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Biochemistry

Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H

(transcription/RNA polymerase/DNA polymerase I/RNA·DNA hybrid)

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ABSTRACT **A plasmid that consists of an 812-base-pair segment containing the replication origin of plasmid ColE1 and of a 1240-base-pair segment containing a β-lactamase gene has been constructed. The plasmid DNA has three principal sites where transcription is initiated *in vitro*. One is located in the ColE1 segment 555 nucleotides upstream from the origin. Most transcription from this site extends past the origin; some of the transcripts form hybrids spontaneously with the template at their 3′ portions. Cleavage of these transcripts by RNase H generates 3′ termini at the origin region. When DNA polymerase I is included in the reaction along with RNA polymerase and RNase H, dAMP or dCMP is added directly onto the cleaved RNA molecules, most of which retain the intact 5′ terminus. The addition of a deoxyribonucleotide to the cleaved RNA can be regarded as the first step of ColE1 DNA synthesis. Once it has served as a primer, the RNA is eliminated from the product by RNase H.**

Closed circular DNA of plasmid ColE1 of *Escherichia coli* can be replicated *in vitro* by a soluble enzyme system (1). Replication starts most frequently at any of the three consecutive nucleotides (dA, dA, and dC) located at a unique position on the DNA (2). No plasmid-encoded protein is required for this replication (3). Initiation of replication can be demonstrated with a mixture of three highly purified *E. coli* enzymes: RNA polymerase, DNA polymerase I, and RNase H (4), an enzyme that creates a 3′-OH end by endonucleolytic cleavage of RNA that is hybridized to DNA (5). Previously we proposed (4) that RNA polymerase synthesizes a transcript that is processed by RNase H and then used as a primer by DNA polymerase I. Here we report the identification of such a transcript and demonstrate that it is processed by RNase H to form the primer. The replication of ColE1 has been recently reviewed (6–8).

MATERIALS AND METHODS

Plasmid DNA. Plasmid pNT7 which contains the replication origin of ColE1 and the β-lactamase gene of the Tn3 transposon was derived from pNT5 (9) and pBR322 (10) as described below. Supercoiled molecules of plasmid DNA were purified as described (4).

Enzymes. *E. coli* RNA polymerase (600 units/mg) was the holoenzyme (4, 11). DNA polymerase I (15,000 units/mg) (12) was a gift of A. Kornberg. RNase H (470,000 units/mg) was prepared as described (13) with the following modifications: The first DEAE-cellulose chromatography was performed at pH 8.9 instead of pH 7.5. An additional DEAE-cellulose chromatography was performed at pH 7.5 without a salt gradient. Sephadex G-50 chromatography replaced glycerol gradient centrifugation. $NaDodSO_4$/polyacrylamide gel electrophoresis (14) of the enzyme preparations showed that the RNA polymerase preparation consisted of the α, β, β′, σ, and ω subunits, and that the DNA polymerase I and RNase H preparations each had a single homogeneous component. No RNase III or other contaminating ribonuclease activity could be detected in these preparations by a gel electrophoretic assay that used [α-^{32}P]AMP-labeled transcripts from λb2 DNA as a substrate (personal communication of M. Rosenberg). Other enzymes were commercial preparations.

Nucleotides. ^{32}P-Labeled nucleotides were obtained from New England Nuclear, Amersham, or ICN. To demonstrate addition of a single dNMP to RNA (Fig. 4) it was necessary to purify both the [α-^{32}P]dNTPs and nonlabeled rNTPs. This was carried out by column chromatography with dihydroxyboryl polyacrylamide (Affi-Gel 601, Bio-Rad) (15).

RNA Synthesis and Addition of Deoxyribonucleotides to RNA. The standard reaction mixture (30 μl) for transcription was 25 mM potassium phosphate buffer, pH 7.4/100 mM KCl/8 mM $MgCl_2$/1 mM dithiothreitol/0.5 mM spermidine/10% (vol/vol) glycerol/3 μg of acetylated bovine serum albumin/40 μM each of four rNTPs (usually including [α-^{32}P]-ATP)/0.3 μg of pNT7 DNA/1 μg of yeast RNA/0.15 unit of RNA polymerase. RNase H (0.04 unit) was added to the reaction mixtures where indicated. When RNA synthesis with concomitant DNA synthesis was examined, 10 or 25 μM each of one to four dNTPs and 0.7 unit of DNA polymerase I were added to the standard reaction mixture. Approximate molar ratios of the enzymes to DNA were 3 for RNA polymerase, 0.02 for RNase H, and 3 for DNA polymerase I. Incubation was for 30 min at 30°C. The reaction was terminated by addition of 0.1 vol each of 200 mM EDTA, 1% $NaDodSO_4$, 500 μg of yeast RNA per ml, and 1 M Tris·HCl (pH 7.9). After phenol treatment, the RNA was precipitated with ethanol.

Electrophoresis. The slab gels used for electrophoresis were 3% or 25% polyacrylamide (acrylamide-to-bisacrylamide ratio was 19:1) in 0.1 M Tris borate, pH 8.3/1 mM EDTA/8 M urea. A linear gradient gel of 2–20% polyacrylamide was also used. The 25% gels were 0.4 mm thick, whereas the others were 1.5 mm thick. The length of the slab gels was 38 cm and their width was 16 or 33 cm. Samples dissolved in 1 mM EDTA/5 M urea were heated at 80°C for 3 min immediately before loading. To recover nucleic acids from the gel, the gel was crushed and shaken for several hours with 10 mM Tris·HCl, pH 8.0/0.2% $NaDodSO_4$. The extract was treated with phenol and the nucleic acids were precipitated with ethanol. Electrophoresis on Whatman no. 1 paper or DEAE paper (Whatman DE 81) was carried out in 1% pyridine/10% acetic acid, pH 3.5 (16).

RESULTS

Construction of Plasmid pNT7. Plasmid pNT5 contains the origin of replication of ColE1 DNA and a β-lactamase structural gene (9). To eliminate a region of pNT5 with unknown nucleotide sequence, the plasmid was digested with *Taq* I enzyme and the largest of the three resulting fragments was joined to the *Taq* I-4 fragment of pBR322 (17). This pBR322 fragment contains the region that encodes the NH_2-terminal portion of β-lactamase, whereas the pNT5 fragment contains the region

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that encodes the COOH-terminal portion and the origin of replication. When *E. coli* W3102 *recA* (18) was transformed to ampicillin resistance with the ligated DNA, we obtained a strain (NT531) containing monomeric molecules of a small plasmid. This plasmid, pNT7, consists of an 812-base-pair segment derived from ColE1 (684 base pairs upstream and 128 base pairs downstream of the origin of replication) and a 1240-base-pair segment composed mainly of the β-lactamase gene. The nucleotide sequences of both segments are known (2, 9, 17, 19).

Effect of RNase H on Transcription of pNT7 DNA. Under standard reaction conditions, transcription of pNT7 DNA by RNA polymerase proceeded at a constant rate for 30 min and then at a gradually decreasing rate for an additional 60 min. In the first 30 min of incubation, about 100 pmol of AMP was incorporated into RNA in a 30-μl reaction mixture. When the reaction mixture also contained DNA polymerase I, RNase H, and the four dNTPs, DNA synthesis occurred on about 20% of the template DNA molecules during a 30-min incubation. When RNase H was omitted, significant DNA synthesis was not observed (4).

The transcripts formed many discrete bands when separated by gel electrophoresis (Fig. 1*A*, lanes 1 and 3). The products that were subjected to further analysis are marked as species I–VII. Their sizes are estimated to be approximately 108, 540, 600, 750, 900, 1200, and 2000 nucleotides, respectively. When RNase H was included, a new product (species VIII) was formed, whereas the amounts of most of the molecules that banded between species II and IV and of some species longer than species IV were markedly decreased (lanes 2 and 4). The yields of RNA molecules per DNA molecule were about 1 for species I, 0.1 for species II, 0.15 for species VI, and 0.25 for species VIII. No band of molecules shorter than 108 nucleotides was detectable irrespective of the presence of RNase H in the reaction (lanes 1 and 2). A band that contained 1% of the radioactivity present as species I (4500 cpm) would have been detected in the autoradiogram or by measurement of the radioactivity in gel slices.

When [γ-^{32}P]ATP was used in the reaction, only species I was labeled (data not shown). This transcript, which starts at position −445 and terminates around position −553 (28), is the only major transcript of the L-strand. (The position of a particular nucleotide is the number of nucleotides that separate it from the origin of DNA replication as defined in ref. 2. The sign + or − indicates whether the position is downstream or upstream of the origin.) When [γ-^{32}P]GTP was used, the other major RNA species were labeled (lanes 5 and 6).

The effect of RNase H on the yields of the various transcripts suggested the possibility that some of the transcripts form an RNA·DNA hybrid with the template. This possibility was tested as follows. Transcription in the absence of RNase H was terminated by passing the reaction mixture through a Sephadex G-50 column. When the excluded fraction (containing transcription products, template DNA, and RNA polymerase) was incubated with RNase H, the amounts of various RNA species were altered in much the same way as occurred when RNase H was added to the transcription reaction itself (Fig. 1*B*, lanes 1 and 2). However, if the excluded fraction was heated for 3 min at 75°C prior to addition of RNase H, no such alteration was observed (lanes 3 and 4).

Sites of Initiation of Transcription. The nucleotide sequences of the 5′ ends of some RNA species whose synthesis is initiated with GTP were determined. [γ-^{32}P]GTP-labeled RNAs were partially digested by various RNases and the resultant oligonucleotides were then separated by gel electrophoresis. The 5′ sequence was deduced from the sizes of the labeled digestion products and the specificity of the nucleases employed.

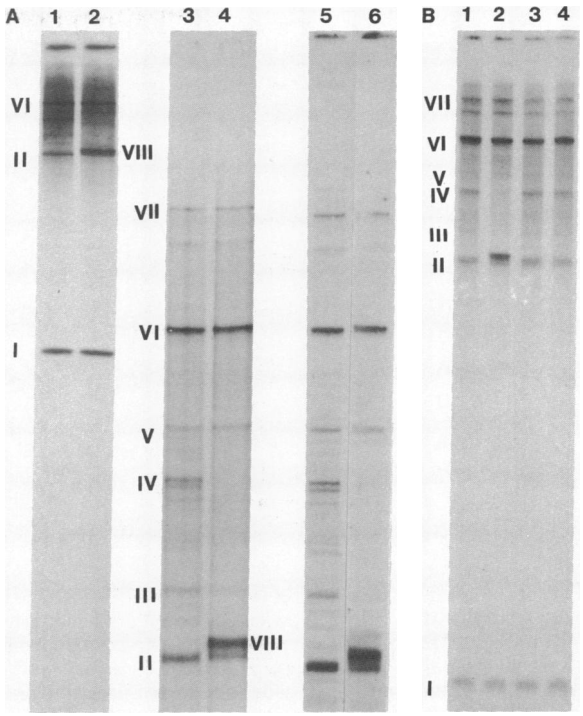

FIG. 1. RNAs synthesized in presence or absence of RNase H (*A*) and products formed by treatment with RNase H after termination of transcription (*B*). (*A*) RNA was synthesized with [α-^{32}P]ATP (1 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) (lanes 1–4) or with [γ-^{32}P]GTP (100 Ci/mmol) (lanes 5 and 6) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of RNase H. Samples 1 and 2 were electrophoresed in a 2–20% polyacrylamide/urea gradient gel at 350 V for 24 hr. Octanucleotide $(Ap)_7Cp$ moved to the bottom. The other samples were electrophoresed in a 3% polyacrylamide/urea gel at 300 V for 22 hr. RNAs used as size standards (data not shown): λ 4S RNA (77 nucleotides) (20), λ 6S RNA (193 nucleotides) (21), *E. coli recA* RNA (1150 nucleotides) transcribed from plasmid pTM-2 (22), a partial *recA* transcript (827 nucleotides) from an *Eco*R1 fragment of pTM-2, and *E. coli* 5S (120 nucleotides) (23), 16S (1550 nucleotides) (24) and 23S (3300 nucleotides) (25) ribosomal RNAs. These were labeled with [α-^{32}P]AMP. Oligoadenylic acids $(Ap)_nA$ (n = 2–10), to which a [5′-^{32}P]pCp residue was added by RNA ligase (26, 27), were also used. A transcript from pNT7 (108 nucleotides) (28) provided an internal marker. (*B*) RNA was synthesized with [α-^{32}P]ATP (5 Ci/mmol) in the absence of RNase H. The reaction mixture was chilled and immediately loaded onto a Sephadex G-50 column (0.8 cm × 9 cm) equilibrated with a buffer containing the same components as the standard reaction mixture but missing the rNTPs, pNT7 DNA, and RNA polymerase. Excluded fractions were pooled and divided into two portions. One portion was heated at 75°C for 3 min (lanes 3 and 4). Each portion was then divided into two aliquots, to one of which RNase H (2 units/ml) was added (lanes 2 and 4). Al the aliquots were incubated at 30°C for 30 min and then analyzed by electrophoresis in a 3% polyacrylamide/urea gel at 300 V for 10 hr.

The analysis showed that the nucleotide sequence at the 5′ end of both species IV and VIII is 5′-G-C-A-A-A-C-A-A-A-A-A-A-A— (Fig. 2). The only region of the template DNA that contains this sequence extends from position −555 towards position 1 (9). Species VI has the 5′-terminal sequence 5′-G-A-U-A-A-A-U-G— (Fig. 2) which is present in pNT7 DNA beginning 36 nucleotides upstream from the first codon for β-lactamase (17) and nowhere else. Thus, species VI must be a messenger RNA for the enzyme. Transcription of species II, III, V, and VII starts at position −555 (data not shown).

The 3′ End of Species VIII RNA. Synthesis of species II begins at position −555 and terminates near the origin of replication. Species VIII starts at the same position but is about 15

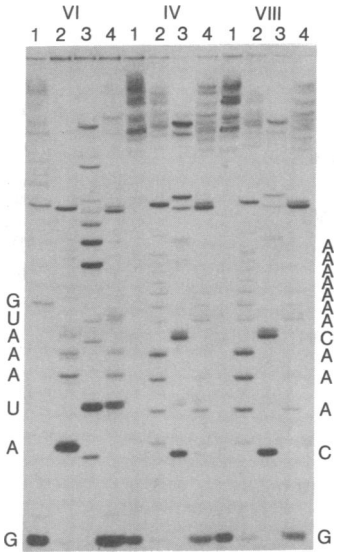

FIG. 2. Partial RNase digests of [γ-^{32}P]GTP-labeled RNAs. RNA species labeled with [γ-^{32}P]GTP, whose electrophoretic patterns are shown in Fig. 1, lanes 5 and 6, were used. RNAs recovered from the gel were digested by various RNases as described (29) except for the increased amounts of enzymes used. Four sets of partial digests by different RNases were made from species IV, VI, and VIII (indicated at the top of the figure). The labeled RNA (about 500 cpm) and carrier tRNA (5 μg) in a 5-μl reaction mixture were digested by 30 ng of RNase T1 in 0.1 M Tris·HCl, pH 7.5/10 mM EDTA (lane 1), by 0.01 unit of RNase U2 in 20 mM sodium citrate (pH 3.5) (30) (lane 2), by 3 ng of RNase A in the same buffer used for RNase T1 digestion (lane 3), or by 0.5 unit of RNase Phy I in 10 mM sodium acetate, pH 5.0/1 mM EDTA (lane 4). Samples were incubated for 30 min at 0°C except for the reaction with RNase Phy I which was carried out at 25°C. The digestion products were separated in a 25% polyacrylamide/urea gel at 2000 V for 5 hr. The assignment of nucleotides is presented to the left (for species VI) and to the right (for species IV and VIII). The additional bands present in some digests are due to slight contamination of the RNAs, one with another.

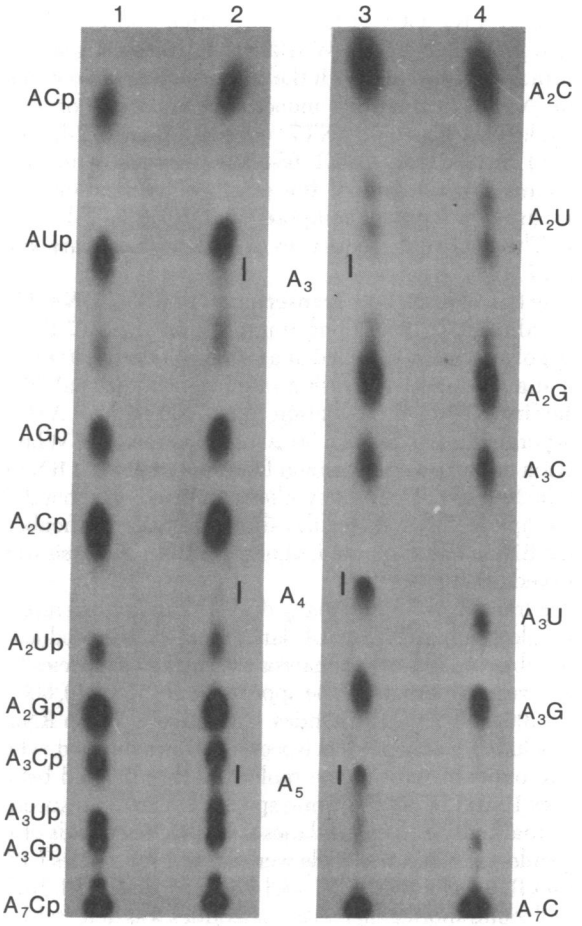

FIG. 3. Separation of oligonucleotides on DEAE-paper. [α-^{32}P]AMP-labeled RNAs (70 Ci/mmol) were prepared in the presence or absence of RNase H and fractionated by gel electrophoresis as described for Fig. 1, lanes 3 and 4. Species II and VIII were recovered from the gel slices. RNA in 5 μl of 10 mM Tris·HCl, pH 8.0/0.1 mM EDTA/5 μg of tRNA/0.2 μg of poly(A) was digested with 0.2 μg each of RNase A and RNase T1 at 37°C for 2 hr. The products were treated with 0.1 M HCl at 37°C for 30 min to break down any cyclic phosphates (16). After drying under decreased pressure, the products were dissolved in 50 mM triethanolamine bicarbonate. Each sample was divided into two equal portions, one of which was treated with bacterial alkaline phosphatase (3 μg at 37°C for 2 hr). Four samples (10,000 cpm each), together with oligonucleotide markers, $(Ap)_nA$ (n = 1–4), were electrophoresed on DEAE-paper (40 × 80 cm) at 1000 V for 18 hr. Lanes 1 and 4 contain products of species II RNA, and lanes 2 and 3 contain those of species VIII RNA. The products in lanes 3 and 4 had been treated with alkaline phosphatase. The spots were assigned to the indicated oligonucleotides (phosphate groups in phosphodiester bonds omitted) by virtue of their known relative mobilities (16). Bars in lanes 2 and 3 represent the positions of spots of nonlabeled A_3, A_4, and A_5. No further effort was made to identify some oligonucleotides present in digests of both species II and VIII. Spots were cut from the paper and their radioactivity was measured. The material recovered from the spot of $(Ap)_2A$ in lane 2 was electrophoresed after treatment by alkaline phosphatase to estimate the radioactivity present as $(Ap)_2A$. For calculation of the molar yield of $(Ap)_3A$ or $(Ap)_4A$, the difference in the radioactivities of the corresponding spots in lanes 1 and 2 was used. Their molar yields were calculated by using the radioactivity (800 cpm) in $(Ap)_2Gp$ (containing 11 mol of phosphate derived from ATP in 1 mol of the 555-nucleotide RNA) as a reference value.

nucleotides longer than species II. Therefore, oligonucleotides present in RNase digests of species VIII, but not in digests of species II, should come from the region of the 3′ terminus of species VIII. Those lacking a 3′-phosphate should include the terminus itself.

Species II and VIII RNAs labeled with [α-^{32}P]AMP were digested to completion with a mixture of RNase A and RNase T1. Each sample was divided into two portions, one of which was treated with alkaline phosphatase. These samples were electrophoresed on DEAE-paper (Fig. 3). Two oligonucleotides were unique to the digest of species VIII. These comigrated with $(Ap)_3A$ and $(Ap)_4A$ (indicated as A_4 and A_5 in Fig. 3), and their mobility was unchanged by treatment with alkaline phosphatase. The amount of radioactivity in the spots corresponded to a molar yield of about 0.15 and 0.5, respectively. In addition, labeled $(Ap)_2A$ was detected by rechromatography of the material in the spot of $(Ap)_2A$ in lane 2 after alkaline phosphatase treatment to eliminate contaminating ApUp. The molar yield was about 0.05. These results indicate that a majority of the molecules present in species VIII must have their 3′ end at one of the rightmost three adenine residues of the sequence -G-A-A-A-A-A-C- at the origin.

Species VIII RNA as a Primer for DNA Synthesis. It has been shown that DNA fragments synthesized in a cell extract have no more than a few ribonucleotides at their 5′ ends (2). We suspected that most of the RNA portion of the primer RNA·DNA complex was eliminated by RNase H (31). We now find that an intact primer that is attached to DNA can be isolated if DNA synthesis is terminated after addition of one or a few deoxyribonucleotides.

To demonstrate addition of dNMP to the primer, the template pNT7 DNA was incubated with RNA polymerase, RNase H, DNA polymerase I, four rNTPs, and one or two of the four [α-^{32}P]dNTPs. Analysis of the products made in the presence

of [α-^{32}P]dATP or [α-^{32}P]dCTP (Fig. 4A) showed the labeled molecules (species A) having a mobility similar to that of species VIII RNA (lanes 1, 4, and 5). This labeling required RNase H (lanes 5 and 6). No such labeling by [α-^{32}P]dTTP or [α-^{32}P]dGTP was detected (lanes 2 and 3). Species A RNA labeled with [α-^{32}P]dCMP was digested with a mixture of RNase A and RNase T1. This yielded an oligonucleotide having the same mobility as $(Ap)_5dC$ upon electrophoresis in a 25% polyacrylamide/urea gel. Digestion with RNase T2 yielded a radioactive product identified as 2'(or 3')-AMP by electrophoresis on Whatman no. 1 paper and on DEAE-paper (data not shown). This indicates that one residue of dCMP was added to the 3' terminus of species VIII RNA to form a species A molecule.

A small amount of radioactivity was also found in unit-length single-strand linear DNA (not seen in Fig. 4A) probably as a result of addition of dNMPs to open-circular templates.

The efficiency of labeling of species A by dAMP or dCMP varied with reaction conditions used. When the RNase H concentration was 10 times higher, the efficiency of labeling by dCMP was decreased and that by dAMP was increased. Labeling by dCMP was decreased to about one-fifth when DNA synthesis was allowed in the presence of rifampicin for 30 min after incubation with RNA polymerase and RNase H for 30 min (data not shown). This indicates that the primer is further

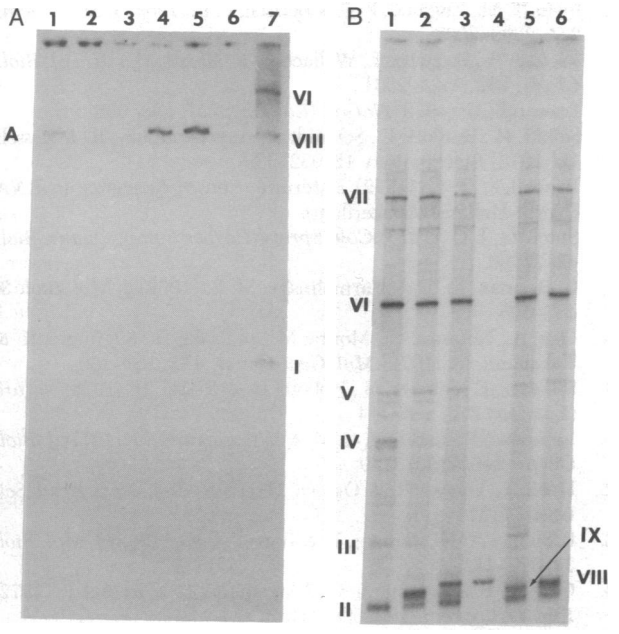

FIG. 4. Products formed by addition of dNTPs onto transcripts. (A) RNA polymerase, DNA polymerase I, and RNase H were added to all reactions except that RNase H was omitted from reaction 6. Radioactive dNTPs (10 μM) added were as follows: [α-^{32}P]dATP to reactions 1 and 6, [α-^{32}P]dTTP to reaction 2, [α-^{32}P]dGTP to reaction 3, and [α-^{32}P]dCTP to reactions 4, 5, and 6. [α-^{32}P]ATP (40 μM) was added to reaction 7. The specific radioactivities were 0.1 Ci/mmol for ATP and 180 Ci/mmol for each dNTP. Nonradioactive dATP was added to reaction 5. Equal volumes (30 μl) of the reaction mixtures were analyzed by electrophoresis in a 2–20% polyacrylamide/urea gradient gel. (B) Synthesis was performed as in A. RNase H was omitted from reaction 1 and DNA polymerase I was omitted from reactions 1, 2, and 6. Reactions 2 and 6 were identical. [α-^{32}P]ATP (1 Ci/mmol, 40 μM) was added to all reactions except for reaction 4, to which [α-^{32}P]dCTP (100 Ci/mmol, 10 μM) was added. Nonradioactive dATP, dGTP, and dCTP (10 μM each) were added to reaction 3; nonradioactive dATP and dGTP (10 μM each), to reaction 4; and 25 μM each of four nonradioactive dNTPs, to reaction 5. Products were analyzed in a 3% polyacrylamide/urea gel.

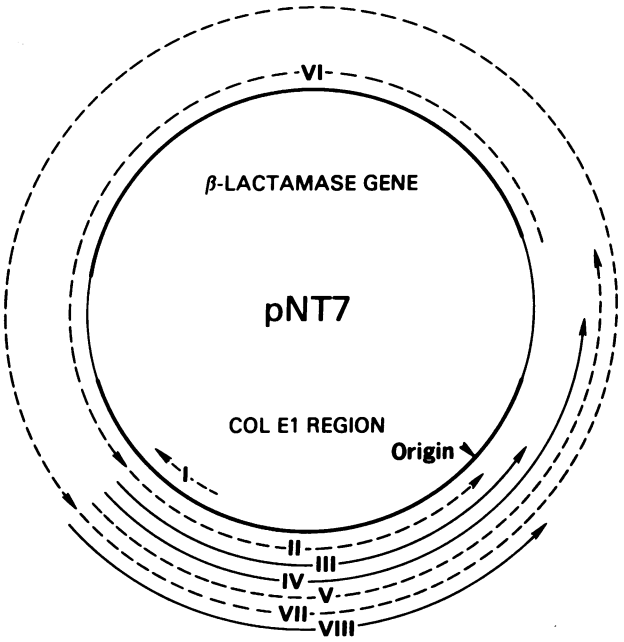

FIG. 5. Transcription patterns of pNT7. pNT7 DNA (inside circle) consists of a region containing the replication origin of ColE1 and of that carrying a β-lactamase structural gene. Continuous lines show transcripts that can be cleaved by RNase H (species III and IV) and the cleavage product (species VIII). Broken lines show transcripts that are not cleaved. The arrowheads indicate the approximate positions of the 3' termini. Many transcripts, most of which initiate at position −555 and terminate between the origin of replication and the promotor of the β-lactamase gene, have been omitted.

cleaved by RNase H in agreement with our previous results (4).

Addition of dNMP to species VIII was also seen when RNA was labeled by [α-^{32}P]ATP in the presence of RNase H, DNA polymerase I, four rNTPs, and the three dNTPs except dTTP (Fig. 4B). DNA synthesis terminated at dCMP at position 6. The products (lane 3) included molecules slightly longer than species VIII that also could be labeled by [α-^{32}P]dCTP (lane 4). The conversion of a considerable amount of species VIII to larger molecules by addition of dNMPs indicates efficient utilization of the RNA as primer.

In a standard reaction mixture for DNA synthesis which included the three enzymes, four rNTPs, and four dNTPs, DNA fragments of an average size of about 100 nucleotides were formed (data not shown; see ref. 4). If RNA was labeled by [α-^{32}P]ATP in the reaction, molecules that moved slightly faster than species VIII were formed (species IX in Fig. 4B, lane 5). These molecules probably arose by cleavage with RHase H of the used primer RNA at or very close to the RNA/DNA junction. In fact, when the product of DNA synthesis was treated with DNase I before electrophoresis, the intensities of some bands above species IX were decreased, whereas that of species IX was increased (data not shown). These bands probably contained DNA products attached to the primer RNA. Cell extracts (1) contain RNase H at approximately 100 times higher concentration than that used here; hence, the elimination of the primer RNA from the DNA product is more complete (2).

DISCUSSION

The transcription patterns of pNT7 DNA are presented in Fig. 5. One of the three initiation sites that govern most *in vitro* transcription of pNT7 DNA is located at position −555. Transcription from this site proceeds towards the origin of replication, frequently extending past the origin and terminating at any of a number of downstream sites. If RNase H is present,

many of these long transcripts are cleaved exactly at the origin. DNA polymerase I can add dAMP and dCMP directly onto the cleaved RNA. Because previous work has shown that replication of ColE1 DNA *in vitro* (2, 4) and *in vivo* (32) initiates most frequently with dAMP or dCMP at the origin, these results indicate that the processed RNA serves as the primer for initiation of ColE1 DNA replication.

Cleavage of the primer precursor by RNase H implies formation of a hybrid between the precursor and the template. Formation of such a hybrid is also indicated by experiments showing that the precursor sediments in a sucrose density gradient together with the template (our unpublished results). Because the cleavage yielded a primer without necessarily altering the 5′ end of the precursor, the region where the precursor forms a stable RNA·DNA hybrid is likely to start near the origin and extend downstream. The fact that RNase H cleavage of the primer that had been used for DNA synthesis yielded RNA of lengths very similar to that of unused primer supports this conclusion. Because a cleavage product containing the 3′ portion of the precursor could not be detected, this portion of the precursor is probably cut at many sites by RNase H.

Remarkably, some transcripts (species V and VII) extend past the origin and yet are not cleaved by RNase H. This finding suggests the presence of a mechanism that determines whether or not the RNA·DNA hybrid is formed during transcription. Because the nucleotide sequence of the region downstream of position 7 can be altered extensively without affecting the specificity of initiation of ColE1 DNA synthesis (2, 32, 33), the DNA structure in the downstream region probably does not play an important role in the mechanism. Transcription termination is an unlikely choice for the mechanism because termination occurs at numerous sites, many of which are separated from the origin by as much as a few hundred nucleotides. Although we cannot specify the mechanism, it is conceivable that there is a region of the template possessing single-stranded character and that this plays a key role in initiating formation of a stable hybrid during transcription. As the RNA polymerase passes through such a region, the transcript might not separate from the template. As a result, all the downstream portion of the transcript could also remain on the template.

No cleavage by RNase H of either species I RNA or the β-lactamase transcript was observed. We also saw no sign of cleavage by RNase H of various RNAs (30–1000 nucleotides) that were transcribed *in vitro* from λb2, λdv, f1 RFI, ΦX174 RFI, and pKY131 (a plasmid containing the origin of replication of *E. coli*, provided by K. Yamaguchi) (our unpublished results). We presume that this reflects the failure of the transcripts to form stable hybrid with their templates and that this failure is a general feature of transcription by RNA polymerase. The precursor of the primer for DNA synthesis appears to be an exception. Nonetheless, the possibility exists that particular transcripts of some DNAs form stable hybrids with their templates. Cleavage of such transcripts by RNase H could play a role in regulation of gene expression.

How could the rate of primer formation be regulated? It seems likely that it will be altered by changes in the rate of initiation of transcription and in the frequency of premature termination of the transcript. The efficiencies of formation of a RNA·DNA hybrid by the transcript and of its cleavage should also affect the rate of primer formation. Because species I RNA is transcribed from the same region where synthesis of the primer initiates (Fig. 5), it is possible that the frequency of species I transcription affects the amount of transcription from the primer promoter. Although we have been unable to find any evidence for such an effect, we do find that the presence of species I RNA inhibits formation of the primer. Furthermore, this RNA only affects the formation of primer by plasmids belonging to the ColE1 incompatibility group (data not shown).

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