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RNA-Linked DNA Fragments *In Vitro**

(*E. coli*/toluene-treated cells/DNA synthesis/discontinuous replication/RNA synthesis/nucleotide sequence)

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ABSTRACT RNA-linked DNA fragments are intermediates in DNA replication in *Escherichia coli* cells made permeable to nucleoside triphosphates by treatment with toluene. Covalent linkage of a short RNA stretch to the 5′ end of the DNA is proved by transfer of ^{32}P from [α-^{32}P]-dNTP to ribonucleotides upon digestion with alkali or pancreatic RNase, and by a small decrease in the molecular size upon alkaline hydrolysis. The ^{32}P transfer experiments reveal a unique structure...p(rPy)p(rA)p(rU or rC)p-(dC)p... at the RNA–DNA junction.

We have shown (1, 2) that a short stretch of RNA is linked to the nascent DNA fragments isolated from exponentially growing cells of *Escherichia coli*. The RNA seems to be removed before the fragments are joined by ligase and become a part of the long DNA strand. These findings suggest that RNA synthesis (transcription) is involved in the initiation of synthesis of these fragments, which would occur at specific sites on the template strands in the discontinuous mode of DNA replication.

To obtain further information on the structure and metabolism of the RNA-linked nascent DNA fragments, we have investigated the synthesis of these molecules in *E. coli* cells made permeable to nucleoside triphosphates by treatment with toluene (3). The results presented here indicate that toluene-treated cells synthesize RNA-linked DNA fragments—as do intact cells—and that the RNA is covalently linked at the 5′ end of the DNA by a phosphodiester bond. Furthermore, unique primary structures at the RNA–DNA junction are demonstrated.

MATERIALS AND METHODS

Unlabeled dNTPs were purchased from Schwarz BioResearch, dT, rATP, DPN from Sigma Chemical Co., and [^{3}H]dTTP (17.4 Ci/mmol) from the Radiochemical Centre (Amersham). [α-^{32}P]dCTP, [α-^{32}P]dGTP, and [α-^{32}P]dATP (14–21 Ci/mmol) were obtained from International Chemical and Nuclear Corp. and treated with periodate (4) to degrade any contaminating ribonucleotides. [α-^{32}P]dTTP (9 Ci/mmol) was prepared as follows: dT was first phosphorylated by *E. coli* dT kinase (5) with [γ-^{32}P]rATP (11.5 Ci/mmol), prepared by a described method (6), and the reaction mixture was treated with periodate to destroy the remaining [γ-^{32}P]rATP. [^{32}P]dTMP was recovered by charcoal treatment and converted to [α-^{32}P]dTTP with unlabeled rATP and dNMP kinase from bacteriophage T4-infected *E. coli*. The labeled dTTP was isolated by paper electrophoresis at pH 3.5, followed by charcoal treatment.

E. coli P3478 *thy*$^+$ (*polA*1′) (7) was grown in Bacto Tryptose Broth to a titer of 6 × 10^8 cells per ml, suspended in 50 mM potassium phosphate buffer (pH 7.6) at a density of 3 × 10^9 cells per ml, and treated with 1% toluene for 15 min at 37°. The standard incubation mixture for DNA synthesis contained 70 mM potassium phosphate buffer (pH 7.6), 13 mM $MgCl_2$, 1.3 mM rATP, 0.3 mM DPN, 33 μM (each) of four dNTPs, and 1.5 × 10^9 toluene-treated cells per ml. The reaction was performed at 30° and terminated by addition of two volumes of an ethanol–phenol mixture (18), and cells were collected by centrifugation. Cells were lysed either by incubation in 0.2 N NaOH–20 mM EDTA–0.5% sodium *N*-lauroyl sarcosinate at 37° for 20 min (method A) or by heating in 0.5% sodium dodecyl sulfate (SDS)–10 mM NaCl–10 mM EDTA–10 mM Tris·HCl (pH 7.6) at 100° for 5 min (method B). Cs_2SO_4 equilibrium centrifugation and alkaline sucrose gradient sedimentation were performed as before (1, 9).

Paper electrophoresis was performed on Toyo no. 50 paper at 60 V/cm in 50 mM sodium citrate buffer at pH 5.0 or 3.5. Two-dimensional paper chromatography was performed on Toyo no. 50 paper in 95% ethanol–1 M ammonium acetate (pH 6.8) 75:30 and saturated $(NH_4)_2SO_4$–1 M sodium acetate 80:18. Thin-layer chromatography was done on an Avicel SF cellulose plate (Funakoshi Pharmaceutical Co.) in isopropanol–HCl–H_2O 65:17:18 or in saturated $(NH_4)_2SO_4$–1 M ammonium acetate–isopropanol 40:9:1. To fractionate oligonucleotides, the sample in 7 M urea–20 mM Tris·HCl (pH 7.6) was applied to a DEAE-cellulose (Whatman DE52) column (0.2 × 30 cm) and eluted with a 60-ml linear gradient of 0–0.25 M NaCl in 7 M urea–20 mM Tris·HCl (pH 7.6). For Dowex-1 chromatography, the sample was applied to a Dowex AG1 X2 (Bio-Rad) column (0.3 × 12 cm) and eluted first by a linear gradient with 20 ml of 4 mM HCl in the mixing vessel and 20 ml of 0.15 M NaCl–10 mM HCl in the reservoir, then with 20 ml of 0.3 M NaCl–12 mM HCl. Gel filtration through Sepharose 2B (Pharmacia) was performed with upward flow of 0.05 M NaCl–10 mM EDTA–5 mM Tris·HCl (pH 8.0).

RESULTS

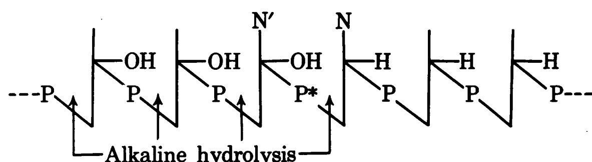
The patterns of alkaline sucrose gradient sedimentation of DNA pulse-labeled with [^{3}H]dTTP for various times (Fig. 1) show that DNA replication in this system occurs by a discontinuous mechanism, as does DNA replication in intact

Abbreviations: SDS, sodium dodecyl sulfate: SSC, standard saline–citrate (0.15 M NaCl–0.015 M sodium citrate).

* This paper is no. X in a series; "Mechanism of DNA Chain Growth." The preceding paper is ref. 1.

cells. Isopycnic analysis (Fig. 2) showed that very nascent DNA in toluene-treated cells has a higher density than does standard *E. coli* DNA, as does nascent DNA of intact cells. Thus RNA-linked DNA fragments seem to be formed as intermediates in DNA replication also in this *in vitro* system.

To test if RNA is linked covalently to the 5′ end of DNA by a 3′:5′-phosphodiester bond, toluene-treated cells were incubated for 5 min in four reaction mixtures, each containing a different dNTP labeled with ^{32}P at the α position. The product of each reaction was hydrolyzed with alkali, and the distribution of ^{32}P in the four 2′(3′)-rNMPs was examined. If covalently linked RNA–DNA molecules are formed with incorporation of the labeled dNTP into the RNA–DNA linkage (the 5′ end of the DNA segment), the ^{32}P originally linked to the 5′ position of that deoxynucleotide should be transferred to the adjacent ribonucleotide (the 3′-terminal nucleotide of the RNA segment) upon alkaline hydrolysis.



As shown in Table 1, transfer of ^{32}P from the α position of 5′-dNTPs to 2′(3′)-rNMPs did occur, indicating the presence of the phosphodiester linkage between ribo- and deoxyribonucleotides in the product. While a relatively large amount of ^{32}P was transferred from [α-^{32}P]dCTP or [α-^{32}P]dGTP to rUMP, and some ^{32}P transfer from dCTP to rCMP, rAMP, and rGMP was found, little other transfer was detected. Thus, unique base sequences exist at the RNA–DNA linkage: (rU)p(dC) is the most common sequence, which occurs at about half of the linkages, and the sequence (rU)p(dG) occurs at about a quarter of the linkages, whereas most of the rest of the RNA–DNA linkages have the structure (rC)p(dC), (rG)p(dC), or (rA)p(dC).

Pancreatic RNase splits the bond between the 3′-pyrimidine ribonucleotide and the 5′ position of the adjacent deoxy-,

TABLE 1. *Distribution of radioactivity in 2′(3′)-rNMPs after alkaline hydrolysis of products formed with [α-^{32}P]dNTP*

[α-^{32}P]dNTP substrate	% of acid-insoluble ^{32}P transferred to: CMP	AMP	GMP	UMP
dCTP	0.08	0.03	0.05	0.34
dATP	<0.01	<0.01	<0.01	0.01
dGTP	<0.01	<0.01	0.01	0.20
dTTP	<0.01	<0.01	<0.01	<0.01

The reaction was performed in four 0.43-ml reaction mixtures for 5 min under the standard conditions, except that one of the four dNTPs was labeled with ^{32}P at the α position as indicated and used at a concentration of 16 μM. The specific activity of dCTP, dATP, dGTP, and dTTP was 9.8, 9.5, 7.0, and 8.9 Ci/mmol, respectively. Cells were lysed by method B and the lysate was subjected to Cs_2SO_4 equilibrium centrifugation. Material banded at densities of 1.40–1.59 g/cm^3 (2.1 to 2.9 $\times$ 10^6 cpm) was dialyzed against 0.1 $\times$ SSC–0.01% SDS, precipitated by ethanol with *E. coli* tRNA (400 μg), and dissolved in 50 μl of 0.3 M KOH. After incubation for 16 hr at 37°, 5 μl of 1% bovine-serum albumin and 3 μl of 60% $HClO_4$ were added and the mixture was centrifuged. The supernatant was neutralized with KOH and the precipitate of $KClO_4$ was removed. The radioactivity of both the supernatant and pellet was measured upon aliquots. The supernatant was subjected to paper electrophoresis at pH 5.0. The rNMP region was cut out, eluted with water, and subjected to a second electrophoresis at pH 3.5. After location of four 2′(3′)-rNMPs under a UV lamp, the paper was cut into 1-cm strips and the radioactivity in each strip was counted. The radioactive nucleotides were eluted from the paper and subjected to thin-layer chromatography, along with authentic 2′(3′)-rNMPs and 5′-dNMPs to confirm identification.

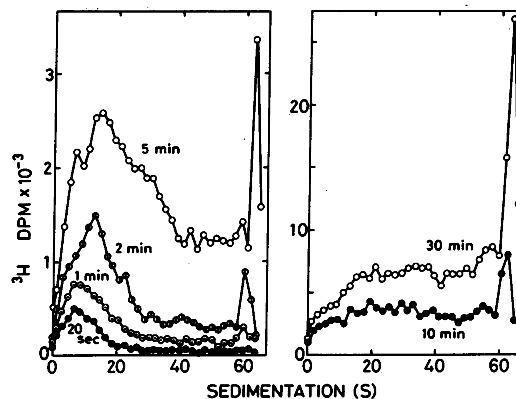


FIG. 1. Alkaline sucrose gradient sedimentation of DNA from toluene-treated cells pulse-labeled with [^{3}H]dTTP. Toluene-treated cells were incubated for 2 min at 30° in the standard reaction mixture (1 ml) with four unlabeled dNTPs. [^{3}H]dTTP (17.4 Ci/mmol) was then added to 2.9 μM, and the reaction was terminated after the times indicated. Cells were lysed by method A and the lysate was subjected to sedimentation through a 5–20% alkaline sucrose gradient made on a 82% sucrose cushion, along with [^{14}C]δA DNA (a 19S internal reference).

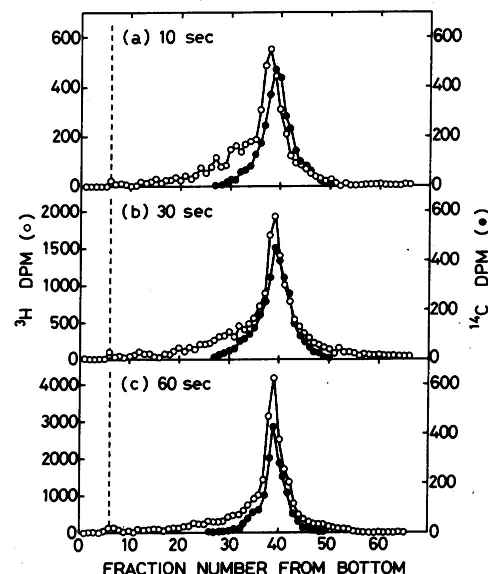


FIG. 2. Cs_2SO_4 equilibrium centrifugation of DNA from toluene-treated cells pulse-labeled with [^{3}H]dTTP. Toluene-treated cells were incubated in the 1-ml standard reaction mixture with [^{3}H]dTTP (3 Ci/mmol) at 30° for the times indicated. Cells were lysed by method B, and the lysate was subjected to equilibrium density centrifugation in a Beckman SW50L rotor, together with denatured *E. coli* [^{14}C]DNA. The vertical *dotted lines* indicate the position of the peak of RNA.

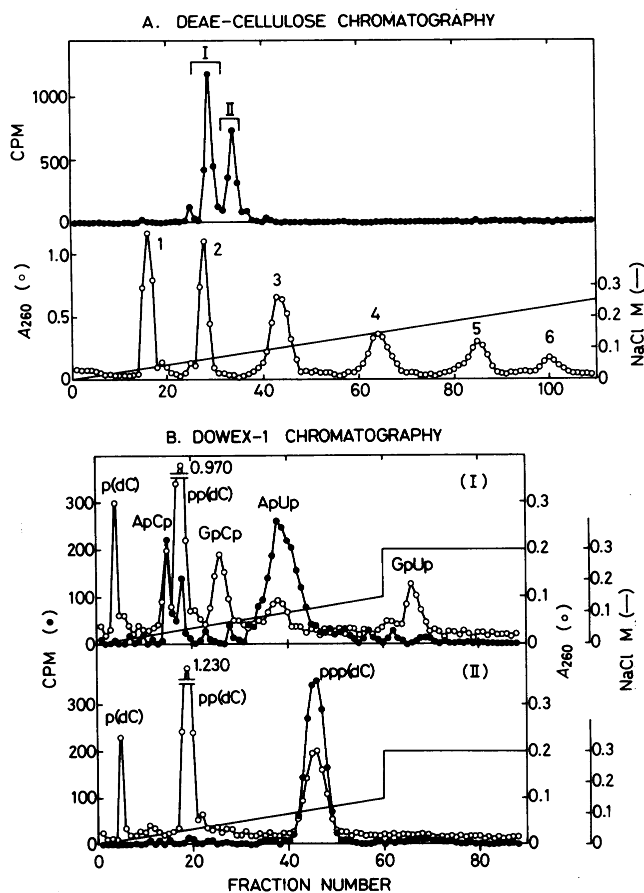
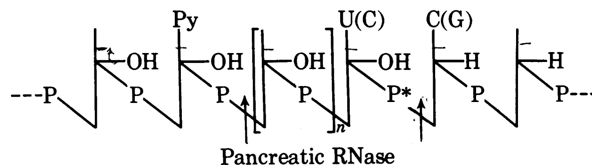
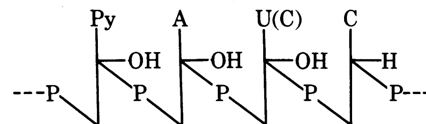


FIG. 3. Identification of radioactive products formed by pancreatic RNase digestion of RNA–DNA molecules synthesized with [α-^{32}P]dCTP. DNA synthesis with [α-^{32}P]dCTP (9.8 Ci/mmol) was performed as in Table 1. Cells were lysed by method B, and the material banding near the density of DNA in Cs_2SO_4 (ρ = 1.40–1.47 g/cm^3; total radioactivity = 3.2 × 10^5 cpm) was dialyzed against 0.1 × SSC–0.01% SDS, and precipitated by ethanol with 300 μg of tRNA as carrier. The sample was dissolved in 50 μl of 20 mM Tris·HCl (pH 7.5)–20 mM NaCl–2 mM EDTA containing 6 μg of pancreatic RNase and incubated at 37° for 2 hr. The mixture was diluted with 2 ml of 7 M urea–20 mM Tris·HCl (pH 7.6) and subjected to chromatography on a DEAE-cellulose–urea column. *Bracketed fractions* (I and II) were diluted 5-fold with water and applied to a Dowex AG1 column along with dCMP and dCDP (*I*) or with dCMP, dCDP, and dCTP (*II*), respectively. The column was eluted as described in *Methods*, and nucleotides were identified by their elution positions and their absorption spectra.

as well as ribonucleotide (10). Therefore, if the RNA–DNA molecules synthesized with [α-^{32}P]dCTP or [α-^{32}]dGTP are digested with pancreatic RNase, radioactive ribonucleotides (oligo- or mononucleotides) will be produced as the result of cleavage of the linkage as (rU)p(dC), (rC)p(dC), and (rU)-p(dG), and at the nearest pyrimidine nucleotides in the RNA.



When the product synthesized in the presence of [α-^{32}P]-dCTP was hydrolyzed with pancreatic RNase and subjected to DEAE-cellulose chromatography, 0.7% of the radioactivity was eluted in the position of dinucleotide (Fig. 3*A*, Peak *I*). Of the radioactivity in this peak, 79% cochromatographed with ApUp on Dowex-1, while 15% was eluted in the region of ApCp; no radioactivity was found in the other possible dinucleotides, GpCp and GpUp (Fig. 3*B I*). 0.46% of the total radioactivity was eluted from DEAE-cellulose between di- and trinucleotides (Fig. 3*A*, Peak *II*); this material was contaminating dCTP (Fig. 3*B II*). No radioactivity was found in mono-, tri-, tetra-, penta-, and hexanucleotides. Thus, ApUp and ApCp are the only major radioactive ribonucleotides produced by the RNase digestion of the product formed in the presence of [α-^{32}P]dCTP. The yields of these radioactive dinucleotides are in rough agreement with the amounts of the (rU)p(dC) and (rC)p(dC) linkages indicated by the alkaline hydrolysis experiments.Thus, on the 5′ side of these linkages is the definite structure (rPy)p(rA)p, and the predominant structure at the RNA–DNA junction is:



In contrast, the sequence on the 5′ side of the (rU)p(dG) linkage seems heterogeneous. After pancreatic RNase digestion of the product synthesized with [α-^{32}P]dGTP as labeled substrate, various sizes of radioactive products from mono- to hexanucleotides were found (Fig. 4). The radioactive material eluted immediately before trinucleotides was contaminating [α-^{32}P]dGTP.

In the ^{32}P transfer experiments described above, the reaction was allowed to proceed for 5 min. The essential fea-

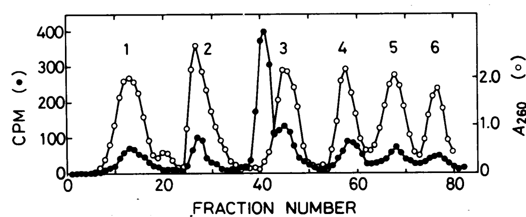


FIG. 4. DEAE-cellulose chromatography of radioactive products formed by pancreatic RNase digestion of RNA–DNA molecules synthesized with [α-^{32}P]dGTP. The experiment was performed as in Fig. 3, except that [α-^{32}P]dGTP (5.5 Ci/mmol) was used as labeled substrate for DNA synthesis.

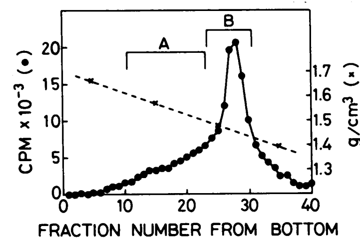


FIG. 5. Cs_2SO_4 equilibrium centrifugation of product synthesized in a 1-min incubation with [α-^{32}P]dCTP. The experiment was performed as in Table 1, except that the reaction was for 1 min with [α-^{32}P]dCTP (14.6 Ci/mmol). *Bracketed fractions* (*A* and *B*) were used for the analysis shown in Table 2.

tures of the results were confirmed with the reaction for 1 min. As shown in Fig. 5, the product of the 1-min reaction with [α-^{32}P]dCTP banded heterogeneously in the Cs_2SO_4 gradient between the densities of RNA and DNA. It was divided into two fractions (*A* and *B*) with relatively high and low densities, and each was hydrolyzed with alkali. As seen from Table 2, 2.82 and 1.10% of the total radioactivity in *A* and *B*, respectively, were transferred to rNMPs. In both fractions, the largest portion of the transferred ^{32}P (78% in *A* and 72% in *B*) was in 2′(3′)-UMP. When the whole product of the 1-min reaction with [α-^{32}P]dCTP was digested by pancreatic RNase and subjected to DEAE-cellulose chromatography, 1.9% of the radioactivity was recovered in dinucleotide; virtually no radioactivity was found in mono-, tetra-, penta-, and hexanucleotides. Of the radioactive dinucleotides, 22% and 77% were ApCp and ApUp, respectively.

From the data of the ^{32}P transfer experiments, the number of total deoxynucleotides per RNA-DNA linkage in the product of the 5-min reaction is calculated to be about 500. If it is assumed that dCMP is contained in three quarters of the RNA-DNA linkages synthesized in the 1-min reaction (as shown for the product of the 5-min reaction), the total number of deoxynucleotides per RNA-DNA linkage is estimated to be about 200 for whole product formed in 1 min, and about 100 and 270 for the product of the 1-min reaction with the high and low densities (*A* and *B* in Fig. 5), respectively. These results are in line with the idea that RNA is linked only to very nascent short DNA, degraded by nuclease action as the DNA segment elongates, and removed completely before ligation of the DNA segments.

In further support of this idea, pancreatic RNase digestion of the product yielded virtually no radioactive dinucleotide (20 cpm in an experiment comparable to that of Fig. 3) when the synthesis with [α-^{32}P]dCTP was followed by incubation in the presence of a 100-fold excess of unlabeled dCTP for 20 min.

Although the ^{32}P transfer experiments prove the RNA-DNA linkage in the product, they do not exclude the possibility that the RNA is located in the middle of the chain or that ribonucleotide sequences are scattered in the molecule. To clarify this point, the molecules of various sizes, synthesized by a 1-min reaction with [^{3}H]dTTP as labeled

TABLE 2. *Distribution of radioactivity in 2′(3′)-rNMPs after alkaline hydrolysis of products formed in a 1-min reaction with [α-^{32}P]dCTP*

	% of acid-insoluble ^{32}P transferred to:			
Fraction	CMP	AMP	GMP	UMP
A	0.23	0.10	0.31	2.18
B	0.18	0.03	0.11	0.78

Fraction A (4.2 × 10^4 cpm) and Fraction B (1.1 × 10^5 cpm) in Fig. 5 were dialyzed against 0.1 × SSC-0.01% SDS and precipitated by ethanol with 600 μg of *E. coli* tRNA. After hydrolysis in 50 μl of 0.3 M KOH at 37° for 15 hr, cold $HClO_4$-soluble supernatant was neutralized, concentrated, and subjected to two-dimensional paper chromatography. The 2′(3′)-rNMP spots were cut out and their radioactivity was counted. In addition, all rNMP spots were eluted and subjected to thin-layer chromatography to confirm their identification.

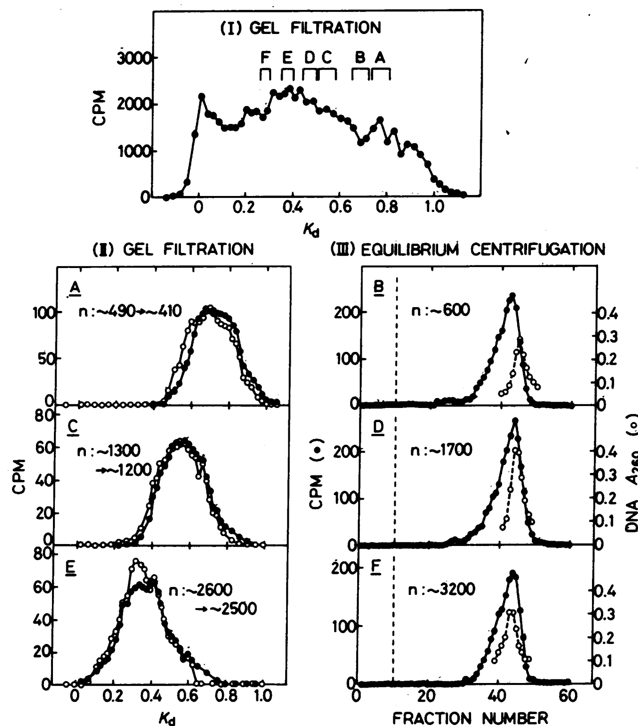


FIG. 6. Fractionation and analysis of RNA-DNA molecules of various sizes. (*I*) Toluene-treated cells were incubated in a 6-ml standard reaction mixture with [^{3}H]dTTP (3 Ci/mmol) for 1 min at 30°. Cells were lysed by method B. The lysate was treated with Pronase E (1 mg/ml, 4 hr at 37°) and then with phenol, and dialyzed against 50 mM NaCl-10 mM EDTA-5 mM Tris·HCl (pH 8.0). After it was heated at 100° for 5 min and quickly chilled, the sample was subjected to gel filtration on a Sepharose 2B column (1.6 × 95 cm). The indicated fractions (*A-F*) were precipitated by ethanol with *E. coli* tRNA as carrier and dissolved in a small volume of 50 mM NaCl-10 mM EDTA-5 mM Tris·HCl (pH 8.0). (*II*) Fractions A, C, and E were divided into two portions: one portion was treated with 0.3 M NaOH at 37° for 4 hr and neutralized. After they were heated at 100° for 5 min and quickly chilled, the untreated (○) and alkali-treated (●) samples were subjected to gel filtration on a Sepharose 2B column (1.6 × 40 cm). (*III*) Fractions *B*, *D*, and *F* were heated at 100° for 5 min, chilled quickly, and subjected to Cs_2SO_4 equilibrium centrifugation in a Beckman SW56Ti rotor with denatured *E. coli* DNA. The *dotted lines* indicate the position of RNA. The chain length (*n*) was estimated from the *K*d values by use of a calibration curve prepared with single-stranded *E. coli* DNA of known chain lengths (Hirose, Okazaki, and Tamanoy, in preparation).

substrate, were isolated by gel filtration on Sepharose 2B, and the change of their size upon alkaline hydrolysis, as well as their density, was analyzed (Fig. 6). Only small decreases in molecular size were observed, excluding the above possibilities. Thus, the RNA is attached to the 5′ end of the DNA. The size of the RNA segments of the molecules of various sizes is suggested by both size change and density analyses to be about 50-100 nucleotides, in agreement with the results obtained with the *in vivo* RNA-DNA molecules (2).

DISCUSSION

Based on studies with intact cells, we have postulated (1, 2) a replication mechanism involving: (*i*) unwinding of the

parental strands; (*ii*) synthesis of short RNA chains along the parental strands beginning at specific initiation signals and stopping at termination signals; (*iii*) extension of the chains by a DNA polymerase in the 5′ → 3′ direction with the RNA as primer; (*iv*) removal of the RNA segments by RNase activity; (*v*) filling the gaps between the DNA fragments by a DNA polymerase; and (*vi*) joining of the DNA fragments by ligase.

The present study performed with toluene-treated cells of a *polA*$^-$ strain, an *in vitro* replication system that closely resembles viable cells, lends strong support for this hypothetical mechanism. It demonstrates that RNA-linked DNA fragments are also synthesized *in vitro* (Figs. 2 and 6) and provides definitive evidence for the covalent attachment of RNA to the 5′ end of the DNA (Tables 1 and 2, Fig. 3). That the attachment of RNA is characteristic of nascent short DNA chains is confirmed (Tables 1 and 2, Fig. 6, and the chase effect of unlabeled dCTP on the ^{32}P transfer from [α-^{32}P]-dCTP).

Furthermore, the ^{32}P transfer experiments (Tables 1 and 2, Fig. 3) reveal that ...p(rPy)p(rA)p(rU)p(dC)p..., with occasional replacement of (rU) with (rC), is a predominant sequence at the RNA–DNA junction. Although the linkage (rU)p(dG) occurs fairly frequently, the sequence at its 5′ side is random. A recent study (ref. 2, Hirose, Okazaki, and Tamanoy, in preparation) has shown that the RNA-linked DNA fragments isolated from growing *E. coli* cells accept ^{32}P from [γ-^{32}P]rATP in the polynucleotide kinase reaction after alkaline hydrolysis. The ^{32}P linked to the 5′ end of the DNA is recovered quantitatively in dCMP upon digestion with pancreatic DNase and venom phosphodiesterase. Moreover, almost the same number of 5′-OH ends of DNA are created by pancreatic RNase digestion as by alkaline hydrolysis. Therefore, most of the *in vivo* RNA–DNA fragments contain the linkage (rPy)p(dC). This remarkable agreement of the *in vitro* and *in vivo* results points to the possibility that ...p-(rPy)p(rA)p(rU or rC)p(dC)p... is a universal sequence of the RNA–DNA junction of the *E. coli* fragments, which may reflect the nucleotide sequence on the template strands designating the termination of synthesis of the RNA segment (and/or the initiation of the DNA segment). The (rU)p(dG) linkage might have a similar significance as the (rPy)p(dC) linkage. However, we think it more likely that this linkage is an *in vitro* artifact, because the sequence in its 5′ side is random and because no dGMP is detected at the RNA–DNA linkage of the *in vivo* fragments.

Wickner *et al.* (11) and Scheckman *et al.* (12) have demonstrated the synthesis of covalently linked RNA–DNA molecules in *E. coli* extracts using M13 and φX DNA as template. The rNMP in the RNA–DNA linkage is largely AMP in the case of M13, whereas all four rNMPs are found in the linkage in the case of φX. Keller (13) has obtained similar results with φX using *E. coli* RNA polymerase and DNA polymerase II from KB cells. Covalently linked RNA–DNA molecules are also synthesized by RNA-dependent DNA polymerase. Flügel and Wells (14) found only (rU)p(dC) and (rA)p(dA) linkages in such molecules formed by avian myelobalastosis virus DNA polymerase. The relationship between these and the present findings is an interesting question to be clarified by future studies.

Synthesis of the RNA-linked DNA fragments in toluene-treated cells occurs in the absence of rNTP other than rATP. While this fact suggests enough endogenous supply of RNA precursors, a labeled rNTP is incorporated into the RNA–DNA molecules when it is added to the reaction mixture. Our results, to be detailed elsewhere, show that [γ-^{32}P]-rGTP is incorporated into the 5′ ends of the RNA-linked DNA fragments and recovered as radioactive ppp(rG)p after alkaline hydrolysis. This finding provides further support for the 5′-terminal location of the RNA segment and indicates that the RNA is formed not by a reaction primed by a preexisting chain, but by *de novo* synthesis. The specific labeling of the 5′ end of the RNA segment by [γ-^{32}P]dGTP would facilitate analysis of its structure and elucidation of the mechanism of its synthesis and degradation.

Our work with *E. coli*, work of Kornberg and coworkers (11, 12, 15) with M13 and φX, and work of Helinski's group with *Col*E$_1$ (16) provide strong evidence for primer functions of RNA in DNA replication. Other possible roles of RNA or RNA synthesis in DNA replication have been suggested by Lark (17) and by Dove *et al.* (18).

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