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Ribonucleotides Covalently Linked to Deoxyribonucleic Acid in T4 Bacteriophage

J. F. SPEYER, J. CHAO, AND L. CHAO
Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268

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Bacteriophage T4 was grown in the presence of labeled uridine. The deoxyribonucleic acid (DNA) of the phage was shown to contain covalently attached ribonucleotides. The label appears not to be internal in the DNA strands. Presumably, it is at the ends of the DNA strands and this may be related to DNA initiation.

Bacteriophages contain either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA): no exceptions are known but for this report. The same rule applies to other viruses, except that some of the tumor RNA viruses have been shown to contain some DNA as well (24, 29, 31). There are two reasons that one might expect some RNA in T4 DNA. We looked, initially, for RNA in T4 because we had observed an inhibition by extracts of T4 phage of in vitro protein synthesis similar to that which we found with polyinosinic·polycytidylic acid [poly(I)·poly(C)] (10). Also, T4 phage can induce interferon (20), which is characteristic of double-stranded RNA (12, 40).

We now favor another reason. DNA synthesis may be initiated by RNA, and some of the hypothetical RNA may remain on the DNA in the T4 phage. This idea about the initiation of DNA synthesis is relatively new but considerable evidence supports it. As mentioned above, some of the RNA viruses also contain a small amount of DNA, some of which appears to be covalently linked to the RNA (24). In addition to their mixed nucleic acids, these viruses contain a "reverse transcriptase"—RNA-dependent DNA polymerase (3, 38). Verma et al. (41) showed that the in vitro product of the reverse transcriptase is DNA covalently linked to RNA. Thus, in this kind of DNA synthesis, RNA acts as template and also as primer, which may explain the mixed nucleic acid content of these viruses. The above authors suggest that in DNA replication, too, the problem of initiating DNA strands may be solved by RNA primers. Initiation is a problem in DNA replication, as none of the known DNA polymerases seem to be able to initiate new chains of DNA (17); this contrasts to RNA polymerase, which does initiate new chains (6, 25). This problem has prompted several groups to see whether DNA replication in vivo is sensitive to an inhibition of RNA synthesis, either by rifampin or streptolydigen, in experiments where DNA replication does not require protein synthesis, which usually depends on messenger RNA (mRNA) synthesis. Brutlag et al. (7) reported that the doubling of single-stranded DNA of phage M13 upon infection of *Escherichia coli* is prevented by inhibition of RNA, but not protein, synthesis. This is not the case with another single-stranded DNA phage, ϕX174 (32). Nevertheless, this DNA, too, is initiated by RNA (*personal communication*, from A. Kornberg, Stanford University). In the replication of the *E. coli* chromosome, there is a time period following a cycle of amino acid starvation when, as was shown by Lark (22), replication is sensitive to an inhibition of RNA, but not protein, synthesis. The replication of extrachromosomal DNA such as plasmids and episomes in *E. coli*, too, is sensitive to even low levels of rifampin (4, 11, 19, 30). Evidently, the replication of this DNA requires transcription. Dove et al. (14) have reached the same conclusion about the in vivo replication of λ phage DNA. In vitro experiments with DNA polymerases known to need both primer and template have shown that RNA as well as DNA can serve both functions (9, 23, 42). Recently, Wickner et al. showed (44) that a soluble enzyme fraction from *E. coli* cells converts single-stranded M13 phage DNA to the double-stranded replicative form. This in vitro conversion is blocked by rifampin. This conversion required not only deoxynucleotide, but also ribonucleotide, triphosphates, and they found a 5′ → 3′ phosphodiester linkage between a deoxynucleotide

and a ribonucleotide in the product. This strongly supports their model of RNA-primed DNA synthesis. Other strong support for this model is the report by D. G. Blair et al. (Fed. Proc. **31**:442, 1972) that the covalently closed circular DNA of an *E. coli Col* factor is sensitive to alkali and ribonucleases, suggesting that there is an RNA link in this structure. The work reported here—RNA covalently linked to T4 DNA—fits the structure expected from the above model for RNA-primed DNA replication, although there is no direct evidence yet that RNA primes or initiates T4 DNA replication.

MATERIALS AND METHODS

Materials. Pancreatic deoxyribonuclease (ribonuclease-free) was purchased from Worthington Biochemical Corp. Pancreatic ribonuclease, T1 ribonuclease, and Pronase (nuclease-free) were purchased from Calbiochem; [5-^3H]uridine (26 Ci/mmole), [CH_3-^{3}H]thymidine (6 Ci/mmole), and [2-^{14}C]thymidine (50 mCi/mmole) were from New England Nuclear Corp. All glassware used was baked at 250 C overnight.

Preparation of phage. A 250-ml culture of *E. coli* B was grown with shaking in M9S (8) in a 2-liter flask at 37 C. At a density of 10^9 cells/ml, a fivefold excess of T4 phage were added. At 5 min after the phage addition, 2 mCi of [5-^3H]uridine (0.1 μmole) was added, and 3 hr later the lysis of the cells was completed by adding 10 ml of $CHCl_3$. Pancreatic deoxyribonuclease, 200 μg (not ribonuclease-free), was added, and the lysate was incubated for 2 hr at 37 C. Cell debris was removed by 10-min centrifugation at 9,000 $\times$ *g*, and the phage were sedimented at 9,000 $\times$ *g* for 3 hr. The phage pellet was resuspended overnight in 50 ml of 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.8) $-$0.15 M LiCl (suspension buffer). The low- and high-speed centrifugations were repeated twice, and the phage were resuspended in 5 ml of the above buffer. The initial phage titer was 10^{11} phage per ml, and the recovery was over 60% through these steps. The homogeneously suspended phage (3-ml portions) were layered on three 50-ml 5 to 30% sucrose gradients containing 0.02 M Tris-hydrochloride (*p*H 7.8) $-$0.15 M LiCl, and were centrifuged for 30 min at 24,000 rev/min in a Spinco SW25.2 rotor at 5 C. Fractions containing 60 drops were collected from the top of the gradient, and the absorbance at 260 nm was measured. The phage peak fractions were combined, dialyzed against the suspension buffer, and sedimented at 17,000 $\times$ *g* for 1 hr. The phage pellet was then resuspended in the above buffer.

Sucrose gradient analysis of T4 DNA. Native T4 DNA was prepared by incubating ^{3}H-uridine-labeled T4 phage with 1% LiDS (lithium dodecyl sulfate) for 3 min at 63 C. The mixture was chilled on ice and was then layered directly on 11 ml of a 5 to 30% sucrose gradient containing 0.02 M Tris-hydrochloride (*p*H 7.8) $-$0.15 M LiCl and centrifuged for 3.5 hr at 35,000 rev/min. Fractions of 30 drops were collected, and the absorbance and acid-insoluble radioactivity were measured. Denatured T4 DNA was prepared by the same procedures as for native DNA except that T4 phage were heated to 100 C for 10 min. Samples were used to determine ribonuclease sensitivity, and acid-insoluble counts were measured.

Thin-layer chromatography of nucleosides. Thin-layer cellulose plates with fluorescent indicator (Eastman Kodak) were used for ascending chromatography. Two solvent systems were used. Solvent 5 contained anhydrous ethanol-*t*-butanol-88% formic acid-water (60:20:5:15). Solvent 11 contained *n*-butanol-formic acid-water (77:10:13).

Isopycnic centrifugation of denatured T4 DNA. Phenol-extracted DNA from [5-^3H]uridine-labeled phage was heated for 10 or 20 min at 100 C in 0.01 M Tris-hydrochloride (*p*H 7.8)$-$0.001 M ethylenediaminetetraacetic acid and chilled in an ice-salt mixture. Solid Cs_2SO_4 was added to this solution to 1.72 molality. Amounts of 10 ml were placed in centrifuge tubes with paraffin oil to fill the tubes. The tubes were centrifuged at 5 C in a Spinco SW36 rotor at 30,000 rev/min for 67 hr. Fractions of 30 drops each were collected from the bottom of the tubes. [5-^3H]uridine-labeled f-2 RNA was used as a marker. Samples were used to determine ribonuclease sensitivity, and acid-insoluble counts were measured.

Acid-insoluble radioactivity. Radioactive materials were precipitated with 5% trichloroacetic acid from solution. The solution was then filtered through 24-mm Whatman GFA glass-fiber discs. These were washed three times each with 5% trichloroacetic acid, water, and 95% ethanol, dried, and counted with a toluene scintillator fluid in a Packard scintillation counter.

RESULTS

T4 phage labeling by [5-^{3}H]uridine. The composition of T4 DNA aids a search for RNA in T4 phage. There is no known path by which the radioactive label of [5-^3H]uridine can be incorporated into T4 (or T2 or T6) DNA. The reason is that in such phage DNA the pyrimidines, for which uridine can be a precursor, are thymine and glucosylated hydroxymethyl-cytosine—both substituted at the 5 position. Thus, the label from [5-^3H]-uridine is lost from both and should not enter such DNA. This is not true for the cytosine-containing DNA from other sources.

Phage were grown as described and purified by three cycles of centrifugation. The phage were then centrifuged on a sucrose gradient (Fig. 1). The absorbance, plaque-forming units, and radioactivity all coincided. Subsequent experiments were performed on such sucrose gradient-purified phage. The phage were then banded in isopycnic centrifugation either on step gradients or on those forming in 48 hr at 30,000 rev/min in a Spinco SW36 rotor at 5 C in CsCl at an initial density of 1.5. In either case, the radioactivity,

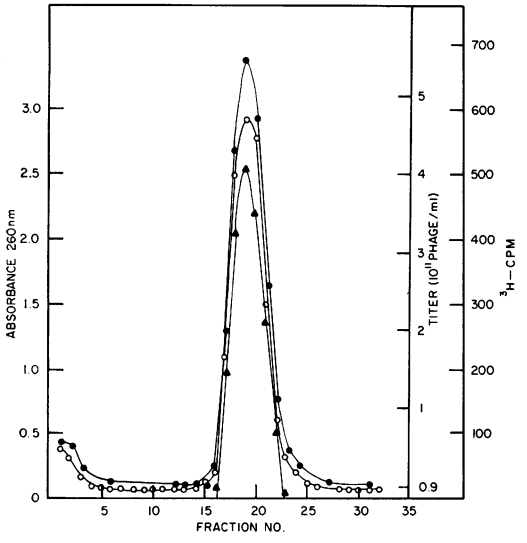

FIG. 1. *Sucrose gradient centrifugation of T4 phage. A 3-ml amount of phage on 50 ml of a 5 to 30% sucrose gradient was centrifuged at 24,000 rev/min for 30 min at 5 C. Fractions of 60 drops each were collected from the top of the gradient. Acid-insoluble radioactivity was determined on 0.1-ml samples. Symbols:* ○, *absorbance at 260 nm;* ●, *^{3}H counts per minute;* ▲, *phage titer.*

plaque-forming units, and absorbance profile coincided similarly to Fig. 1. These results show no sign of any impurity and suggest that the radioactivity is associated only with the phage. However, the total quantity of label incorporated was very small. The phage had 1,400 counts per min per A_{260} (~10″) from a lysate that was grown in the presence of 10 μCi of uridine per ml. We estimate that an equivalent quantity of labeled thymidine would have produced phage more than 1,000 times as radioactive. It is thus important to show that the phage radioactivity is due to uridine in RNA and not due to an unknown radioactivity impurity that is incorporated into the phage.

RNA content of the phage. No acid-insoluble radioactivity was lost from the uridine-labeled phage by treatment with pancreatic ribo- or deoxyribonuclease. Phage labeled by [^{3}H]thymidine were equally resistant. Presumably, the uridine-labeled material is inside the phage. To release the inside contents, the phage were heated to 100 C for 20 min. This treatment is known to denature the major protein of the phage coat (36). The DNA of the phage was released as judged by the increase in viscosity and the susceptibility to pancreatic deoxyribonuclease of the acid-precipitable radioactivity from [CH_3-^{3}H]thymidine-labeled phage.

Table 1 shows the nuclease, Pronase, and alkali susceptibility of uridine- or thymidine-labeled denatured phage. Only 30 to 40% of the uridine label was susceptible to alkali or to T1 or pancreatic ribonuclease, whereas about 60 to 70% was sensitive to pancreatic deoxyribonuclease. This suggests that over half of the label enters DNA. It may be that some of the cytidines in DNA are not hydroxymethylated, or the label is not completely at the 5 position. Alternatively, this label might be in single internal ribonucleotides in the DNA; subsequent experiments have made this an unlikely possibility. The uridine-labeled phage DNA was completely solubilized by combined digestion of deoxyribonuclease and ribonuclease. The thymidine label was susceptible only to deoxyribonuclease, as expected. Pronase did not release any acid-soluble counts in either case. None of the experiments described so far was restricted to T4; the same observations were made when T2 phage instead of T4 was used.

The alkali-sensitive moiety of the T4 appears to be RNA because it is due to labeled uridine and cytidine. This was shown by digesting labeled phage with 0.3 N KOH for 30 min at 100 C. The solution was acidified with 5% Cl_3CCOOH and filtered. The Cl_3CCOOH-soluble fraction was heated to 50 C for 15 min and was then filtered

TABLE 1. *Nuclease, Pronase, and alkali susceptibility of uridine- or thymidine-labeled denatured T4 phage*[a]

Addition	^{3}H-uridine T4 phage		^{3}H-thymidine T4 phage	
	Counts/min	Loss of radioactivity (%)	Counts/min	Loss of radioactivity (%)
None	2,355	—	2,112	—
Deoxyribonuclease	860	64	53	98
Ribonuclease	1,512	36	2,209	0
Deoxyribonuclease + ribonuclease	22	99	—	—
Pronase	2,305	2	1,963	2
KOH	1,330	44	2,156	0

[a] The purified T4 phage was heated to 100 C for 30 min in 0.02 M Tris-hydrochloride (*p*H 7.8)-0.15 M LiCl and then chilled on ice. Deoxyribonuclease (100 μg/ml), pancreatic ribonuclease (20 μg/ml), or Pronase (100 μg/ml) was added and incubated for 1 hr at 30 C. Deoxyribonuclease was used in the presence of 0.001 M Mg^{2+}. Alkaline hydrolysis was carried out in 0.2 N KOH for 20 min at 100 C. Acid-insoluble radioactivity was determined.

through Norit A charcoal. The presumptive ribonucleotides were eluted from the charcoal with 50% ethanol in 1 N NH_4OH. The eluate was dried. It was then dissolved in 0.1 M Tris-hydrochloride (*p*H 8.5) and treated with 0.1 mg of *E. coli* alkaline phosphatase/ml for 2 hr at 37 C. The resulting material should contain nucleosides. This material was concentrated by flash evaporation and chromatographed as mentioned in Materials and Methods with two solvent systems. The only radioactivity recovered from the chromatography with either solvent corresponded to the two locations of cytidine and uridine, which were equally labeled. The recovery of radioactivity associated with only these two two ribonueleosides from the charcoal eluate indicates that no other alkali-labile nucleotides were present in the DNA. This, together with the solubilization of the same percentage of the total radioactivity from the DNA by either ribonuclease or alkali, leads to an inescapable conclusion: the radioactivity is indeed in uridine or cytidine and in ribonucleotide linkage, and is not due to some unknown radioactive impurity that might have been present in the [5-^3H]uridine that was used in the growth of the phage.

Association of the RNA with the DNA of T4. Uridine-labeled DNA was released from purified phage by 1% LiDS (63 C, 3 min). This native DNA was then centrifuged in a sucrose gradient. The result (Fig. 2) shows that the ribonuclease-sensitive uridine label parallels the absorbance profile. Thus, the RNA is attached to the DNA. Is this RNA attached to the DNA by covalent bonds or by hydrogen bonds? The latter possibility can be readily examined by denaturing the DNA. If the RNA is attached by hydrogen bonds, then it should separate from denatured DNA in isopycnic gradient centrifugation. The results in Fig. 3 show that most of the RNA, as measured by ribonuclease sensitivity of the uridine label, remained with the DNA. Thus, the RNA is probably covalently linked to the DNA. The ribonuclease sensitivity of this material also argues against attachment of the RNA to the DNA by hydrogen bonds, because such RNA-DNA hybrids are known to be resistant to ribonuclease (5). Only about 20% of the radioactivity in the experiment (Fig. 3) was ribonuclease-sensitive. We do not know why this was less than in Table 1. In identical experiments performed on T4 DNA isolated from infected cells, we found that most of the label (~70%) was ribonuclease-sensitive, and 90% of that banded at the density of denatured DNA with the remainder banding as RNA. It may be that some of the covalently linked RNA is split from the DNA during the heat denaturation. Sucrose gradient analysis of heat-denatured DNA from T4 phage gave variable results. In all experiments, the peak of DNA contained 30 to 50% ribo-

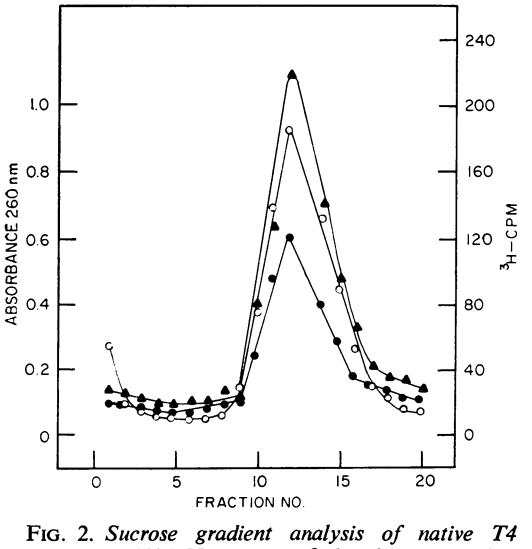

FIG. 2. *Sucrose gradient analysis of native T4 DNA. Native T4 DNA was purified and centrifuged as described. Samples from the gradient were used to determine ribonuclease sensitivity, and acid-insoluble counts were measured. Symbols: ○, absorbance at 260 nm; ▲, ^3H counts per minute of control; ●, ^3H counts per minute after 30 min of incubation with 20 μg of ribonuclease/ml at 37 C.*

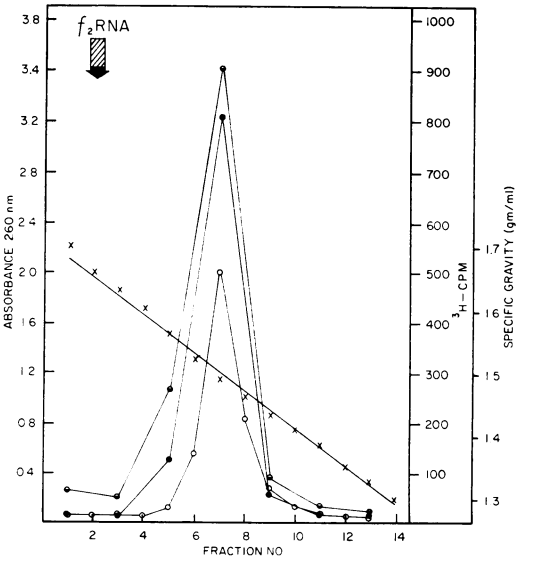

FIG. 3. *Ribonuclease sensitivity of denatured T4 DNA after isopycnic centrifugation in Cs_2SO_4. Symbols: ○, absorbance at 260 nm; ×, specific gravity; ◒, ^3H counts per minute of control; ● ^3H counts per minute after 30 min of incubation with 20 μg of ribonuclease/ml at 37 C.*

nuclease-sensitive label; however, there was a variable amount of ribonuclease-sensitive material near the top of the gradient. This may have been produced by breakage during the denaturation at 100 C for 10 to 20 min (*p*H 7.8).

We do not think that the RNA in T4 DNA is internal for the following reason. It is known that this DNA does not fracture in alkaline sucrose gradients (1). If it contained RNA inside the chain, it would break with alkali as was the case with the colicin E_1 supercoiled DNA (Blair et al., Fed. Proc. **31**:442, 1972). Also, ribonuclease treatment has no effect on the sedimentation of denatured T4 DNA. In this experiment, [*2-*^{14}*C*]-thymidine-labeled T4 DNA was first heat-denatured and then treated with 40 μg of ribonuclease/ml for 30 min at 37 C in 0.02 M Tris-hydrochloride (*p*H 7.8). This had no effect on the sedimentation behavior of T4 DNA in neutral sucrose gradient centrifugation. This experiment should detect a single internal ribonucleotide per DNA molecule. Since the experiment was negative, the RNA in T4 DNA probably is not internal. Presumably, the RNA is at the end of the DNA; however, we have not yet done any work on this question.

DISCUSSION

There is no clear picture of how the DNA of T4 phage is replicated. It is known that T4 phage codes for its own DNA polymerase (2). It is also known that the chief function of this polymerase is replication rather than repair or recombination (33). In addition, about a dozen other T4 gene products are also required for DNA replication; the function of only half of these is known (17). Nothing is known about the in vivo initiation of this DNA replication. There is evidence both for and against a preferred origin of replication (13, 26). The published models for the replication of T4 DNA predate, and thus do not include, current thoughts about initiation by RNA. Some of these models propose that concatemers of T4 DNA are produced, perhaps by circularizing a parental strand (15, 43), and that a parental strand primes the DNA synthesis (16, 18). However, most workers have not found a covalent link between parental and progeny DNA (15, 21, 43), though thymine incorporation into parental DNA has been reported (27). Circles of T4 DNA have not been observed in vivo. The sedimentation behavior of progeny DNA indicates that it is much larger, 400 and 200*S*, than mature DNA (63*S*) from phage particles (15). It is not clear how this large material is generated, whether by replication or recombination, or if it truly represents super-sized concatemers. The mature phage DNA contains circularly permuted ends (34, 39), and it was proposed by Streisinger et al. (35) that these permutations are due to withdrawing "head full" portions of mature DNA from a larger (or longer) precursor. Okazaki et al. (28) have shown that small fragments of DNA are the first product of DNA synthesis in T4-infected and uninfected cells. They have recently shown that these precursor fragments, too, are initiated by RNA (37). Presumably, that RNA is not found in phage because we can detect no internal RNA in T4 phage DNA.

Irrespective of the details of these models, one might expect that, if RNA is a primer, this RNA initiation point would be removed before the DNA is packaged. Our finding of RNA in T4 phage is thus surprising, even if RNA were known to initiate T4 DNA synthesis. One might expect unpackaged T4 DNA to be a better source of such material. This may be true. In preliminary experiments, we found alkali-labile, ribonuclease-sensitive, acid-insoluble label in DNA from [*5-*^{3}*H*]uridine-labeled T4-infected cells. The labeled material sediments with the large progeny DNA (15) in sucrose gradients, has the chromatographic properties on hydroxyapatite that differentiate T4 DNA from RNA and *E. coli* DNA, and cosediments with denatured T4 DNA in isopycnic gradients. This material may be a better source of DNA-linked RNA than the mature phage DNA because its specific activity is greater. It may be that the RNA content of the mature DNA is fortuitous and not necessarily homogeneous in a DNA population. There is an indication of a slight difference between the absorbance profile and the uridine radioactivity in the isopycnic centrifugation shown in Fig. 3. This difference is much less than that of free RNA and denatured DNA, but it may indicate a population of DNA which is heterogeneous in RNA content. If only a small minority of the DNA of phage contain RNA, then the failure of ribonuclease to change the sedimentation behavior of denatured T4 DNA cannot be taken as evidence that the RNA is at the ends of the DNA and not in the middle. This consideration has to qualify our interpretation of that experiment.

We have looked in vain for a period in the in vivo replication of T4 DNA that is rifampin-sensitive and chloramphenicol-resistant. Our experiment was similar to that of Brutlag et al. (7) with M13 phage-infected cells, except that T4 was used. The T4 DNA synthesis and also T4 phage production become rifampin-resistant at the times, or a little sooner, when these processes become chloramphenicol-resistant. Since a dozen

T4 gene products are needed for DNA synthesis, all of which require mRNA synthesis, this experiment does not rule out a role of RNA in the initiation of T4 DNA replication. Thus, unfortunately, T4 does not lend itself to the M13 type of experiment. We can say that, if RNA plays this role in T4, it is stable, or its synthesis is not inhibited by rifampin.

It should be possible to design other in vivo and in vitro experiments to determine whether RNA initiates DNA replication. What we have found is a structure that may be the initiation point. Future work will try to find where this RNA is located and why it is there.

ACKNOWLEDGMENT

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