol was added at 8 or 15 min after infection. The samples were pulse-labeled with 14C-

chloramphenicol was added at 8 min (A) and at 15 min (B) after infection. The number below each gel strip shows the time of initiation of a 4-min pulse labeling period. The band designations are those previously described (13).
time in cultures treated at 8 min or 15 min (Fig. 5, A and B). Thus, the addition of chloramphenicol before (at 8 min) or after (at 15 min) the beginning of DNA synthesis does not qualitatively change the pattern of RNA species shown by the autoradiogram. The time of appearance of Class III is between 8 and 12 min and is in good agreement with results published by Sirbasku and Buchanan (13) for a normal infection in the absence of inhibitors.

DNA and Class III RNA Synthesis in Permissive and Nonpermissive Hosts Infected with Bacteriophage T5 am I (DNA Polymerase)—The experiments presented above suggest that DNA synthesis is not required for the synthesis of Class III RNA in T5 infection. However, since it is not yet clear that protein synthesis is the only metabolic target of chloramphenicol in the T5 system, one might argue that the results obtained by this approach might reflect an imbalanced regulation of transcription with the aberrant formation of Class III RNA in the presence of this antibiotic. For this reason we decided to compare the synthesis of Class III RNA when DNA synthesis was controlled by use of a mutant of T5 (T5 am I), which can undergo a normal infective cycle in the permissive host E. coli F sup+ but fails to induce a DNA polymerase in the nonpermissive host, E. coli F.

A comparison has been made of the labeling of RNA and DNA from its precursors in the permissive and nonpermissive host infected with T5 am I (Fig. 6). The synthesis of RNA in the permissive host continues up to 48 min without any marked change. The situation is different in the nonpermissive host where RNA synthesis breaks sharply after 12 min. Although in these experiments the incorporation of 14C-precursors was cumulative starting with the beginning of infection, the same phenomenon was observed when RNA was pulse-labeled at certain intervals after infection (the results are not presented here).

The synthesis of DNA in the permissive host starts between 8 and 12 min and continues without significant change up to 48 min. In the nonpermissive host there is no significant incorporation of 14C-adenine into DNA during 48 min of infection. Since the inhibition of DNA synthesis in E. coli F infected with T5 am I was complete, we are assured that the mutation is not “leaky” and that the results of analysis of classes of RNA obtained in the nonpermissive host will be truly represented by the conditions of the experiment stated.

Analysis of Pulse-labeled Bacteriophage T5 am I in Permissive and Nonpermissive Hosts by DNA-RNA Hybridization Technique—In order to determine whether Class III RNA synthesis and DNA replication are linked in the T5 system, RNA was pulse-labeled between 12 and 16 min after infection of the permissive and nonpermissive host with T5 am I and was analyzed by the technique of DNA-RNA hybridization-competition (Fig. 7). Unlabeled 6-min RNA was only partially competitive with 12- to 16-min labeled RNA obtained from the permissive host (Fig. 7A). When 6 min unlabeled RNA was mixed with 36-min unlabeled RNA, the competition was complete. Class III RNA is therefore made in the permissive host between 12 and 16 min.

![Fig. 6.](image)

**Fig. 6.** RNA and DNA synthesis in *Escherichia coli* F and *Escherichia coli* F sup+ infected by bacteriophage T5 am I (DNA polymerase). The incorporation of radioactive adenine into RNA and DNA was measured by a procedure previously described (13). The following symbols indicate: ○—○, RNA synthesis in permissive host (*E. coli* F sup+); ●—●, RNA synthesis in nonpermissive host (*E. coli* F); Δ—Δ, DNA synthesis in permissive host; ■—■, DNA synthesis in nonpermissive host.

![Fig. 7.](image)

**Fig. 7.** DNA-RNA hybridization-competition analysis of RNA pulse labeled at two different time periods in the permissive and nonpermissive host after infection with T5 am I. The hybridization mixture contained, in 1 ml, 5 μg of T5 DNA bound to a nitrocellulose membrane, 11 μg of pulse labeled RNA, and unlabeled RNA as indicated. A, the competition of 12 to 16 min pulse labeled RNA extracted from the permissive host by unlabeled 6-min RNA prepared from T5st(--) infected cells (●—●) and by mixed competitor containing a fixed amount of 6-min RNA (0.34 mg) and variable amounts of 36-min RNA from T5st(--) infection (Δ—Δ). B, the same experiment described in A with the exception that the 12- to 16-min labeled RNA was prepared after infection of the nonpermissive host. C, the competition of 28- to 32-min pulse-labeled RNA extracted from the permissive host by the same two types of competitor described under A. D, the competition of 28- and 32-min pulse-labeled RNA prepared from the nonpermissive host by the two types of competitor described under A.
When a comparable experiment was carried out with the nonpermissive host, it was not clear whether the difference between two curves at the plateau level of radioactivity remaining in hybrid form (unlabeled 6-min versus 6-min plus 36-min RNA) was of quantitative significance (Fig. 7B). In any event the amount of Class III RNA formed during the 12- to 16-min time period would be small. Therefore the relative amount of Class III RNA made at a later time period in the infective cycle was analyzed. Both the permissive and nonpermissive strains of E. coli F were infected with phage T5 am I. The RNA was pulse-labeled with H-adenine between 28 and 32 min and was analyzed by the hybridization-competition technique. In Fig. 7C one can see that unlabeled 6-min RNA (Class I and Class II) is a poor competitor for pulse-labeled RNA obtained from the permissive host. When 36-min RNA (Class III in addition to Class II RNA) was mixed with 6-min RNA, the amount of labeled RNA retained in hybrid form decreases rapidly. In Fig. 7D a similar hybridization-competition is presented with T5 am I RNA labeled between 28 and 32 min in the nonpermissive host. As one can see, the competition is greater with the 36 min RNA than with 6 min RNA. These results suggest that sequences of Class III RNA are synthesized not only in the permissive host but also in the nonpermissive host as late as 32 min after infection, and that, in fact, the relative amount of this Class of RNA increases in each instance at the later time period. However, at both time periods the relative amount of Class III RNA synthesized in the nonpermissive host is substantially less than that formed in the permissive host. Thus, although DNA synthesis is linked quite probably to the formation of Class III RNA in minimal quantities, it is probably not required for the production of this class of RNA in minimal amounts.

Since the relative amount of Class III RNA formed in the nonpermissive host infected with T5 am I is so small, we next attempted to determine whether all species of Class III RNA were present in a sample isolated from this source. In this case the manner of labeling and competition with unlabeled RNA were reversed. RNA pulse-labeled between 28 and 32 min in a normal T5st(0) infection of E. coli F was hybridized to T5 DNA in the presence of unlabeled RNA extracted either from the permissive or nonpermissive cells 32 min after infection with bacteriophage T5 am I (Fig. 8). Both preparations of unlabeled competitor from T5 am I-infected cells compete completely with the normal labeled late RNA from T5-infected cells. This experiment supports the belief that all species of Class III RNA are formed in the nonpermissive host. Although the amount of Class III RNA formed during a pulse period between 12 and 16 min is relatively small (Fig. 7B), the results of the experiment shown in Fig. 8 indicate that after 32 min a substantial amount of this type of RNA has accumulated.

Activity of "Lysozyme" in T5st(0)-infected E. coli F and in T5 am I-infected Nonpermissive and Permissive Hosts—It is well known that in the case of bacteriophage T2 and T4, lysozyme is a representative of the late proteins and that DNA synthesis is required for its production (25). Comparable information is not available for lysozyme induced in E. coli F infected with T5st(0). However, from the kinetics of its appearance during the infective cycle one may conclude that lysozyme is representative of a Class III protein. McCorquodale and Buchanan (10) have shown that the appearance of several of the early enzyme activities occurs during the period of Class II protein synthesis starting at 5 min. Class III protein synthesis is known to commence at 12 to 14 min. As shown in Fig. 9 lysozyme activity first appears at 12 min and therefore may be classified as a Class III function.

With this information in mind we then tested for the appearance of this activity in the permissive and nonpermissive hosts infected with T5 am I in order to determine whether DNA synthesis is a prerequisite for lysozyme synthesis (Fig. 9). It may be seen that activity appears in either instance at approximately 12 min and that DNA synthesis, which does not occur in the nonpermissive host, is not required for the synthesis of this Class III protein. This evidence thus supports our conclusions drawn from other experiments that in the case of T5 the production of Class III RNA as viewed in its qualitative aspect is not dependent on DNA synthesis.

![Fig. 8. DNA-RNA hybridization-competition analysis of "late" pulse-labeled T5st(0) RNA with unlabeled T5 am I RNA extracted from permissive and nonpermissive host at 32 min after infection. Pulse labeling was performed between 32 and 36 min. The hybridization mixture contained in 1 ml 5 µg of T5 DNA bound to nitrocellulose membrane, 5 µg of labeled T5st(0) RNA, and, in the quantity indicated, unlabeled T5 am I RNA extracted at 32 min from the permissive (O--O) and nonpermissive host (●—●).](http://www.jbc.org/)

![Fig. 9. The course of appearance of lysozyme activity after bacteriophage infection. The assay was carried out as described under "Experimental Procedure." ●—●, activity in extracts from T5st(0)-infected E. coli F; ▲—▲, activity in extracts from T5 am I-infected E. coli F sup+; ○—○, activity in extracts from T5 am I-infected E. coli F.](http://www.jbc.org/)
Our results show that the replication of DNA is not a prerequisite for the transcription of Class III RNA in T5 infection. However, the relative amount of this RNA synthesized under conditions that do not allow replication of the T5 genome is quite small. When DNA replication was permitted to take place even for a few minutes before addition of chloramphenicol to inhibit DNA synthesis, the rate of Class III RNA production was maintained at the higher control level. This phenomenon could be explained by an increase in the relative amount of the late genes available for the transcription and hence for formation of Class III RNA or by the increase early in the third period of a protein or proteins necessary for Class III transcription, for example, a new initiation factor for RNA polymerase. As mentioned previously, DNA synthesis is required for formation of late RNA in the normal T4 infection (14-16). However, of particular interest is the report by Riva, Casino, and Geiduscheck (33) that transcription of the late genes can be uncoupled from replication under certain conditions in T4 infection. They postulate that newly replicated segments of T4 DNA are transcribed by RNA polymerase for synthesis of late RNA but become unavailable as templates once they are assembled into longer units by action of an induced ligase. Parental DNA may also be made competent for transcription of late genes by selective action of nuclease.

The relationship of DNA synthesis to formation of late proteins in T5-infected cells has been examined by us and by Dr. D. J. McCorquodale and his colleagues. They have compared by the technique of disc electrophoresis the synthesis of Class III proteins in a permissive and nonpermissive host after infection with an amber mutant of T5 with a mutation in the gene for DNA polymerase. As far as they could determine all late proteins were synthesized in both cases.

In our experiments we have compared the formation of lysozyme, a presumed representative of the Class III proteins. The rate of synthesis of lysozyme was so nearly the same in the presence of 0.2 M hydroxyurea. We conclude therefore that the drug at this concentration does not specifically affect DNA synthesis but is generally toxic to cellular metabolism.

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