Proc. Nat. Acad. Sci. USA
Vol. 70, No. 12, Part II, pp. 3744–3748, December 1973

Size and Base Composition of RNA in Supercoiled Plasmid DNA

(*Escherichia coli*/RNase-sensitive DNA/DNA replication/colicinogenic factor E_1/polymerase of Rous sarcoma virus)

PETER H. WILLIAMS*, HERBERT W. BOYER†, AND DONALD R. HELINSKI*

* Department of Biology, University of California, San Diego, La Jolla, Calif. 92037; and † Department of Microbiology, University of California, San Francisco Medical Center, San Francisco, Calif. 94122

Communicated by Clifford Grobstein, August 20, 1973

ABSTRACT **The average size and base composition of the covalently integrated RNA segment in supercoiled $ColE_1$ DNA synthesized in *Escherichia coli* in the presence of chloramphenicol (CM-$ColE_1$ DNA) have been determined by two independent methods. The two approaches yielded similar results, indicating that the RNA segment in CM-$ColE_1$ DNA contains GMP at the 5′ end and comprises on the average 25 to 26 ribonucleotides with a base composition of 10–11 G, 3 A, 5–6 C, and 6–7 U.**

Evidence has been obtained for a direct role of RNA in the initiation of replication of the DNA of the bacteriophages λ (1), M13 (2–4), φX174 (5), T4 (6, 7), and T7 (8), the *Escherichia coli* chromosome (9, 10), the plasmids $ColE_1$ (11, 12), F1 (13), and plasmid 15 (14) of *E. coli*, and a plasmid of *Salmonella pullorum* (15). In the *in vitro* conversion of M13 and φX174 single-stranded phage DNA to the double-stranded RFII form, it has been demonstrated that RNA serves a primer role and is covalently joined to the newly synthesized DNA strand (3, 16). A similar role for RNA in the initiation of synthesis of covalently closed, circular $ColE_1$ DNA was proposed to explain the finding of a substantial level of RNA-containing supercoiled $ColE_1$ DNA molecules in *E. coli* cells synthesizing this plasmid DNA in the presence of chloramphenicol (17). The majority of molecules possess the RNA at a single site in one of the two complementary DNA strands; the RNA occurs with equal probability in either of the two complementary strands (17). The generation of these alkali- and RNase-sensitive $ColE_1$ molecules in the presence of chloramphenicol (CM-$ColE_1$ DNA) depends upon $ColE_1$ DNA synthesis (17) and is prevented by rifampicin, an inhibitor of RNA polymerase (11, 12). It was proposed that RNA serves normally as a primer for the initiation of $ColE_1$ DNA synthesis and that inhibition of protein synthesis by chloramphenicol interferes with the removal of the priming RNA, resulting in its adventitious covalent integration into supercoiled DNA (17).

In this communication the average size and overall base composition of the RNA in the CM-$ColE_1$ DNA molecules is determined by analysis of the products of digestion of the RNA segment and by analysis of the products of the repair synthesis of the single-strand gap produced by RNase digestion of the CM-$ColE_1$ DNA.

MATERIALS AND METHODS

Bacterial Strain and Growth Medium. The *E. coli* K-12 strain JC411 $Thy^-(ColE_1)$ used in these experiments has been described previously (18). The low-phosphate medium (19) used to grow the bacteria contained 1.5 g of KCl, 5.0 g of NaCl, 1.0 g of NH_4Cl, 12.1 g of Tris (base), 246 mg of $MgSO_4$, 27 mg of KH_2PO_4, 2 g of glucose, 2 mg of thymine, and 4 g of phosphate-free bactopeptone per liter at pH 7.4.

Growth of Cells and Labeling of DNA. Cells were grown at 37° with aeration by shaking from a 2% inoculum of an overnight culture in the same medium. For ^{32}P-labeling of normal supercoiled $ColE_1$ DNA, carrier-free $H_3{}^{32}PO_4$ was added to 400- to 600-ml cultures at levels of 25–40 μCi/ml at the time of inoculation. Chloramphenicol-generated supercoiled $ColE_1$ DNA was labeled by adding either $H_3{}^{32}PO_4$ (40–60 μCi/ml) or [^3H]thymine (3 μCi/ml) immediately upon, or 2–2.5 hr after, addition of solid chloramphenicol at concentrations between 150 and 300 μg/ml to growing cultures at a cell density of 3×10^8 cells per ml. Cells were harvested about 18 hr after the addition of chloramphenicol.

Purification of Supercoiled $ColE_1$ DNA. Cleared lysates were prepared as previously described (20) and subjected to ethidium bromide–cesium chloride buoyant density centrifugation (21) to give a discrete band of supercoiled DNA (22). Two or three successive bandings in dye-buoyant density gradients were necessary to remove virtually all of the labeled RNA in the case of ^{32}P-labeled cultures. Ethidium bromide was extracted with cold CsCl-saturated isopropanol, and the CsCl was removed by dialysis against TESP [50 mM Tris·HCl (pH 8)–5 mM EDTA–50 mM NaCl–50 mM K_2HPO_4]. The supercoiled $ColE_1$ DNA was then sedimented through neutral sucrose density gradients, either 5–20% in a Spinco SW 50.1 rotor at 45,000 rpm for 160 min at 15°, or 15–50% in a Spinco SW 27 rotor at 25,000 rpm for 17 hr at 4°. Sucrose gradients contained 50 mM Tris·HCl (pH 8)–0.55 M NaCl–5 mM EDTA. The DNA was finally recovered from the sucrose gradient fractions by precipitation at −18° with two volumes of ethanol after addition of 0.3 N sodium acetate and 50 μg/ml of carrier tRNA.

Analysis of Alkali- and RNase-Sensitive $ColE_1$ DNA. Incubation of purified DNA samples at pH 13 or with RNase A and analysis of this treated DNA on neutral 5–20% sucrose gradients were performed as described by Blair *et al.* (17).

Determination of Release of Ethanol-Soluble Radioactivity by Alkaline and RNase Digestion. Purified ^{32}P-labeled normal or CM-$ColE_1$ DNA was redissolved in TES [50 mM Tris·HCl (pH 8)–5 mM EDTA–50 mM NaCl] after precipitation with ethanol. For alkaline digestion, 100 μl of $ColE_1$ DNA (3 to 4 $\times 10^5$ ^{32}P cpm) in TES were added to 100 μl of 0.6 N KOH

Abbreviations: $ColE_1$, colicinogenic factor E_1; CM-$ColE_1$ DNA, $ColE_1$ DNA synthesized in the presence of chloramphenicol; RSV, Rous sarcoma virus.

and incubated at 37° for 22–24 hr. As a control an equal amount of DNA was diluted with TES and incubated similarly. For RNase treatment, $MgCl_2$ (final concentration 20 mM) and 5 μl of RNase H solution were added to 100 μl of DNA (3 to 4 × 10^5 ^{32}P cpm) in TES; after incubation at 37° for 1 hr the reaction was terminated by the addition of Pronase at 2 mg/ml in TES. Incubation was continued overnight at room temperature. The DNA was then irreversibly denatured by raising the pH to 13 with 0.2 M KH_2PO_4–1.0 N NaOH and immediately neutralizing with 1 N HCl. RNase A (pretreated by incubation at 100° for 5 min) was added at about 3 mg/ml and incubation continued at 37° for 5 hr, followed by the addition of Pronase at 2 mg/ml for a further 30 min at 37°. At the end of the digestion reactions, sodium acetate and tRNA (final concentrations of 0.3 M and 50 μg/ml, respectively) were added to the 200-μl reaction mixtures, followed by the addition of 400 μl of cold ethanol. After storage at −18° overnight, the DNA precipitates were pelleted by centrifugation at 16,300 × *g* for 20 min in a Sorvall HB4 swinging-bucket rotor. Radioactivity in supernatants and precipitates was counted in 10 ml of Triton scintillation fluid [1 liter of toluene, 600 ml of Triton X-100, 168 ml of water, and 3.5 g of 2,5-diphenyloxazole (PPO)].

Electrophoretic Separation of Products of Alkaline Digestion. Purified ^{32}P-labeled CM-$ColE_1$ DNA was redissolved after ethanol precipitation in 20 μl of 0.3 N KOH and incubated in a sealed glass capillary tube for 18 hr at 37°. Samples of ^{32}P-labeled tRNA were similarly treated to monitor the complete hydrolysis of RNA to monoribonucleotides. Digests were fractionated by high voltage electrophoresis at pH 3.5 on DEAE–cellulose paper (Whatman DE81), as described by Sanger and Brownlee (23).

Preparation of RNase-Gapped DNA. Purified ^3H-labeled CM-$ColE_1$ DNA was incubated for 60 min at 37° in a 500-μl reaction mixture containing 30 μg of $ColE_1$ DNA, 30 μg of RNase H, 33 mM Tris·HCl (pH 7.5), 2 mM EDTA, 11 mM $MgCl_2$, and 5% glycerol (v/v). The reaction was terminated by the addition of 12.5 mM EDTA. To the reaction mixture were added 400 μg/ml of RNase A and incubated at 37° for 90 min, followed by the addition of more RNase A at a concentration of 600 μg/ml and further incubation for 90 min. Treatment was terminated by incubation for 30 min at 37° with 2.5 mg/ml of Pronase and the reaction mixtures were run to equilibrium in ethidium bromide–cesium chloride buoyant density gradients to separate RNase-resistant supercoils from RNase-nicked molecules.

Incorporation of Single ^{32}P-labeled Deoxyribonucleotides into RNase-Gapped $ColE_1$ DNA. Twenty-five-microliter reaction mixtures contained about 50 ng of Rous sarcoma virus (RSV) DNA polymerase, 50–200 ng of $ColE_1$ DNA, 0.5 to 2.5 × 10^6 cpm of a single [α-^{32}P]deoxyribonucleoside triphosphate at 2–4 μM, and the other three unlabeled deoxyribonucleoside triphosphates at 10 μM each, 100 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, and 2% mercaptoethanol. Incubation was at 37° for 60 min, and the reaction was terminated by addition of 100 μl of cold 0.1 M sodium pyrophosphate. Five microgram of T7 DNA was added to each reaction mixture as carrier and cold perchloric acid was added to a final concentration of 6%. The mixtures were decanted onto 24-mm diameter glass fiber filters and the liquid was removed by vacuum aspiration. Filters were washed with about 100 ml of cold 3.5% perchloric acid, 20 ml of 2 N HCl, and 20 ml of 95% ethanol, and dried. Radioactivity was determined in Omnitol (4 g of Omnifluor per liter of toluene).

TABLE 1. *Release of nucleotides by alkaline and RNase digestion of CM-$ColE_1$ DNA**

Treatment	Ethanol-precipitable radioactivity, 10^5 cpm	Ethanol-soluble radioactivity	
		cpm	% of total plasmid DNA
	Normal $ColE_1$ DNA		
Control	4.26	49	0.011
Alkali	4.26	47	0.011
RNase	4.23	44	0.010
	CM-$ColE_1$ DNA		
Control	4.41	39	0.009
Alkali	4.48	420	0.096
RNase	4.41	343	0.079

* In this preparation of supercoiled CM-$ColE_1$ DNA approximately 35% of the molecules contained RNA, as indicated by the conversion of this proportion of molecules to the open-circular DNA form upon treatment with RNase or alkali. Conditions of alkali or RNase treatment are given in *Materials and Methods.*

Total Incorporation of ^{32}P-Labeled Deoxyribonucleotides into RNase-Gapped $ColE_1$ DNA. Reactions were performed as described above except that all four α-^{32}P-labeled deoxyribonucleoside triphosphates were present and the total reaction volume was 50 μl. The reaction mixtures were dialyzed overnight at room temperature against 50 mM Tris·HCl (pH 7.5)–300 mM sodium acetate and the DNA was precipitated with ethanol and redissolved in water.

Complete Digestion of DNA and Analysis of Products. Five-microliter portions of solutions of DNA containing 5 to 15 × 10^3 ^{32}P cpm from the incorporation of all four labeled deoxyribonucleotides were dried under vacuum on polyethylene and redissolved in 10 μl of 20 mM Tris·HCl (pH 7.5)–10 mM $MgCl_2$. Ten microliters of 1.6 mg/ml of pancreatic DNase I were added and the mixtures were incubated in capillary tubes at 37° for 3 hr. The reaction mixtures were again dried under vacuum, redissolved in 20 μl of 0.2 mg/ml of snake venom phosphodiesterase in 20 mM Tris·HCl–10 mM $MgCl_2$–10 mM ATP (pH 7.5) and incubated for 1 hr at 37°. The reaction mixtures were subjected to electrophoresis as described by Boyer *et al.* (24).

Ligase Treatment. E. coli ligase at a final concentration of 50 units/μl was added to 5 μl of a solution of DNA (5 to 15 × 10^3 ^{32}P cpm) in which all four labeled deoxyribonucleoside monophosphates were incorporated. The total reaction volume of 100 μl also contained 0.3 M Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 32 μM NAD, 50 μg/ml of BSA, and 10 mM $(NH_4)_2SO_4$. Incubation was for 2 hr at 37° and the reaction was terminated by incubation with 2 mg/ml of Pronase for 30 min at 37°.

Reagents and Enzymes. [*Methyl*-^3H]thymine (40–60 Ci/mmol) and carrier-free $H_3{}^{32}PO_4$ in HCl were obtained from New England Nuclear Corp., Boston, Mass., [α-^{32}P]deoxyribonucleoside-5′-triphosphates were from International Chem-

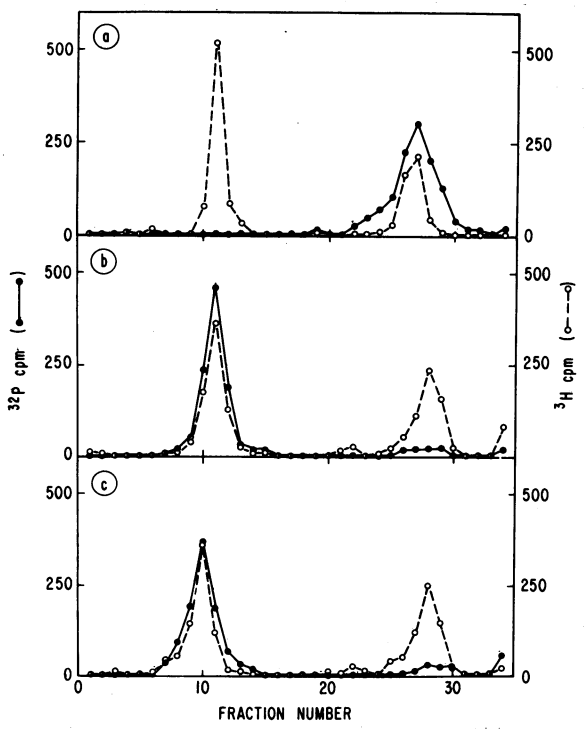

FIG. 1. Ligase sealing of RNase-gapped CM-ColE$_1$ DNA after incorporation of [α-^{32}P]deoxyribonucleotides. Samples of DNA (*a*) before ligase treatment, (*b*) after ligase treatment, and (*c*) after ligase treatment and incubation of the DNA for 16 hr at room temperature at pH 13, were analyzed on alkaline (0.3 N NaOH) 5-20% sucrose density gradients. Centrifugation was carried out in a Spinco SW 50.1 rotor at 45,000 rpm for 100 min at 4°. Alkali-nicked, ^{3}H-labeled CM-ColE$_1$ DNA (17) was added to each sample as a marker. The level of ^{3}H in the RNase-gapped DNA was insignificant. Ten-drop fractions were collected from the bottom of the gradients directly in Triton scintillation fluid.

ical and Nuclear Corp., Irvine, Calif. (specific activities were: dCTP, 22.5 Ci/mmol; dATP, 19.5 Ci/mmol; dGTP, 14.5 Ci/mmol; and dTTP, 9.5 Ci/mmol), unlabeled deoxyribonucleoside triphosphates were from Sigma, St. Louis, Mo., and ribonuclease A (beef pancreas, 3000 units/mg; code RASE), deoxyribonuclease I (bovine pancreas, 2000 units/mg; code DPFF) and venom phosphodiesterase I (potency 0.3; code VPH) were all from Worthington Biochemical Corp., Freehold, N.J. The RNase H used in this work was the generous gift of Dr. Walter Keller; the enzyme preparation (50 mg of protein per ml) was purified from KB cells. Two preparations of Rous sarcoma virus DNA polymerase (10 μg of protein per ml), purified as described by Faras *et al.* (25), were generously supplied by Drs. Tony Faras and John Bishop. The specific activity of each preparation was 0.1 units/mg. *E. coli* ligase was kindly provided by Drs. Paul Modrich and Robert Lehman.

TABLE 2. *Electrophoretic separation of the products of alkaline digestion of CM-ColE$_1$ DNA*

Digestion products*	^{32}P cpm	% of total radioactivity†	No. of ribonucleotides per RNase-sensitive molecule
2′(3′)-GMP	493.3	0.086	10.9
2′(3′)-AMP	136.2	0.023	2.9
2′(3′)-CMP	260.6	0.045	5.7
2′(3′)-UMP	290.6	0.051	6.5
P$_i$	239.3	0.042	—
Total monophosphates	1180.7	0.206	26.0

* Products were identified as described in *Materials and Methods.*

† 1.8 × 10^6 cpm of alkali-digested ^{32}P-labeled CM-ColE$_1$ DNA were applied to the DEAE-cellulose paper. Values represent percentage of total radioactivity in the RNase-sensitive fraction (32%) of CM-ColE$_1$ DNA molecules. The DNA preparation employed was different from that used in Table 1.

RESULTS

The Size of the RNA Segment. As a first step in the characterization of RNA-containing supercoiled ColE$_1$ DNA, the size of the RNA segment was estimated by quantitation of the ethanol-soluble radioactivity released by exhaustive alkaline and RNase digestions of CM-ColE$_1$ DNA. Table 1 shows data from an experiment in which 35% of the supercoiled CM-ColE$_1$ DNA molecules were sensitive to nicking by alkali or RNase A. Only about 0.01% of the total radioactivity remained in the supernatant after ethanol precipitation when normal ColE$_1$ DNA had been incubated at pH 13 or at pH 8 in the presence of RNase H plus RNase A. Since the ColE$_1$ DNA molecule comprises approximately 12,700 deoxyribonucleotides, 0.01% of the total radioactivity represents about one nucleotide per molecule. When CM-ColE$_1$ DNA was incubated at pH 8, the same 0.01% background level was observed in the supernatant after precipitation. However, incubation in alkali or RNase treatment produced about a 10-fold increase in ethanol-soluble radioactivity.

TABLE 3. *Incorporation of deoxyribonucleotides into RNase-treated CM-ColE$_1$ DNA: Use of a single labeled deoxyribonucleotide plus three unlabeled deoxyribonucleotides**

Exp. No.	Polymerase prep.†	pmol of [^{32}P]dNMP incorporated per pmol of RNase-sensitive CM-ColE$_1$ DNA			
		dGMP	dAMP	dCMP	dTMP
1	A	11.5	3.2	6.6	5.1
2	A	8.9	3.0	4.4	6.5
3	A	—	—	4.7	—
	B	—	—	5.8	—
4	B	10.0	—	—	6.2
5	B	8.9	3.1	5.7	6.4
	Average	9.8 ± 1.1	3.1 ± 0.1	5.5 ± 0.3	6.1 ± 0.2

* In each experiment one of the four deoxyribonucleotides was α-^{32}P-labeled and the other three deoxyribonucleotides were unlabeled. The level of radioactivity incorporated into the RNase-gapped molecules in each case was 1 to 12 × 10^3 cpm. Reaction mixtures without enzyme or DNA, or having untreated DNA as the substrate, gave no significant level of incorporation of labeled deoxyribonucleotides. The CM-ColE$_1$ DNA preparation employed here is distinct from the one used in Tables 1 and 2.

† The two polymerase preparations employed are described in *Materials and Methods.*

Within the limits of the experimental measurements, approximately 100% of the DNA was recovered by precipitation in every case. In the experiment shown in Table 1, RNase H plus RNase A solubilized about 0.07% of the total radioactivity in the DNA above background, or 0.20% of the radioactivity in the molecules that can be nicked by alkali or RNase A. This is the equivalent of 25 nucleotides per sensitive CM-ColE$_1$ DNA molecule. Alkali treatment releases slightly more material, representing 0.25% of the RNA-containing molecules. In several other experiments, involving different preparations of CM-ColE$_1$ DNA, this value varied from 0.24 to 0.26%. The discrepancy between the amounts of material released by RNase and by alkali in these experiments is largely due to the release of ^{32}P-labeled inorganic phosphate by alkali treatment, as is indicated in Table 2.

Identification of the Products of Alkaline Hydrolysis of CM-ColE$_1$ DNA. Purified CM-ColE$_1$ DNA labeled with ^{32}P was alkali-treated and fractionated by high voltage electrophoresis. The alkali-released radioactive material migrated as five discrete spots, which were identified as the four ribonucleotide 2′(3′)-monophosphates and inorganic phosphate. The results of an experiment involving a CM-ColE$_1$ DNA preparation in which 32% of the molecules were sensitive to nicking by alkali are shown in Table 2. The total radioactivity released represented 0.25% of the RNA-containing CM-ColE$_1$ DNA, but that in the four nucleotide spots comprised 0.21% of these molecules, equivalent to an average of 26 ribonucleotides per RNA-containing plasmid molecule. The proportions of the individual ribonucleotides are shown in Table 2.

Incorporation of α-^{32}P-Labeled Deoxyribonucleoside Monophosphates into RNase-Gapped CM-ColE$_1$ DNA. Confirmation of the average ribonucleotide composition determined by electrophoretic methods was achieved by two procedures previously used to analyze the single-strand termini generated by the *E. coli* RI and RII restriction endonucleases (24, 26). The ribonucleotides that confer RNase sensitivity on supercoiled CM-ColE$_1$ molecules were first removed by digestion with RNase H plus RNase A. The gapped molecules were then used as a primer template for RSV polymerase and deoxyribonucleotides.

Table 3 shows the results of the first procedure in which the incorporation of each radioactive nucleotide in the presence of the other three unlabeled nucleotides into RNase-gapped DNA was determined. Essentially similar results were obtained with a second preparation of CM-ColE$_1$ DNA. The results, indicating an average of 9.8 GMP, 3.1 AMP, 5.5 CMP, and 6.1 UMP per RNA-containing CM-ColE$_1$ DNA molecule, are similar to the results of the previous experiment (Table 2).

The second procedure involved the incorporation of all four α-^{32}P-labeled deoxyribonucleoside triphosphates into RNase H- plus RNase A-gapped CM-ColE$_1$ DNA by the RSV DNA polymerase, followed by hydrolysis of the DNA to nucleoside-5′-monophosphates by exhaustive digestion with pancreatic DNase I and venom phosphodiesterase I. The 5′-monophosphates were fractionated by electrophoresis and the radioactivity in each nucleotide determined. Table 4 shows the results obtained using two separate preparations of CM-ColE$_1$ DNA. Both DNA preparations gave similar results, and in each case the total and composition of ribonucleotides determined by this procedure were similar to the results summarized in Tables 2 and 3.

TABLE 4. *Incorporation of all four labeled deoxyribonucleotides into RNase-treated CM-ColE$_1$ DNA*

DNA preparation†	pmol of [^{32}P]dNMP incorporated per pmol of RNase-sensitive CM-ColE$_1$ DNA*				
	dGMP	dAMP	dCMP	dTMP	Total
1	10.8	3.1	5.1	5.9	24.9
2	10.6	2.7	5.3	6.5	25.1

* Approximately 750–5000 cpm were recovered in each deoxyribonucleotide spot after electrophoresis and elution. As a control for recovery of released deoxyribonucleotides, uniformly ^{32}P-labeled λ DNA, digested under the same conditions, yielded the expected ratio of deoxyribonucleotides after electrophoresis.

† Two gapped CM-ColE$_1$ DNA preparations, purified from different cultures of bacteria, were employed in these experiments.

To test the efficiency of the RNase H and RNase A treatment in removing all ribonucleotides from the RNA-containing CM-ColE$_1$ DNA, and the effectiveness of the RSV DNA polymerase in catalyzing the incorporation of deoxyribonucleotides corresponding to all of the removed ribonucleotides, the DNA samples were treated with *E. coli* ligase after incorporation of labeled deoxyribonucleotides. Fig. 1 shows the profiles of alkaline sucrose gradient sedimentation velocity analyses of ligase-treated CM-ColE$_1$ DNA that was recovered from the reaction mixture prior to nuclease digestion. It is clear that ligase converted all the DNA from the open form, which sedimented as denatured single-stranded DNA in the gradient (Fig. 1*a*), to the covalently closed form (Fig. 1*b*). Extensive incubation under alkaline conditions that are sufficient to nick a molecule containing a single ribonucleotide (27) failed to cause opening of any of the ligated molecules (Fig 1*c*), indicating a total absence of ribonucleotides. These data indicate that RSV DNA polymerase completely replaced nucleotides in the gap in CM-ColE$_1$ DNA after prior removal of all ribonucleotides by the action of RNase H and RNase A.

Incorporation of Individual Labeled Nucleotides in the Absence of the Other Three Nucleotides. The first nucleotide(s) to be incorporated by RSV DNA polymerase into RNase-gapped ColE$_1$ DNA was determined by measurement of incorporation in a reaction mixture containing a single deoxyribonucleoside triphosphate. In the case of two different RNase-gapped preparations of CM-ColE$_1$ DNA, only dGTP was incorporated to any significant extent, equivalent to three residues of dGMP per molecule of gapped CM-ColE$_1$ DNA. The level of incorporation of the other three deoxyribonucleotides was essentially equivalent to background levels of radioactivity. These data indicate the presence of GMP at the 5′-terminus of the RNA segment in the CM-ColE$_1$ DNA.

DISCUSSION

The covalent linkage of a small segment of RNA to DNA has been demonstrated in several systems *in vivo* in addition to the ColE$_1$ plasmid, including the chromosomal DNA of *E. coli* (28, 29) and cultured mammalian cells (30, 31), and the DNA of bacteriophages T4 (6, 7) and T7 (8). Further-

more, covalently closed circular molecules of mitochondrial DNA (27, 32) have been presumed to contain covalently integrated ribonucleotides from their sensitivity to nicking by alkali and RNase. Cogent support for attributing the role of primer to such small RNA pieces, at least in the case of certain of these DNA replication systems, comes from the ability of inhibitors of RNA synthesis to block the replication of the DNA molecules *in vivo* under conditions where protein synthesis is inhibited (2, 11–15). Similarly, the proposal that the ribonucleotides present at a single site in CM-ColE$_1$ DNA molecules represent primer RNA (17) is supported by the reported inhibition of CM-ColE$_1$ DNA synthesis by rifampicin (11, 12).

Two independent estimates have been made of the average size of the RNA segment in CM-ColE$_1$ DNA. A combination of RNase H and pancreatic RNase A converts the equivalent of about 0.2% of the total radioactivity in ^{32}P-labeled CM-ColE$_1$ containing ribonucleotides to products that are ethanol-soluble (Table 1). Assuming that the specific activity of the ^{32}P-labeled nucleotides in the RNA and DNA moieties of the ColE$_1$ molecule are equal, this represents about 25 nucleotides out of the total 12,700 in the molecule. Similar results were obtained with alkali treatment.

Measurement of levels of RSV DNA polymerase-directed incorporation of each individual α-^{32}P-labeled nucleoside triphosphate in the presence of the other three unlabled triphosphates into RNase-gapped molecules enabled estimates to be made of the number of moles of each nucleotide incorporated per mole of DNA (Table 3). All four nucleotides were incorporated, and the total number and composition of nucleotides obtained by this procedure agreed well with the results of electrophoretic analysis of the released ribonucleotides of uniformly ^{32}P-labeled CM-ColE$_1$ DNA and CM-ColE$_1$ DNA molecules that were labeled with all four deoxyribonucleotides after removal of the RNA segment. The agreement is particularly significant in view of the utilization of different preparations of CM-ColE$_1$ DNA for each approach. Data from these three different approaches indicate that the average base composition of the 25–26 nucleotide segment of integrated RNA is 10–11 G, 3 A, 5–6 C, and 6–7 U. RSV DNA polymerase clearly incorporates deoxyribonucleotides sufficient to fill completely, but not in excess of, the gap generated by RNase treatment in the CM-ColE$_1$ molecules, since ligase is able to seal the product.

Grossman *et al.* (27) determined that alkali sensitivity of supercoiled mitochondrial DNA is due to approximately 10 ribonucleotides per molecule distributed at several sites in both strands of the DNA. The RNA attached to nascent DNA fragments in *E. coli* has been estimated, on the basis of density shifts in $CsSO_4$ equilibrium gradients, to be 50–100 nucleotides in length (29). While the average number of 25–26 ribonucleotides characterized in this study is derived from one strand of any particular DNA molecule, the population of CM-ColE$_1$ DNA molecules contains ribonucleotides in either DNA strand with equal probability (17). It is not known whether the same number or composition of ribonucleotides is present in each strand, but clearly the data indicate that the RNA segments from the two strands are not completely complementary over the entire length of the RNA segment. There is no evidence of course to rule out the possibility that much larger primer molecules initiate DNA synthesis, but that in conditions of chloramphenicol inhibited protein synthesis only an average of 25 bases remains in the supercoiled molecules. The finding of only GMP at the 5′-terminus of the RNA segment in CM-ColE$_1$ DNA is of interest in that GMP has also been identified at the 5′-terminus of the RNA linked to nascent DNA fragments purified from exponentially growing *E. coli* cells (29).

We thank Drs. Anthony Faras, John Abelson, and Howard Goodman for valuable discussions during the course of this work. We are indebted to Drs. Anthony Faras, John Bishop, and Walter Keller for their generous provision of enzymes, and Drs. Howard Goodman and John Abelson for the use of their facilities. This work was supported by U.S. Public Health Service Research Grant AI-07194 and National Science Foundation Research Grant 6B-29492.

1. Dove, W. F., Inokuchi, H. & Stevens, W. F. (1971) in *The Bacteriophage Lambda*, ed. Hershey, A. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 747–771.
2. Brutlag, D., Schekman, R. & Kornberg, A. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2826–2829.
3. Wickner, W., Brutlag, D., Schekman, R. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 965–969.
4. Staudenbauer, W. L. & Hofschneider, P. H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1634–1637.
5. Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L. L. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2691–2695.
6. Speyer, J. F., Chao, J. & Chao, L. (1972) *J. Virol.* **10**, 902–908.
7. Buckley, P. J., Kosturko, L. D. & Kozinski, A. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3165–3169.
8. Miller, R. C. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1082–1086.
9. Lark, K. G. (1972) *J. Mol. Biol.* **64**, 47–60.
10. Messer, W. (1972) *J. Bacteriol.* **112**, 7–12.
11. Clewell, D. B., Evenchik, B. G. & Cranston, J. W. (1972) *Nature New Biol.* **237**, 29–31.
12. Clewell, D. B. & Evenchik, B. G. (1973) *J. Mol. Biol.* **75**, 503–514.
13. Kline, B. C. (1973) *Biochem. Biophys. Res. Commun.* **50**, 280–288.
14. Messing, J., Staudenbauer, W. L. & Hofschneider, P. H. (1972) *Nature New Biol.* **238**, 202–203.
15. Kline, B. C. (1972) *Biochem. Biophys. Res. Commun.* **46**, 2019–2025.
16. Westergaard, O., Brutlag, D. & Kornberg, A. (1973) *J. Biol. Chem.* **248**, 1361–1364.
17. Blair, D. G., Sherratt, D. J., Clewell, D. B. & Helinski, D. R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2518–2522.
18. Clewell, D. B. & Helinski, D. R. (1970) *Biochemistry* **9**, 4428–4440.
19. Pinkerton, T. C., Paddock, G. & Abelson, J. (1973) *J. Biol. Chem.* **248**, 6348–6365.
20. Katz, L., Kingsbury, D. T. & Helinski, D. R. (1973) *J. Bacteriol.* **114**, 577–591.
21. Radloff, R., Bauer, W. & Vinograd, J. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 1514–1521.
22. Bazaral, M. & Helinski, D. R. (1968) *J. Mol. Biol.* **36**, 185–194.
23. Sanger, F. & Brownlee, G. G. (1967) in *Methods in Enzymology* eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. XII, part A, pp. 361–381.
24. Boyer, H. W., Chow, L. T., Dugaiczyk, A., Hedgpeth, J. & Goodman, H. M. (1973) *Nature New Biol.* **244**, 40–43.
25. Faras, A. J., Taylor, J. M., McDonnell, J. P., Levinson, W. E. & Bishop, J. M. (1972) *Biochemistry* **11**, 2334–2342.
26. Hedgpeth, J., Goodman, H. M. & Boyer, H. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3448–3452.
27. Grossman, L. I., Watson, R. & Vinograd, J. (1973) *Fed. Proc.* **32**, 529 Abstr.
28. Sugino, A. & Okazaki, R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 88–92.
29. Sugino, A., Hirose, S. & Okazaki, R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1863–1867.
30. Sato, S., Ariake, S., Saito, M. & Sugimura, T. (1972) *Biochem. Biophys. Res. Commun.* **49**, 827–834.
31. Fox, R. M., Mendelsohn, J., Barbosa, E. & Goulian, M. (1973) *Fed. Proc.* **32**, 529 Abstr.
32. Miyake, M., Koide, K. & Ono, T. (1973) *Biochem. Biophys. Res. Commun.* **50**, 252–258.