

Origin of replication of colicin E1 plasmid DNA

(cleavage map/nucleotide sequence/polynucleotide kinase/primer)

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ABSTRACT Cleavage maps of colicin E1 plasmid DNA and its smaller derivative, pNT1 DNA, were constructed by using restriction endonucleases. The nucleotide sequence of a region that contains the origin of replication was determined. The site of the nucleotide from which DNA replication is initiated was determined with 6S L-fragments, the DNA fragment first made on colicin E1 plasmid DNA. The fragments were labeled with [γ - 32 P]ATP and polynucleotide 5'-hydroxyl-kinase (ATP:5'-dephosphopolynucleotide 5'-phosphotransferase, EC 2.7.1.78) at the 5'-OH groups which were uncovered by alkali treatment. The site is one of three consecutive nucleotides, dA, dA, and dC, located at a unique position. One or a few rA residues were found to be attached to some of the DNA molecules. The transition from the primer RNA to DNA occurs in a region consisting of a segment of five A residues. Both sides of this segment are rich in G and C.

Colicin E1 plasmid (Col E1) is an *Escherichia coli* plasmid with a molecular weight of approximately 4 million (1). *In vitro* replication (2, 3) of the plasmid DNA is initiated at a specific region on the H strand by the formation of a 6S L-fragment (4-6). Synthesis of 6S L-fragments depends on prior RNA synthesis by *E. coli* DNA-dependent RNA polymerase but translation of the RNA is not required (3). The RNA is assumed to serve as a primer for the DNA synthesis.

In order to locate the origin where DNA synthesis of a 6S L-fragment is initiated, the nucleotide sequence of the region containing the origin was determined and the transition point from RNA to DNA on the fragment was located within this sequence.

MATERIALS AND METHODS

Plasmids. Col E1 is the plasmid in *E. coli* A745 (2). Plasmid pNT1 was isolated by transformation of *E. coli* C600 *thy* with an *EcoRI* digest of plasmid pML21 (Col E1-*kan*) (7). This plasmid is probably identical to pVH51 (8).

Enzymes. The restriction endonucleases used were *Hae* II, *Hae* III, *Hha* I, *Hinf*I and *Hpa* II prepared as described (9) and *EcoRI* (Miles). DNA was digested in 20 mM Tris-HCl, pH 7.9/10 mM MgCl₂/5 mM dithiothreitol/bovine serum albumin, 500 μ g/ml (60 mM NaCl was added for digestion with *EcoRI*). The 5' ends of polynucleotides were phosphorylated with ATP or [γ - 32 P]ATP (about 1 Ci/ μ mol; ICN) and polynucleotide kinase (ATP:5'-dephosphopolynucleotide 5'-phosphotransferase, EC 2.7.1.78) prepared as described (10) in 50 mM Tris-HCl, pH 9.5 (buffer at pH 8.0 was also used, as indicated)/10 mM MgCl₂/5 mM dithiothreitol at 0° or 37°. Phosphorylation by exchange of 5'-P_i groups was minimized by the use of the low temperature (11) and the high pH (12). When phosphorylation of *Hae* II/*Hae* III generated fragments (approximately 5 pmol of 5' termini per ml) of pNT1 DNA was performed at 0° for 20 min, the extent of labeling was less than 2% of that of the fragments previously treated with *E. coli* alkaline phosphatase (Worthington). The absence of enzyme activities that degrade RNA or remove 5'-[32 P]P_i-groups of RNA

in the kinase and *Hae* III preparations was shown by using 5'- 32 P-labeled yeast tRNA (5 pmol of 5' termini per ml) under the conditions of the respective treatments of 6S fragments. Nucleic acids were digested with snake venom phosphodiesterase (Worthington) in 30 mM Tris-acetate, pH 8.8/30 mM magnesium acetate.

Cleavage Maps and Nucleotide Sequences. The maps were constructed by analysis of fragments formed by digestion of the plasmid DNAs with restriction enzymes. The nucleotide sequence of 5'- 32 P-labeled DNA was determined as described (13).

Labeled 6S L-Fragments. Cell extracts were made from *E. coli* NT525 *pnp rns end* (14). A reaction mixture (4) (0.3 ml) containing Col E1 DNA (10 μ g/ml) and [α - 32 P]dTTP (about 10 Ci/mmol) was incubated for 45 min at 30°. The reaction mixture also contained glycerol (10% vol/vol) and spermidine (2 mM) to stimulate synthesis of replicative intermediates containing 6S fragments and suppress further replication of the intermediates (3, 4). After addition of 0.1 volume each of 3 M sodium acetate (pH 7.5) and 0.2 M EDTA containing 2.5% (wt/vol) sodium dodecyl sulfate, the solution was shaken with chloroform/isoamyl alcohol, 24:1 (vol/vol), and centrifuged at 10,000 \times g for 10 min. The DNA that was precipitated with ethanol was dissolved in 0.3 ml of buffer A (10 mM Tris-HCl, pH 8.0/0.2% sodium dodecyl sulfate) and applied to a column (0.9 \times 15 cm) of Sepharose 2B. Sodium acetate, pH 6.0, was added to the excluded fraction to 0.3 M and the DNA was precipitated with ethanol. The precipitate was dissolved in 0.3 ml of buffer A, heated at 90° for 2 min to release 6S fragments from replicative intermediates, and cooled quickly (4). The sample was applied to a column (0.7 \times 17 cm) of Sepharose 2B. The DNA in the fractions with *K_d* values of 0.3 to 0.7, representing approximately 75% of the labeled DNA, was pooled. The labeled DNA had an average sedimentation constant of approximately 6 S. NaCl was added to 0.5 M and the solution was heated at 64° for 5 hr. The solution was applied to a column (0.7 \times 0.2 cm) of hydroxylapatite (Bio-Rad, HTP). After a washing with 0.01 M sodium phosphate, pH 6.8, single-stranded DNA was eluted in approximately 0.8 ml of 0.17 M phosphate. This step eliminated 6S H-fragments that had hybridized to 6S L-fragments. The fragments were annealed at 64° for 5 hr with slightly more than an equivalent amount of H-strands of Col E1 DNA in a solution containing approximately 0.5 M CsCl. The annealed DNA was applied to a column of hydroxylapatite and washed with 0.17 M phosphate. The hybridized DNA was eluted in about 0.8 ml of 0.4 M phosphate and freeze-dried. The DNA was dissolved in 0.1 ml of buffer A and applied to a column (0.7 \times 15 cm) of Sephadex G-50. The DNA that was excluded from the column was precipitated with ethanol together with 50 μ g of tRNA.

3 H-Labeled 6S fragments were similarly prepared from 6 ml of a reaction mixture containing [*methyl*- 3 H]dTTP (about 1 Ci/mmol); 6S fragments that were eluted from the second column of Sepharose 2B were self-annealed and applied to a column of hydroxylapatite. NaCl was added to 0.5 M to the

Abbreviation: Col E1, colicin E1 plasmid.

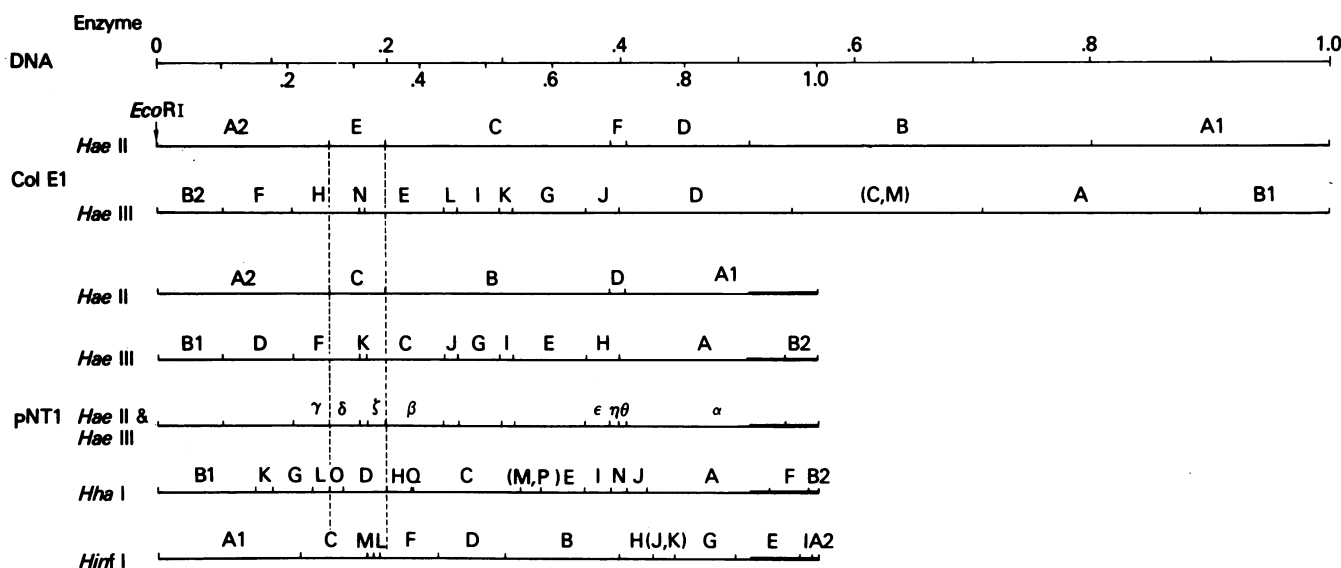


FIG. 1. Cleavage maps for Col E1 and pNT1 DNAs. The segments formed are named alphabetically in order of decreasing size. The two segments generated by digestion with *EcoRI* are given the number 1 or 2 for small or large, respectively. The segments that have ends formed by *Hae II* and *Hae III* are named by the Greek alphabet in order of decreasing size. The region indicated by the bold line is non-Col E1 DNA that is present in pML21 (8). The maps are oriented with respect to the single *EcoRI* site, and the 5' end of the L-strand is assigned to the left end (6).

material eluted from the column with 0.17 M phosphate and the solution was applied to a column (0.7 × 0.4 cm) of nitrocellulose (15). The column was washed with 10 mM Tris-HCl, pH 8.0/0.5 M NaCl/1 mM EDTA to remove RNA, and DNA was eluted with 1 mM Tris-HCl, pH 8.0, and freeze-dried. After phosphorylation in various ways with [γ - ^{32}P]ATP and polynucleotide kinase, these fragments were hybridized to H-strands of Col E1 DNA as described above.

Other Methods. Gel electrophoresis to fractionate native DNA fragments was carried out in a slab gel of 6% polyacrylamide (5.7% polyacrylamide and 0.3% methyl bis-acrylamide) with 90 mM Tris-borate, pH 8.3/1 mM EDTA. Polyethyleneimine-cellulose thin-layer chromatography was performed as described (16).

RESULTS

Cleavage Maps and Nucleotide Sequences. Cleavage maps for Col E1 and pNT1 DNAs are presented in Fig. 1 (experimental details will be published separately by H. Ohmori and J. Tomizawa). Some cleavage maps of Col E1 DNA have been published (17). In this paper, the segments referred to are those of pNT1. Because the origin of replication assigned by electron microscopic examination of replicative intermediates is located approximately 17% of the length from the left end of Col E1 DNA (6), it probably lies in the *Hae II*-C segment.

The nucleotide sequence of the *Hae II*-C segment was determined by using the 5'- ^{32}P -labeled polynucleotides shown in Fig. 2. An example of autoradiographs of chemically modified polynucleotides used for determination of the sequence is shown in Fig. 3. The sequence of the complementary strand, whenever available, provided confirmation. The sequence of the *Hae II*-C segment is presented in Fig. 4.

Location of the 5' Ends of 6S L-DNA. A sample of 6S L-fragments labeled with [α - ^{32}P]dTMP was hybridized to H-strands of Col E1 DNA. The DNA was cleaved with a mixture of *Hae II* and *Hae III* and fractionated (Fig. 5a). Radioactive bands were found at the position of the *Hae III*-K, ζ , and β fragments. Radioactivity of the *Hae III*-K band was approximately 40% of that of the ζ band (data not shown). A band was not observed at the position of the δ fragment. These results

show that the 5' end of 6S L-DNA is located in the δ segment or in the *Hae III*-K segment very close to its left end.

In the following experiments the site of 5'-OH groups of the DNA uncovered by alkali treatment of ^3H -labeled 6S L-fragments was determined. First, the fragments were phosphorylated and then treated with alkali and the uncovered 5'-OH groups of the DNA were labeled with ^{32}P . The labeled DNA was hybridized to H-strands of Col E1 DNA, digested with *Hae III*, and fractionated. The results shown in Fig. 5b indicate that polynucleotides of 14, 15, and 16 residues (named N_{14} , N_{15} , and N_{16} , respectively) were the major components. The bands of N_{12} and N_{13} contained few percent of the total radioactivity and those found around the position of N_{60} contained several percent. Relative intensity of three major bands varied with different preparations of 6S L-fragments. When the labeled DNA was cleaved by *Hpa II* instead of *Hae III*, polynucleotides of 54, 55, and 56 residues were the major components (data not shown). These results suggest that most of the labeled 5' ends are located at 14, 15, or 16 residues from the right end of the δ segment. If so, the 5' ends of these fragments should be a dC,

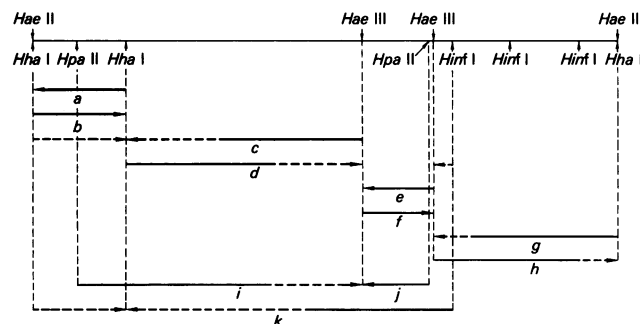


FIG. 2. Labeled strands used for determination of the nucleotide sequence of the *Hae II*-C segment. An arrow indicates a 5'- ^{32}P -labeled strand aligned in the 5'-to-3' direction. The strands *a*, *b*, *e*, *f*, *g*, and *h* were obtained by the strand separation technique (13); other strands were obtained by secondary cleavage of 5'- ^{32}P -labeled double-stranded DNA. The strands indicated by a discontinuous arrow, which were obtained simultaneously were not used. The sequence of the discontinuous portion of an arrow was not determined.

Table 1. Radioactive label in 5' ends of fragments

5'-dNMP	cpm		
	N ₁₄	N ₁₅	N ₁₆
dA	<3	159	240
dG	<3	5	<3
dC	100	<3	<3
dT	<3	<3	<3

The polynucleotides in the three major bands shown in Fig. 5b were separately extracted (13). They were precipitated with ethanol together with 50 μg of denatured calf thymus DNA and digested for 1 hr with snake venom phosphodiesterase (0.1 unit in 10 μl of reaction mixture). The 5'dNMPs were separated by thin-layer chromatography with 1 N acetic acid and 0.3 M LiCl (16). Radioactivity of each spot of 5'dNMPs was measured.

dA, or dA residue, respectively. The results presented in Table 1 show that this is the case.

To study the structure at the 5' ends, 6S L-fragments that had been treated with ATP and polynucleotide kinase were labeled at the 5' ends after having been treated with alkali (sample 2) or alkaline phosphatase (sample 3). These labeled preparations were hybridized to H-strands of Col E1 DNA, cleaved with *Hae* III, and examined (Fig. 6, channels 1 to 3). These preparations gave major bands at N₁₄, N₁₅, and N₁₆. The label found in sample 1, which had not been treated with alkali or alkaline phosphatase, was less than one-fifth of that found for sample 2, indicating that phosphorylation by the phosphate exchange reaction had a minor role in the labeling of sample 2. The longer polynucleotides were more radioactive than the shorter ones for sample 3 and *vice versa* for sample 2, indicating that alkali treatment shortened the length of some of polynucleotides to be labeled. For a preparation of 6S L-fragments treated with alkaline phosphatase and then labeled more heavily, minor bands at N₁₇ and N₁₈ were seen (channel 5). These minor bands were not seen for the preparations treated with alkali before or after labeling (channels 4 and 6). These bands remained after treatment with RNase A and RNase T₁ (channel 7). Digestion of the polynucleotides N₁₇ and N₁₈ with snake venom phosphodiesterase (oligonucleate 5'-nucleotidohydrolase, EC 3.1.4.1) gave predominantly [5'-³²P]AMP by two-dimensional thin-layer chromatography (16) (data not shown). These results indicate that some 6S L-fragments have one or a few rA residues at the 5' ends. The bands obtained with the sample that had been treated with alkaline phosphatase and alkali before labeling (channel 4) show that 5' ends of DNA portions of the fragments were located almost exclusively at the three consecutive nucleotides.

DISCUSSION

Alkali treatment of 6S L-fragments exposed 5'-OH groups that had been covered. The uncovered 5' end of the molecules is either dA, dA, or dC located at a unique position. AMP was detected at the 5' ends of some of 6S L-fragments. A search for 2'(3')rNMPs formed by alkaline hydrolysis of 6S L-fragments labeled with one of [α-³²P]dNMPs has shown that rA-dA and rA-dC linkages were the predominant ribonucleotide-deoxyribonucleotide linkages (R. E. Bird and J. Tomizawa, unpublished data).

Because synthesis of 6S L-fragments necessitates prior RNA synthesis by DNA-dependent RNA polymerase with all four rNTPs (2) and translation of the RNA is not required (3), synthesis of the DNA molecules probably depends on an RNA primer that contains all four rNMPs. In this case, the presence

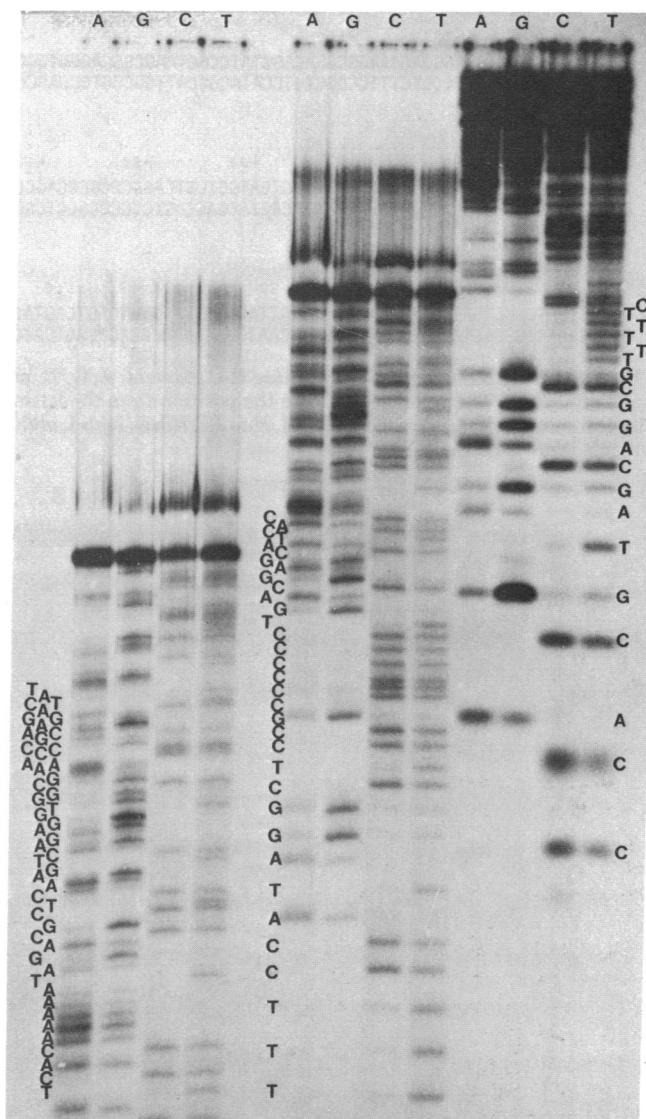


FIG. 3. Radioautograph for determination of the sequence of strand c. The δ fragments that had been treated with alkaline phosphatase were labeled with [γ-³²P]ATP and polynucleotide kinase. They were cleaved with *Hha* I, and the larger fragments were used. Samples A and G were methylated with 0.05 M dimethyl sulfate for 50 and 15 min, respectively, at 20°. After treatment of sample A in 0.1 M HCl at 0° for 60 min and of sample G in 10 mM sodium phosphate, pH 7.0, at 90° for 15 min, polynucleotides were cleaved by heating at 90° for 30 min in 0.1 M NaOH. Sample C in 2.5 M NaCl and sample T in H₂O were treated with 18 M hydrazine at 20° for 30 and 20 min, respectively. Polynucleotides were cleaved by heating at 90° for 60 min in 0.5 M piperidine. The products were fractionated by electrophoresis in a 30 × 40 × 0.15 cm slab gel of 20% polyacrylamide/7 M urea (13). Three sets of samples were run at 20 V/cm for 48, 24, and 7 hr, respectively.

of DNA molecules with a few or no ribonucleotide residue attached means that most or all of the residues of the primer were removed from the DNA product. On the other hand, most of the deoxyribonucleotide residues from which DNA replication was initiated were preserved.

The transition from the primer RNA to 6S L-DNA occurs in a region consisting of a segment of five A residues. The 5' side of the segment, one or two turns of the helix away, is extremely rich in G and C. The 3' side is also rich in G and C. It has been shown that transcription by RNA polymerase tends to terminate in a region rich in A/T pairs and that the region one turn before this region is rich in G/C pairs (cf. 18 and 19). The structural

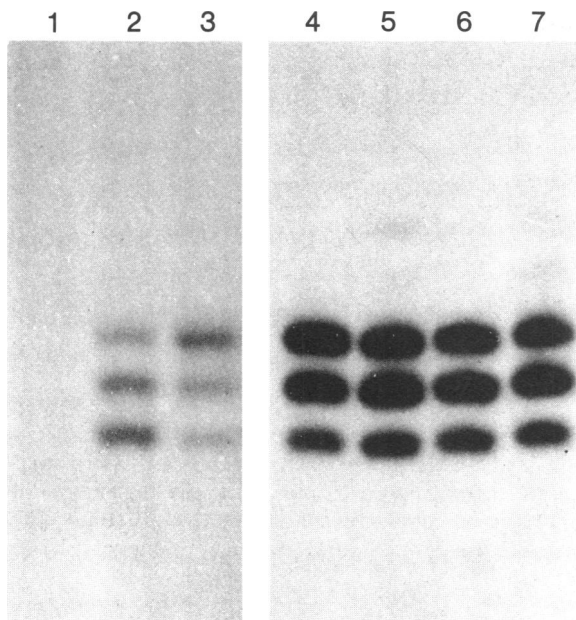


FIG. 6. Portions of electropherograms of *Hae* III cleavage products of 6S L-fragments variously labeled with ^{32}P at their 5' ends. A sample of ^3H -labeled 6S L-fragments (approximately 0.7 pmol) was divided into three equal portions. These samples were treated before labeling as follows: sample 1, 5' ends masked with phosphate; sample 2, masked fragments treated with alkali; sample 3, fragments treated with alkaline phosphatase (1 $\mu\text{g}/\text{ml}$) for 30 min at 37° followed by deproteinization. The DNA in each sample was purified and labeled with ^{32}P as described in the legend to Fig. 5. The labeled DNA that was hybridized to H-strands of Col E1 DNA was digested with *Hae* III. Another preparation of ^3H -labeled 6S L-fragments (approximately 1 pmol) was used for samples 4 to 7. The preparation was treated with alkaline phosphatase followed by deproteinization. A fourth of the material (sample 4) was incubated with 0.3 M NaOH at 37° for 16 hr. Three-fourths of the material (samples 5, 6, and 7) was not treated with alkali. These samples were applied to a column of Sephadex G-50, and DNA was eluted with 1 mM Tris-HCl, pH 8.0, and freeze-dried. The DNA was then phosphorylated with [γ - ^{32}P] ATP and polynucleotide kinase (60 units/ml) at 37° for 20 min in a reaction mixture (40 and 120 μl) at pH 8.0. Fresh kinase (60 units/ml) was added and incubation was continued for an additional 20 min before addition of EDTA to 20 mM. A third of the sample (sample 6) that had not been treated with alkali was treated with NaOH as above. To these samples, 50 μg of tRNA was added and nucleic acids were precipitated with ethanol and dissolved in 0.1 ml of buffer A. After passage through a column of Sephadex G-50, DNA was hybridized to H-strands of Col E1 DNA and digested with *Hae* III. After digestion, the sample that had not been treated with alkali was divided into two portions (samples 5 and 7); sample 7 was treated with 1 μg each of RNase A and RNase T₁ for 30 min at 37° . Approximately 0.01 pmol of 6S L-fragments was placed on each channel. Electrophoresis in a 20% polyacrylamide/7 M urea gel was carried out for 14 hr. Portions of radioautographs which cover the nucleotides of 12 to 21 residues are presented.

site is separated from the origin of replication by approximately 300 nucleotides.

Some Col E1 DNA molecules formed *in vivo* in the absence of protein synthesis contain a segment of ribonucleotides (25). The nucleotide sequences of a portion of such segments have been reported (26). We do not find such sequences in the *Hae* II-C segment or in an additional 900 base pairs in the *Hae* II-B segment. On the other hand, we had found that, in some molecules, ribonucleotides exist in the region of which the sequence has been determined (unpublished data).

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