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In Vivo Production of an RNA–DNA Copolymer after Infection of *Escherichia coli* by Bacteriophage T4

(initiation/hybridization/DNA replication/chloramphenicol/density gradient)

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ABSTRACT **An RNA–DNA copolymer was isolated from *Escherichia coli* infected with bacteriophage T4. The RNA and DNA are covalently linked, and in the same polynucleotide strand. The DNA of the copolymer hybridizes specifically to the left strand of phage T4 DNA. The copolymer is produced in cells infected with amber mutants of phage T4 deficient in DNA replication and is not inhibited by the addition of chloramphenicol.**

Recent investigations by several groups of workers have suggested that RNA may play a direct role in the initiation of DNA replication. Chang and Bollum showed that polyribonucleotides could function as primers for the *in vitro* synthesis of DNA, catalyzed by certain mammalian DNA polymerases.* Brutlag, Schekman, and Kornberg found that the conversion of M13 DNA to the replicative form *in vivo* was sensitive to rifampicin (1). In partially purified, soluble extracts of *Escherichia coli*, this conversion was also sensitive to rifampicin and required ribonucleoside triphosphates.† They showed, further, that the newly synthesized DNA from this *in vitro* system contained covalently linked ribonucleotides. They suggested that RNA primes synthesis of complementary phage DNA in this system.

This paper describes the isolation and characterization of an RNA–DNA copolymer (copolymer) synthesized *in vivo* in *E. coli* B infected with bacteriophage T4. The copolymer, which is composed of RNA and DNA covalently linked in the same strand, contains phage-specific DNA that is complementary to the left (l)-strand of phage T4 DNA.

MATERIALS AND METHODS

E. coli strain B-23, bacteriophage T4 BO_1^r ("wild type"), TCG medium, density (BrdU) and isotope labeling, and CsCl and sucrose gradient analyses have been described (2). T4D amber mutants A453 (gene 32), B22 (gene 43), and N82 (gene 44) were isolated and characterized by Epstein *et al.* (3). Intracellular DNA was isolated and extracted by the sodium dodecyl sulfate–Pronase–phenol method (4). Cs_2SO_4 density gradients were prepared by addition of 1.15 ml of sample solution to 1.35 ml of saturated Cs_2SO_4 at 25° in water. All experiments were performed at 37° at a cell density of 3×10^8/ml.

The preparative separation of T4 DNA strands was accomplished as described by Guha and Szybalski (5). The l-strand is that strand from which immediate early and early T4 messenger RNAs are transcribed (6). Hybridization to DNA on nitrocellulose membranes was performed according to the method of Denhardt (7).

EXPERIMENTAL RESULTS

Discovery of a Phage T4 RNA–DNA Copolymer. During the course of experiments designed to investigate very early stages of T4 DNA replication, an unusual phenomenon was observed. *E. coli* B cells were infected with ^{32}P- and BrdU-labeled (heavy) phage T4. 3 min after infection, [^3H]dT was added. 5 min after infection the intracellular DNA was extracted and analyzed by CsCl density gradient centrifugation. The results are shown in Fig. 1. [^3H]dT-labeled material appeared near the bottom of the gradient, well separated from the parental (HH) location. Virtually all the newly incorporated [^3H]dT was found at this location in the gradient. Analysis of intracellular DNA from samples taken at later times after infection (6, 7, and 8 min) revealed a significant decrease in the proportion of [^3H]dT-labeled material near the bottom of the gradients, as if this substance were of a transient nature. Similar experiments, performed with nondensity-labeled phage T4 and addition of [^3H]BrdU instead of [^3H]dT gave an identical result: newly incorporated label was found near the bottom of the gradients shortly after infection but decreased at the time when replication of the parental phage DNA could be detected.

Sucrose gradient sedimentation (not documented here) of the intracellular DNA extracted from samples taken very early after infection, when nearly all the newly incorporated label appeared at the bottom of the CsCl gradients, showed that the [^3H]dT- or [^3H]BrdU-labeled material was composed of very small fragments of molecular weight about 1×10^6.

In the following experiments, we shall refer to the "dense" material, synthesized very soon after infection with phage T4 and isolated from the bottom of CsCl density gradients as the RNA–DNA copolymer (copolymer).

In order to determine whether the copolymer contained DNA or if the label had, somehow, become incorporated into some other substance, [^3H]dT-labeled copolymer was isolated from CsCl and incubated with DNase, RNase, and alkali. The results are shown in Table 1. These data clearly show that the [^3H]dT in the copolymer fraction does, indeed, reside in DNA, since it was completely sensitive to DNase and resistant to RNase and alkali.

Hybridization of RNA–DNA Copolymer to the Separated Strands of Phage T4 DNA. Since the copolymer did contain DNA, a question was raised about its origin. Was it complementary to phage DNA?

Abbreviations: Copolymer, RNA–DNA copolymer; l- and r-strands, left and right strands of T4 DNA, respectively; CM, chloramphenicol.

* Chang, L. M. & Bollum, F. J. (1972) *Fed. Proc.* **31**, 846 Abstr.

† Wickner, W., Brutlag, D. & Kornberg, A. (1972) *Fed. Proc.* **31**, 441 Abstr.

TABLE 1. *DNase, RNase, and alkali sensitivity of RNA–DNA copolymer*

Treatment	% Hydrolysis	
	^3H*	^{32}P†
DNase‡	92.0	91.9
RNase‡	8.8	0
Alkali§	0	—

* Copolymer labeled with [^{3}H]dT.

† ^{32}P-labeled DNA isolated from mature phage was added as an internal reference.

‡ RNase and DNase reaction mixtures contained (2.0 ml total volume): 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffer (pH 7.3); 5 mM $MgCl_2$; and [^{3}H]dT-labeled copolymer fraction. The RNase mixture contained 100 μg of pancreatic RNase A; the DNase mixture contained 100 μg of pancreatic DNase I. Incubations were for 60 min at 37°. Aliquots were precipitated with 0.3 M Cl_3CCOOH and counted.

§ Copolymer fraction was incubated with 1.0 M KOH for 18 hr at 37°, then precipitated with 0.3 M Cl_3CCOOH and counted.

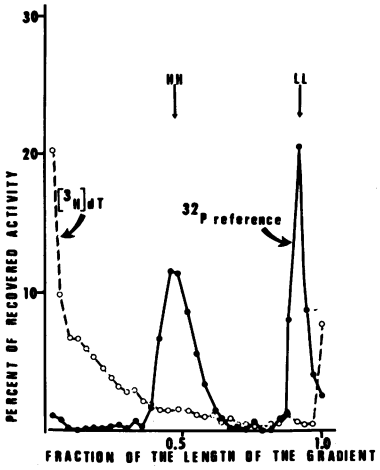

FIG. 1. Synthesis of the RNA–DNA copolymer in cells infected with phage T4. *E. coli* B-23 were infected with ^{32}P- and BrdU-labeled (heavy) T4 phage. The multiplicity of infection was 10. 3 min after infection, [^{3}H]dT was added to give a specific activity of 5 Ci/g. 5 min after infection, cells were chilled in 0.15 M NaCl–15 mM EDTA (pH 7.6). The intracellular DNA was extracted by the sodium dodecyl sulfate–Pronase–phenol method (3) and fractionated by CsCl density gradient centrifugation in the presence of ^{32}P-labeled (light) reference DNA from phage T4. Each fraction was precipitated with 0.3 M Cl_3CCOOH and counted. The *solid line* represents ^{32}P and the *dashed line* represents [^{3}H]dT. The position of the heavy parental DNA is indicated by the (HH) *arrow*, while the light reference position is indicated by the (LL) *arrow*. The bottom of the gradient is to the left. Note that nearly all the newly incorporated [^{3}H]dT is present in the copolymer fraction near the bottom of the gradient. Little, if any, of the ^{32}P-labeled parental DNA can be detected in the copolymer fraction.

The copolymer was isolated from CsCl density gradients, as described previously, and hybridized to the separated l- and right (r)-strands of phage T4 DNA. One sample was a portion of the [^{3}H]dT-labeled copolymer analyzed in Table 1. Another sample was obtained from a similar experiment in which *E. coli* cells infected with phage T4 were labeled from 4.5 to 5 min with [^{3}H]BrdU. In addition, for comparison of hybridization of the copolymer to that of newly replicated progeny phage DNA, [^{3}H]BrdU-labeled DNA, banding close to the location of parental phage DNA, was isolated from a CsCl density gradient of intracellular DNA obtained from this same experiment 9 min after infection.

The hybridization results are shown in Table 2. These data show that the copolymer not only contains DNA complementary to phage T4 DNA, but also that this DNA exhibits a considerable bias for the l-strand (Table 2). This same bias was evident whether the copolymer was labeled with [^{3}H]dT or with [^{3}H]BrdU. On the other hand, newly replicated progeny phage DNA, isolated from near the parental location in the gradient, was complementary to both strands of phage T4 DNA, confirming the previous observations of Kozinski (8) (Table 2).

Physical Properties of the RNA–DNA Copolymer. Previous experiments involved the isolation of the copolymer from near the bottom of CsCl density gradients. RNA is characteristically found at this location in such gradients. This suggested that the copolymer might somehow be associated with RNA, which would account for its density in CsCl.

The banding characteristics of the copolymer were compared with those of RNA, by analysis in Cs_2SO_4 density gradients sufficiently dense to band both RNA and DNA as distinct peaks. [^{3}H]dT-labeled copolymer was isolated from cells infected with phage T4 by CsCl density gradient centrifugation. Reference DNA from ^{32}P-labeled phage T4 was mixed with the sample and the mixture was centrifuged in a Cs_2SO_4 density gradient. A second gradient containing [^{3}H]uridine-labeled RNA and reference DNA from ^{32}P-labeled phage T4

TABLE 2. *Hybridization of RNA–DNA copolymer to separated strands of phage T4 DNA*

Sample	Cpm hybridized to DNA on filters				Ratio $^3H/^{32}P$		
	l-strand†		r-strand†				
	^3H	^{32}P‡	^3H	^{32}P‡	l-strand	r-strand	Bias*
[^{3}H]dT copolymer	139	26	30	29	5.4	1.0	5.4
[^{3}H]BrdU copolymer	973	146	53	54	6.7	0.98	6.8
[^{3}H]BrdU from parental location	333	59	204	26	5.7	7.8	0.72

* The bias equals the ratio of ($^3H/^{32}P$) for the l-strand divided by the ratio of ($^3H/^{32}P$) for the r-strand.

† The background of radioactivity nonspecifically adsorbed to blank filters was subtracted from the counts hybridized to the l- and r-strand filters.

‡ ^{32}P-labeled phage T4 DNA from mature phage was added to the hybridization mixture as an internal reference.

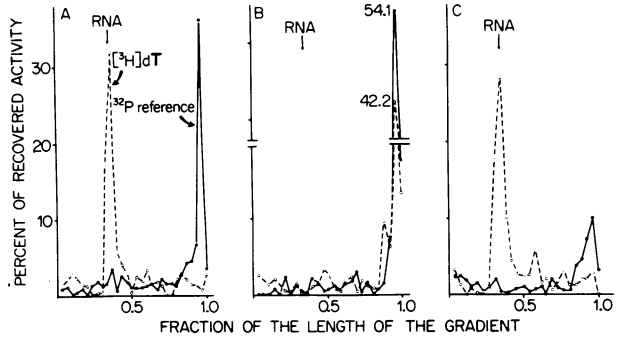

FIG. 2. Physical characterization of the phage T4 RNA–DNA copolymer. [3H]dT-labeled copolymer was isolated from a CsCl density gradient as described in the text. The bottom of each gradient is to the left. (*A*) A portion of the [3H]dT-labeled copolymer fraction was analyzed in a Cs_2SO_4 density gradient in the presence of 32P-labeled reference DNA from phage T4. A parallel gradient, containing [3H]uridine-labeled RNA was centrifuged in the same rotor. The location of the RNA reference in the parallel gradient is indicated by the *arrow*. (*B*) [3H]dT-labeled copolymer was incubated with 20 μg of pancreatic RNase A per ml for 60 min at 37°, then analyzed by Cs_2SO_4 density gradient centrifugation. (*C*) [3H]dT copolymer fraction was heated for 10 min at 100°, chilled on ice, then analyzed by Cs_2SO_4 density gradient centrifugation. The *solid line* represents 32P-labeled reference DNA from phage T4; the *dashed line* represents [3H]dT-labeled copolymer.

was centrifuged in the same rotor. The results of this analysis are shown in Fig. 2*A*. The [3H]dT-labeled copolymer and the [3H]uridine-labeled RNA banded at the same location in the gradients, well separated from the 32P-labeled reference DNA. This result supported the assumption that DNA and RNA were associated in the copolymer. These data suggest, further, that RNA must be the major component, since the overall density of this material was very similar to that of RNA in the gradient.

To further substantiate this possibility, another portion of the [3H]dT-labeled copolymer was incubated with RNase and then analyzed by Cs_2SO_4 density-gradient centrifugation. If DNA and RNA were associated in this material, hydrolysis with RNase should have a dramatic effect on the [3H]dT-labeled portion of the complex. The final density of the labeled DNA portion would yield some information as to the mode of association of the DNA and RNA. If the DNA were hydrogen-bonded to the RNA, hydrolysis with RNase would destroy only that portion of the RNA not directly involved in hydrogen bonding with DNA. The resulting RNA–DNA hybrid, which is resistant to RNase, would have a density in Cs_2SO_4 intermediate between that of DNA and RNA. The same result would be expected of a structure containing DNA covalently attached to the RNA and "looped back" by hydrogen bonding. A linear, covalent attachment of RNA and DNA, on the other hand, should yield pure DNA after RNase treatment. The [3H]dT-labeled DNA product would, in this case, coband with reference DNA in a density gradient. The result of the Cs_2SO_4 analysis after incubation with RNase is shown in Fig. 2*B*.

After RNase hydrolysis, the [3H]dT-labeled DNA portion of the complex cobanded with 32P-labeled reference DNA from phage T4. This result confirms that the copolymer contained RNA and DNA and indicates that the components are associated in a linear copolymer.

An additional experiment showed that the bond between RNA and DNA in the copolymer was covalent. Denaturation of the copolymer followed by analysis by Cs_2SO_4 density gradient centrifugation would cause no change in the density of the copolymer if RNA and DNA were covalently linked in the same strand. If RNA and DNA were joined by a non-covalent bond, denaturation should release the DNA moiety, allowing it to coband with reference DNA in the gradient. [3H]dT-labeled copolymer, isolated as in the preceding experiment, was mixed with 32P-labeled reference DNA from phage T4, heat denatured for 10 min at 100°, and chilled on ice. The mixture was analyzed by Cs_2SO_4 density gradient centrifugation. The result is shown in Fig. 2*C*. Heat denaturation caused no change in the original density of the copolymer (indicated by the *arrow*). This result supports a covalent attachment of RNA and DNA in the copolymer.

Additional information, obtained from DNA–DNA hybridization experiments with the copolymer, substantiate the conclusions that have been made about the structure of the copolymer. Previous results have shown that the copolymer specifically hybridized to the l-strand of phage T4 DNA. It could be argued, however, that since RNA is the major component of the complex, the hybridization results (Table 2) were due primarily to the hybridization of the RNA component to the DNA on the membranes. If this were the case, the DNA moiety of the copolymer would hybridize to the DNA on the membrane, exhibiting the same strand specificity as the RNA component, regardless of its own strand specificity. Removal of the RNA by RNase hydrolysis could result in one of the following: (*a*) the DNA might not hybridize at all because it is too small or because it depends on the RNA for hybridization to DNA on the membrane; (*b*) lack of hybridization of the DNA could be observed because the RNA and the DNA moiety are hydrogen-bonded and resistant to RNase. If such a structure were incubated with membrane-bound DNA without prior heat denaturation, no hybridization could occur. Upon heat denaturation of such a hydrogen-bonded structure, the DNA could hybridize to the *opposite* strand of phage T4 DNA. On the other hand, the RNA and DNA could be covalently attached and the DNA could be hydrogen bonded by folding back on itself. In this case, after RNase hydrolysis, no hybridization might be observed, even after heating, because such a structure would have a tendency to rapidly

TABLE 3. *Hybridization bias of native, denatured, and RNase-treated RNA–DNA copolymer*

Copolymer	Bias*
Native†	3.9
Denatured‡	3.9
Treated with RNase§	3.8

* Bias was calculated as in Table 2.

† Heated 32P-labeled reference DNA was added to native [3H]dT-labeled copolymer before hybridization.

‡ A mixture of 32P-labeled reference DNA and [3H]dT-labeled copolymer was heated to 100° for 10 min before hybridization.

§ A mixture of 32P-labeled reference DNA and [3H]dT-labeled copolymer was digested with pancreatic RNase A before hybridization, as described in footnote ‡ of Table 1.

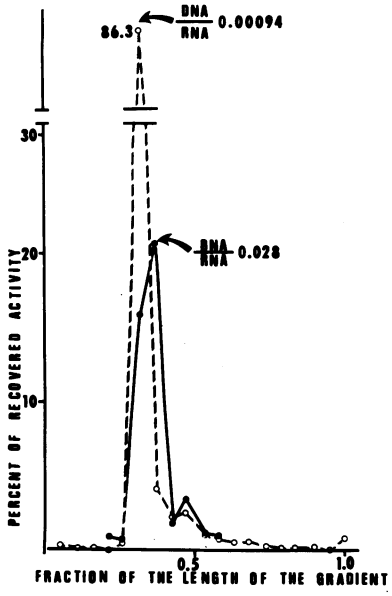

FIG. 3. Proportion of RNA and DNA in the phage T4 copolymer. *E. coli* B-23 was infected with a multiplicity of 10 phage T4. At 3 min after infection, [^{3}H]adenine was added to give a specific activity of 100 Ci/g ([^{3}H]adenine is incorporated into both RNA and DNA). 5 min after infection, the infected cells were chilled in 0.15 M NaCl–15 mM EDTA. The intracellular nucleic acids were extracted by the sodium dodecyl sulfate–Pronase–phenol method (4), then fractionated by Cs_2SO_4 density gradient centrifugation. Part of each fraction of the gradient was precipitated with 0.3 M Cl_3CCOOH and counted (*dashed line*), and represents the total incorporation of [^{3}H]-adenine in both RNA and DNA. The remainder of each fraction was incubated with 1.0 M KOH for 18 hr at 37°. This procedure hydrolyzes RNA. These fractions were neutralized and precipitated with Cl_3CCOOH. After three additional washes with Cl_3CCOOH, each fraction was counted (*solid line*). This peak represents the DNA component of the RNA–DNA copolymer. Note that this peak is displaced by one fraction from the major peak of RNA, because of the less dense DNA component of the copolymer. This represents a 5% density difference in this type of gradient. The copolymer then, contains approximately 5% DNA and 95% RNA. The ratios (DNA/RNA) in this graph represent the counts in DNA (after alkaline hydrolysis; about 100) to the total counts (99% RNA; 10^4–500) in the respective peak fractions. This emphasizes the fact that the DNA of the copolymer does cause a discrete density shift.

renature; or (*c*) the DNA could hybridize to the DNA on the membrane, exhibiting the same strand specificity as before RNase hydrolysis, because RNA and DNA are present in the same strand.

Portions of the copolymer were hybridized to the l- and r-strands of phage T4 DNA in the native state, or after heat denaturation, after incubation with RNase. The results are shown in Table 3. The copolymer hybridized only to the l-strand of phage T4 DNA, with equal efficiency both with and without heat denaturation and after treatment with RNase. These data confirm the results from previous experiments: RNA and DNA are covalently attached and located in the same polynucleotide chain. Furthermore, they establish that the DNA component of the copolymer is single stranded.

A previous experiment (Fig. 2*A*) showed that the copolymer banded at approximately the RNA location in a Cs_2SO_4 density gradient. For a more accurate determination of the proportions of RNA and DNA in the copolymer, it was essential to have an internal RNA reference in the Cs_2SO_4 gradient analysis of the copolymer. This was accomplished by labeling infected cells with [^{3}H]adenine between 3 and 5 min after infection. The intracellular nucleic acids were centrifuged in a Cs_2SO_4 density gradient. After collection, part of each fraction was precipitated with Cl_3CCOOH and counted. Since the bulk of the [^{3}H]adenine-labeled material precipitable by Cl_3CCOOH at this time after infection is RNA, this procedure yielded an internal reference for the location of RNA in the gradient. Another portion of each fraction was incubated with alkali to hydrolyze the RNA. This treatment revealed the location of the copolymer in the gradient, since the DNA component was also labeled with [^{3}H]adenine (Fig. 3). The majority of the [^{3}H]adenine-labeled material bands at the location in the gradient characteristic of RNA. After alkaline hydrolysis of the fractions collected from the gradient, a moiety was observed that showed a displacement of one fraction from the major RNA peak in the gradient. This peak of alkali-resistant material represents the RNA–DNA copolymer. In this case, the displacement of the copolymer from the majority of RNA in the gradient, caused by the small DNA component of the copolymer, indicates that DNA comprises about 5% of the total mass of the copolymer.

Synthesis of the Copolymer under Conditions Nonpermissive for DNA Replication; DNA$^-$ Ambers and Protein Inhibition by CM. The evidence presented thusfar, has shown that a copoly-

TABLE 4. *Hybridization of RNA–DNA copolymer from cells infected by amber mutants deficient in DNA synthesis and from cells infected by wild-type phage in the presence of chloramphenicol*

	Cpm hybridized to DNA on filters								
	T4 l-strand*		T4 r-strand*		*E. coli* DNA*		Ratio ^{3}H/^{32}P		
Copolymer	^{3}H	^{32}P‡	^{3}H	^{32}P‡	^{3}H	^{32}P‡	l-strand	r-strand	Bias†
am A453	243	293	1	220	—	—	0.83	0.005	150.0
am B22	162	178	9	191	—	1	0.91	0.048	19.0
am N82	1360	482	60	302	4	—	2.82	0.20	14.0
CM 0–8 min	169	1219	49	791	—	—	0.14	0.062	2.2
CM 0–30 min	116	146	3	62	34	—	0.80	0.005	15.0

* The background of radioactivity nonspecifically adsorbed to blank filters was subtracted from the cpm hybridized to the l-, r-, and *E. coli* filters.

† The bias was calculated as in Table 2.

‡ ^{32}P-labeled phage T4 DNA was added as an internal reference.

mer, consisting of RNA and DNA covalently linked in the same polynucleotide strand, is produced soon after infection of *E. coli* B with bacteriophage T4. The DNA of the copolymer is complementary to phage T4 DNA and specifically hybridizes to the l-strand. If synthesis of the copolymer were phage controlled, mutations that limit or totally abolish phage DNA replication might restrict or abolish its formation. To explore this possibility, cultures of *E. coli* B-23 were infected with each of three amber mutants of phage T4: *am* A453 (gene 32), *am* B22 (gene 43), and *am* N82 (gene 44). The multiplicity of infection was 10. 3 min after infection, [^{3}H]dT was added, and 5 min after infection, the intracellular DNA was isolated and analyzed by CsCl density-gradient centrifugation. These gradients (not documented here) were nearly identical to those from previous experiments (Fig. 1): [^{3}H]dT-labeled copolymer was found near the bottom of each gradient. The copolymer was isolated from each gradient and hybridized to the separated strands of phage T4 DNA and to *E. coli* DNA. The results are shown in Table 4. Copolymer from cells infected by each phage T4 amber mutant hybridized preferentially to the l-strand of phage T4 DNA, as had been observed with the copolymer isolated from cells infected with wild-type phage T4. Little, if any, bacterial DNA could be detected in these samples.

These results suggested that the host could be, in some way, responsible for the synthesis of the copolymer. This possibility was explored by addition of chloramphenicol (CM) to cells before phage infection. This prevents all protein synthesis and, subsequently, any synthesis of phage-induced enzymes after infection. CM (150 μg/ml) was added to *E. coli* B 2 min before infection. 3 min after infection with phage T4 (multiplicity of 10), [^{3}H]dT was added. Samples were obtained 8 min and 30 min after infection, and the intracellular DNA was analyzed by CsCl density gradient centrifugation. Just as in all previous experiments, [^{3}H]dT-labeled copolymer was detected near the bottom of the gradients, 8 and 30 min after incubation in the presence of CM.

The copolymer was isolated from each of the gradients and hybridized to phage T4 l- and r-strands and to host DNA. These results are shown in Table 4. The data show that this DNA, synthesized in the absence of phage-directed protein synthesis, was, nevertheless, complementary to phage T4 DNA and specific for the l-strand.

In addition, the data in Table 4 show that the copolymer incubated 30 min in the presence of CM contained some DNA complementary to the *E. coli* DNA. This suggested that a bacterial copolymer is also synthesized.

DISCUSSION

This paper describes the formation and physical properties of an RNA–DNA copolymer synthesized in *E. coli* B infected with bacteriophage T4. The copolymer can be detected shortly after infection with phage T4 and appears to decrease at the time when parental phage DNA replication begins. It is synthesized in cells infected with three different amber mutants of phage T4, deficient in DNA replication: *am* A453 (gene 32), *am* B22 (gene 43), and *am* N82 (gene 44). The formation of the copolymer is not inhibited by the addition of CM before infection, suggesting that copolymer synthesis is mediated by host-coded enzymes. Hybridization studies have shown that the copolymer is complementary to phage T4 DNA and, furthermore, that it is specifically hybridized to the l-strand. This is the same strand from which "pre-early" and early messenger RNA is transcribed (5).

Physical analyses have shown that the copolymer contains RNA and DNA and that the RNA is the major component, comprising about 95% of the structure. The RNA and DNA components are covalently linked and in the same polynucleotide strand.

The function of the copolymer remains undetermined. However, recent studies by several groups of investigators suggest that RNA may have a direct role in the initiation of DNA synthesis in several different organisms (ref. 1,*,†). Although the phage T4 copolymer was not found associated with the parental DNA matrix, the fact that it is complementary to phage T4 DNA necessitates such an association at some time after infection. The procedures used for the extraction of the copolymer could have detached it from phage DNA. Alternatively, the copolymer could represent the degradation product of some larger structure. Further studies of certain pre-replicative structures found in cells infected with phage T4 may serve to elucidate the function of phage T4 RNA–DNA copolymer.

Preliminary transformation experiments (L. A. McNicol, unpublished results) have shown that the phage T4 copolymer is genetically competent.

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