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# Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA

(DNA replication/RNA polymerase/DNA polymerase I/RNase H/incompatibility)

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**ABSTRACT** **Transcription of ColE1 DNA by RNA polymerase *in vitro* starts at two sites in a region required for maintenance of the plasmid. Certain transcripts that start at one of the sites can be cleaved by RNase H and then act as primers for DNA replication. Transcription from the other site produces a RNA ≈108 nucleotides long (species I or RNA I). Transcripts analogous to the primer and RNA I of ColE1 are produced when p15A or small derivatives of two other ColE1-compatible plasmids, CloDF13 and RSF1030, are used as template. If purified RNA I is added to the transcription reaction containing RNase H, formation of primer is inhibited. Each RNA I can inhibit primer formation by the plasmid that specifies it but has no effect on primer formation by heterologous templates. Thus, the inhibition of primer formation by RNA I is incompatibility specific. Because RNA I does not inhibit initiation or propagation of transcription or the processing of preformed precursors, the step that is sensitive to inhibition is probably formation of the hybrid between the primer precursor and the template. This hybrid is the required substrate for RNase H. Experiments with recombinant plasmids show the region that determines the specificity of response to RNA I to be >300 base pairs upstream of the origin of DNA replication.**

Studies on the maintenance of various bacterial plasmids have defined two possibly related phenomena. One is that the number of molecules of a given plasmid found in the cell is relatively fixed. The other is that certain plasmids, said to be members of the same incompatibility group, are not stably maintained together in the same host cell. In principle, any incompatibility-specific mechanism that regulates replication or segregation of the plasmid molecules could be responsible for these two effects. To date, however, no biochemical reaction has been shown to be a basis for such regulation.

The plasmid ColE1 displays both copy number control and incompatibility. There is some evidence that a region of the plasmid 200 to 600 base pairs (bp) upstream of its origin of replication regulates replication and provides a function required for incompatibility (1). In addition, it has been reported that a small insertion in this region alters the copy number of the ColE1-related plasmid pMB8 (2). Using a small derivative of ColE1, we have demonstrated that *in vitro* transcription starts at two sites within the region (3). Transcription from one of these sites yields products that can be processed by RNase H to give a 555-nucleotide primer for DNA replication. Transcription from the other site proceeds on the opposite strand and terminates very close to where the primer initiates to yield a RNA ≈108 nucleotides long (species I or RNA I).

There are a number of small plasmids whose replication shares with that of ColE1 certain features such as a requirement for DNA polymerase I and for RNA polymerase (4). These plasmids include pI5A (5), RSF1030 (6), and CloDF13 (7). As shown here, each of these plasmids specifies transcripts which are analogous to the two ColE1 transcripts in both size and function. Furthermore, the structure of the origin regions of these plasmids, as shown by nucleotide sequence analysis, is remarkably similar to that of ColE1 (to be published elsewhere). Nonetheless, each plasmid defines an incompatibility group which is different from that of ColE1 and of the other.

*A priori* it seemed likely that the specificity of the incompatibility mechanism would be mediated by a protein encoded by one of the origin region transcripts. Therefore, we have compared the nucleotide sequences of the origin of the various plasmids. Although our analysis does not exclude every possibility, it appears unlikely that analogous proteins that could provide the requisite specificity are encoded by these transcripts (to be published elsewhere). This led us to consider the possibility that a transcript is directly involved in regulation of replication. Such a role for RNA I has been suggested by studies of mutant plasmids in which the region that specifies RNA I is deleted (8) or altered (9). This interpretation, however, ignored the fact that the region specifies a second transcript. In this report, we show directly that RNA I is an incompatibility-specific inhibitor of primer formation.

## MATERIALS AND METHODS

**Plasmids.** Plasmids pNT2 (10), pNT5 (10), and pNT7 (3) are derivatives of ColE1; p15A (5), CloDF13 (6), and RSF1030 (7) have been described. To obtain plasmids with less-complex transcription patterns, smaller derivatives of RSF1030 and CloDF13 were constructed as follows. Plasmid pST10 was made by self-ligation of *Hae* II-digested RSF1030 DNA. It consists of two segments of the RSF1030 genome, the smaller containing the origin of replication and the larger containing the β-lactamase gene and a region upstream of the origin. Two segments, corresponding to the *Hin*dII D and F (11) fragments of RSF1030, were removed from pST10 by *Hin*dII digestion and self-ligation. The plasmid thus obtained was treated with *Bst*EII and *Pvu* II followed by S1 nuclease and then T4 DNA ligase to generate pST19 which has 3 kilobase pairs (kb) of DNA. Plasmid pDF105 was constructed by ligation of *Hae* II-digested CloDF18 DNA to a *Hae* II fragment containing the β-lactamase gene of pNT2 (10). This 4.3-kb plasmid contains a portion of the CloDF13 genome which includes the origin of replication (ref. 12; unpublished data). The construction of recombinant plasmids containing segments of ColE1 and RSF1030 or of ColE1 and p15A will be described below.

**Transcription.** Conditions used for transcription by RNA polymerase in the presence or absence of RNase H have been described, as has the procedure for isolating nucleic acid from the reaction mixtures (3). Unless otherwise stated, reaction products were separated by electrophoresis in slab gels of 3%

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Abbreviations: bp, base pair(s); kb, kilobase pair(s).

polyacrylamide/8 M urea as described (3). To prepare RNA I, plasmid DNA was transcribed in 1 ml of a reaction mixture containing [5,6-$^3$H]UTP (1 mCi/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels), plasmid DNA (60 $\mu$g/ml), RNA polymerase (30 units/ml), and four rNTPs (200 $\mu$M). After incubation for 90 min at 37°C, nucleic acids were isolated as described (3), dissolved in dimethyl sulfoxide (50% in water), and electrophoresed in an 8% polyacrylamide/8 M urea gel. After electrophoresis, the nucleic acids were stained with ethidium bromide, and RNA I was eluted from a gel slice (3). To test the effect of RNA I on primer formation, an appropriate amount (0–2.0 $\mu$g/ml) of purified RNA I was added to a standard reaction mixture of reduced volume (10 $\mu$l).

**DNA Synthesis.** The ability of DNA polymerase I to add dAMP and dCMP to transcripts was measured as described (3).

## RESULTS

**Inhibition of Primer Formation by RNA I.** Transcripts obtained *in vitro* by using pNT7 DNA as template are diagrammed in Fig. 1 (3). This plasmid is a small derivative of ColE1 which has ColE1 incompatibility and is of known nucleotide sequence (3, 10). Of particular interest are RNA I and a set of transcripts that begin near the termination site for RNA I and extend toward the origin of replication. Among this set are transcripts (such as RNAs III and IV) that can be cleaved by RNase H to form RNA VIII, the primer for DNA replication (3), and other transcripts (such as RNAs II, V, VII) that are insensitive to RNase H.

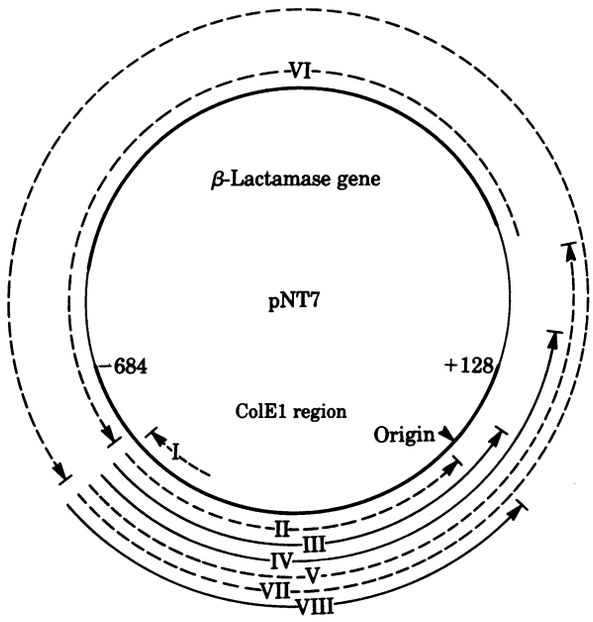


FIG. 1. Transcription pattern of pNT7 DNA. pNT7 (inside circle) consists of an 812-bp segment of ColE1 (684 bp upstream and 128 bp downstream of the origin of DNA replication) coupled to a 1240-bp segment containing a $\beta$-lactamase gene. Three sites are used to initiate the majority of the *in vitro* transcripts made by RNA polymerase (3). One site is in the ColE1 segment 555 nucleotides upstream of the origin. Transcription from this site frequently terminates just before the origin (RNA II) or just before (RNA V) or beyond the $\beta$-lactamase gene (RNA VII). Other transcripts—for example, RNAs III and IV—terminate at various sites between the origin and the $\beta$-lactamase gene. RNase H cleaves certain transcripts that extend past the origin—for example, RNAs III and IV—to yield RNA VIII which can act as a primer for DNA synthesis by DNA polymerase I. We refer to the region that governs initiation of these transcripts as the primer promoter. The major transcript (RNA VI) of the $\beta$-lactamase gene extends from just ahead of the gene to a termination signal (10) that is present in the primer promoter. Another major transcript (RNA I) (13, 14) starts 447 nucleotides upstream of the origin and terminates near the site where the primer transcripts start on the opposite strand. Transcripts insensitive to RNase H are indicated by a dashed line.

We find that the addition of purified RNA I to the pNT7 transcription reaction alters this pattern of products but does not substantially affect the total yield of RNA. In the presence of RNase H, the yield of primer and of RNA II decreased as increasing amounts of RNA I were added whereas the yield of RNAs V and VII increased (Fig. 2*A*). No effect on the synthesis of RNA I was observed. In the absence of RNase H, in which case the primer is not formed, the addition of RNA I reduced the yield of the known primer precursors and of RNA II and again increased the yield of RNAs V and VII (Fig. 2*B*).

In a standard transcription reaction that includes RNase H (30°C for 30 min), the yield of RNA I is about one molecule per template molecule; the yield of primer is one-fourth of this (3). The concentration of RNA I required to reduce the yield of primer by 50% is 0.5 $\mu$g/ml (15 nM). This corresponds to two molecules of RNA I per template molecule. When the concentration of RNA polymerase or of template DNA is decreased, the same concentration of RNA I still causes a 50% reduction in primer yield (data not shown).

**Primer Formation by Other Plasmids.** We have examined the products obtained when the DNAs of p15A, the CloDF13 derivative pDF105, and the RSF1030 derivative pST19 are transcribed in the presence and absence of RNase H. The tran-

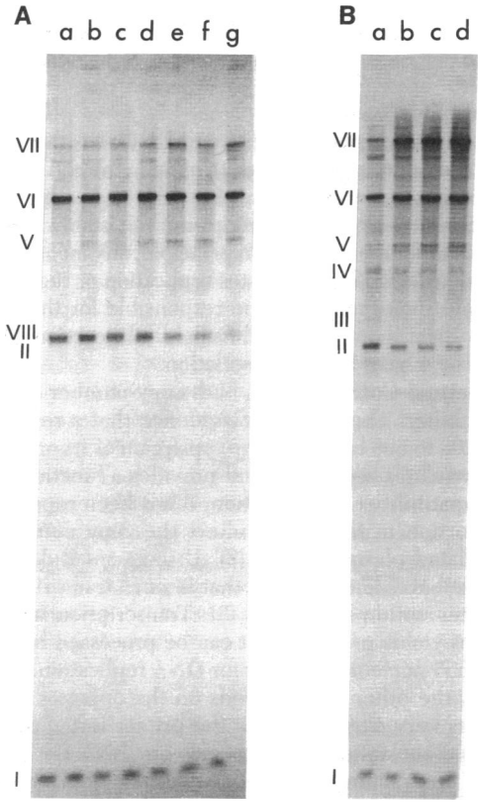


FIG. 2. Effect of RNA I on transcription of pNT7 DNA. RNA synthesis was carried out with RNA polymerase (5 units/ml), pNT7 DNA (10 $\mu$g/ml), and the four rNTPs (each at 40 $\mu$M) including [$\alpha$-$^{32}$P]ATP (1 Ci/mmol) with (*A*) or without (*B*) RNase H (1.5 units/ml). The amount of purified pNT7-RNA I added per ml of reaction mixture was as follows: in *A*, a, none; b, 0.06 $\mu$g; c, 0.13 $\mu$g; d, 0.25 $\mu$g; e, 0.5 $\mu$g; f, 1.0 $\mu$g; g, 2.0 $\mu$g; in *B*, a, none; b, 0.05 $\mu$g; c, 1.0 $\mu$g; d, 2.0 $\mu$g. Roman numerals indicate the position of the corresponding RNA species.

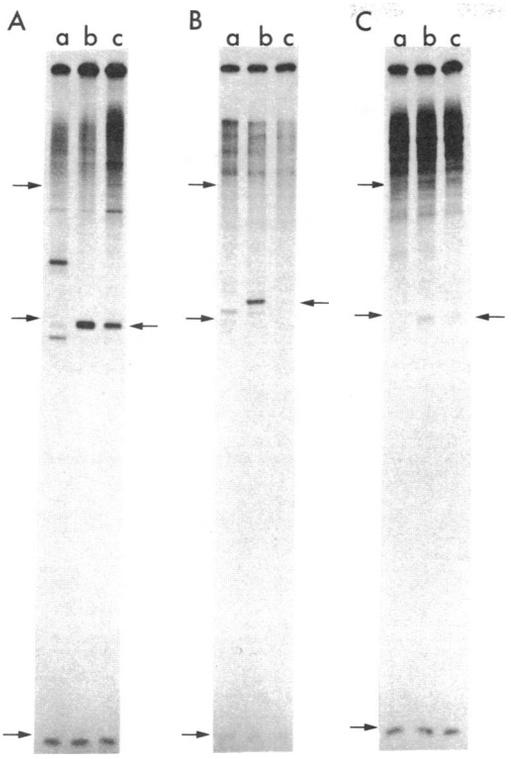


FIG. 3. Effect of RNase H and of homologous RNA I on transcription of various plasmid DNAs. The template DNAs used were: (*A*) p15A (10 μg/ml); (*B*) pDF105 (15 μg/ml); (*C*) pST19 (15 μg/ml). RNA was labeled by [α-$^{32}$P]ATP (1 Ci/mmol) in the absence (lanes a) or the presence (lanes b and c) of RNase H. Homologous RNA I (2.0 μg/ml) was added to reaction c. The molarity of each template DNA was roughly equivalent and all reactions were analyzed on the same gel; thus, the relative amount of a given RNA species should indicate the approximate relative yield of the RNA for each template. The left-pointing arrows indicate the position of the RNA species formed by cleavage with RNase H. The right-pointing arrows indicate the positions of pNT7-RNA VI (1150 nucleotides), RNA VIII (555 nucleotides), and RNA I (108 nucleotides), in order from the top.

scription patterns (Fig. 3) show certain similarities to the pNT7 DNA pattern. In particular, each plasmid specified an RNA species 530–590 nucleotides long whose formation depended on the presence of RNase H (compare lanes a and b). When DNA polymerase I was present, DNA replication started by using the cleaved RNA as a primer (data not shown). Except in the case of p15A, each primer was formed from precursor molecules whose synthesis was arrested (but may not have terminated) at many positions downstream of the origin of DNA replication, as observed with pNT7 (3). The p15A precursors were arrested at a relatively unique position about 100 nucleotides downstream of the origin (details of these results will be published elsewhere).

With each template we also observe a short RNA species (100–110 nucleotides long). Such a short *in vitro* transcript of RSF1030 DNA has been reported (9). The nucleotide sequences at the 5′ ends of the p15A and pST19 short transcripts (data not shown) indicated that, in each case, synthesis started at a unique site 400–480 nucleotides upstream of the origin of DNA replication. The estimated size of the transcripts indicated that each terminated just before the site where primer synthesis started on the opposite strand. As in the case of pNT7, we refer to each short RNA as species I RNA or RNA I. We distinguish them by a prefix indicating the plasmid that specified the RNA (e.g., p15A-RNA I).

When any of the species I RNAs was added to a transcription reaction that contained RNase H and had a homologous DNA as template, formation of primer was inhibited (compare lanes b and c). At the same concentration, the extent of inhibition by each RNA I varied significantly; the RNA I of pDF105 was the most active and that of pST19 was the least active. Although relatively poor, the slight inhibition observed with pST19-RNA I at this concentration was reproducible. The RNA I of RSF1030 also had the same low activity for inhibition of primer formation on pST19 and on RSF1030 itself (data not shown).

We also examined whether or not the species I RNAs inhibit primer formation on heterologous templates. The effect of the species I RNAs of p15A, pDF105, and pST19 on pNT7 primer formation is shown in Fig. 4*A*. With this heterologous template, no inhibition was observed when the RNAs were present at concentrations sufficient to inhibit primer formation on a homologous template. Similarly, the addition of pNT7-RNA I had no effect on primer formation by p15A, pDF105, and pST19 (Fig. 4*B*). We have tested six other combinations of various species I RNAs and heterologous templates and consistently have observed the absence of detectable inhibition (data not shown).

**The Region of the Plasmid that Determines Sensitivity to RNA I.** To define the region of pNT7 DNA that determines susceptibility to inhibition by RNA I, we constructed two hybrid

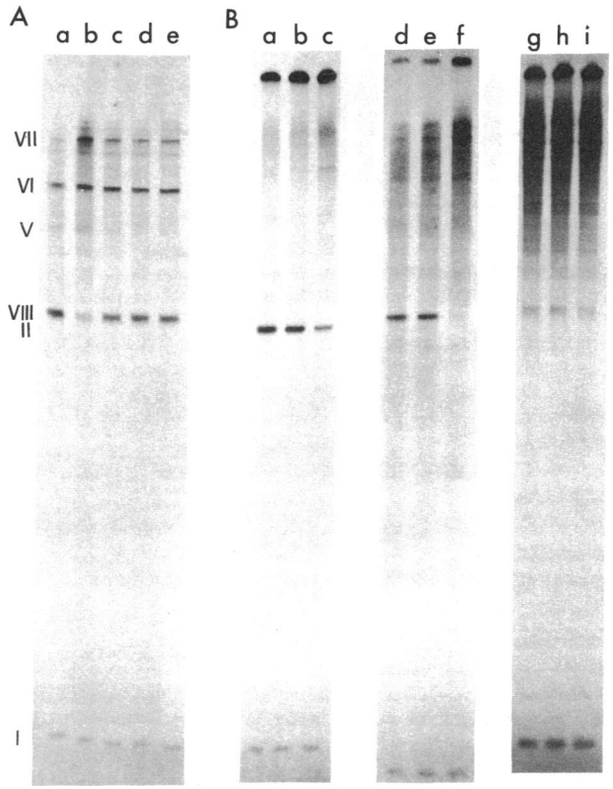


FIG. 4. Specificity of inhibition of primer formation by RNA I. (*A*) Effect of various species I RNAs on primer formation on pNT7 DNA. The template DNA was pNT7 (10 μg/ml). Species I RNAs (2.0 μg/ml) were added as follows: a, none; b, pNT7-RNA I; c, p15A-RNA I; d, pDF105-RNA I; e, pST19-RNA I. All reactions contained [α-$^{32}$P]ATP (1 Ci/mmol) and RNase H. (*B*) Effect of pNT7-RNA I on primer formation on heterologous template DNAs. The DNAs used were: a–c, p15A (10 μg/ml); d–f, pDF105 (15 μg/ml); g–i, pST19 (15 μg/ml). pNT7-RNA I (2.0 μg/ml) was added to reactions b, e, and h. Homologous RNA I (2.0 μg/ml) was added to reactions c, f, and i. RNA was synthesized as in *A*. Arrows indicate the position of primer RNA formed by cleavage with RNase H.

plasmids from pNT5 (the immediate precursor of pNT7) and pST10. One plasmid (pST11) was made by joining a *Hae* II fragment containing the primer promoter and β-lactamase gene of pNT5 to a *Hae* II fragment containing the origin of pST10; the other (pST14) had the reverse arrangement. The recombination point in the origin region was a *Hae* II site located 188 bp upstream of the origins of both pNT5 and pST10. The incompatibility of these hybrid plasmids is that of the plasmid that donated the region upstream of the *Hae* II site (data not shown). Primer formation on the hybrid that had the upstream region of pNT5 was inhibited by pNT7-RNA I but not by pST10-RNA I (Fig. 5, lanes a–c); primer formation on the reciprocal hybrid was suppressed by pST10-RNA I but not by pNT7-RNA I (Fig. 5*A*, lanes d–f).

To delimit this region further, we constructed pFT104, a plasmid that had the origin of P15A and the primer promoter of pNT7. The two were joined at a *Hin*fI site 313 bp upstream of the origin of pNT7 (294 bp upstream of the origin of p15A). The hybrid plasmid had the incompatability properties of pNT7 (data not shown). Primer formation on pFT104 was inhibited by pNT7-RNA I but not by p15A-RNA I (Fig. 5*B*). We conclude that the region upstream of the *Hin*fI site determines the specificity of susceptibility to RNA I.

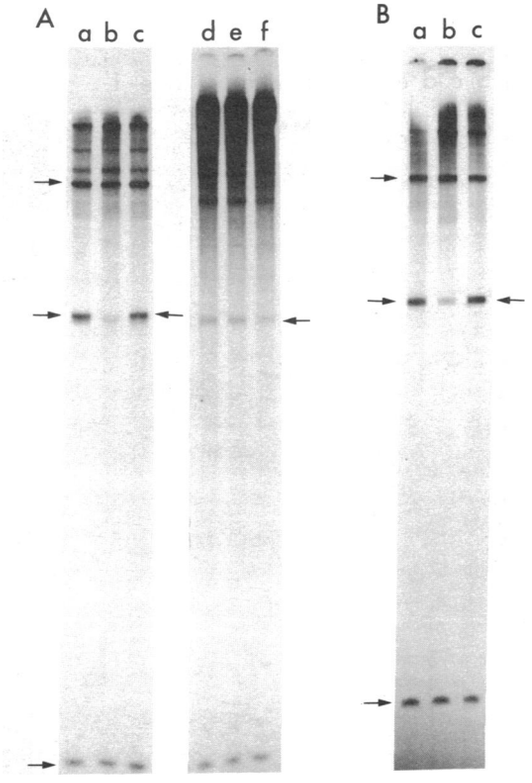


FIG. 5. Effect of RNA I on transcription of hybrid plasmid DNAs. (*A*) Transcription of ColE1-RSF1030 hybrids. RNA was synthesized on pST11 DNA (2.5 kb, 10 μg/ml) (lanes a–c) and pST14 DNA (5.5 kb, 25 μg/ml) (lanes d–f) with [α-$^{32}$P]ATP (1 Ci/mmol) in the presence of RNase H. The amount of RNA polymerase was increased 2-fold in the reactions containing pST14 DNA. Species I RNAs (2.0 μg/ml each) were added as follows: lanes b and e, pNT7-RNA I; lanes c and f, pST19-RNA I. (*B*) Transcription of a ColE1-p15A hybrid. RNA was synthesized as in *A*. The template was pFT104 (2.1 kb, 10 μg/ml). pNT7-RNA I and P15A-RNA I (2.0 μg/ml each) were added to reactions b and c, respectively. The left-pointing arrows indicate the position of the RNA species formed by cleavage with RNase H. The right-pointing arrows indicate the positions of pNT7-RNA VI, RNA VIII, and RNA I as in Fig. 3.

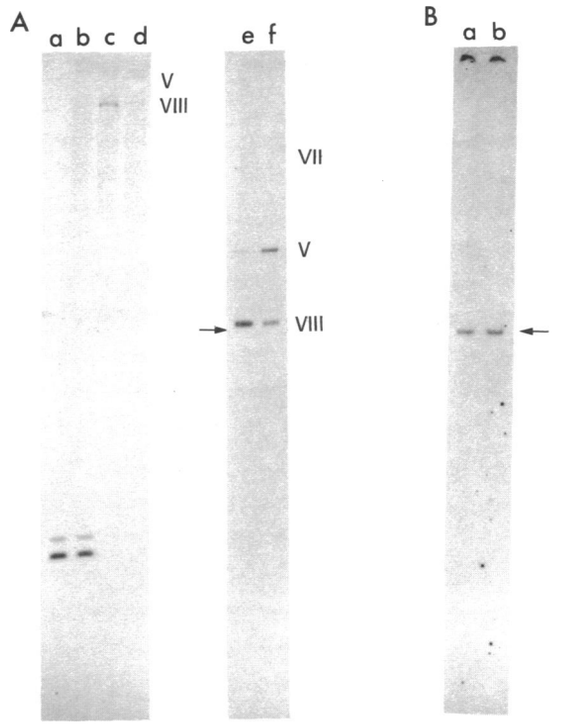


FIG. 6. Effect of RNA I on initiation and elongation of pNT7 transcripts (*A*) and on addition of dCMP to primer by DNA polymerase I (*B*). (*A*) RNA was synthesized in reaction mixtures containing GTP (400 μM), CTP (10 μM), and [α-$^{32}$P]ATP (2 μM, 40 Ci/mmol) for 20 min at 30°C; after addition of rifampicin (10 μg/ml), incubation was continued for an additional 15 min. pNT7-RNA I (2.0 μg/ml) was added to reaction b at the beginning of incubation. After adjustment of CTP to 40 μM and ATP to 2 mM and addition of UTP (40 μM) and RNase H, reactions c–f were incubated further for 20 min with (reactions d and f) or without (reactions c and e) pNT7-RNA I. The products isolated from equal volumes of the various reaction mixtures were applied to a 10% polyacrylamide/8 M urea gel (a–d). Because longer RNA molecules enter the 10% gel poorly, the products of reactions identical to c and d were analyzed on a 3% gel (e and f). The major product in lanes a and b is an RNA of 32 bases, as estimated from the behavior of standard RNAs of known size in the same gel (not shown). The total amount of radioactivity in the major bands (RNAs V and VIII) in lanes e and f was about 70% of the amount found in the two major products of the first-stage reaction (lane a). RNA VII is seen poorly in this figure. The absence of RNA II in lanes e and f (expected position marked by an arrow) was confirmed by a longer run on a second gel in which RNAs II and VIII were clearly resolved (data not shown). (*B*) RNA was labeled by [5,6-$^{3}$H]UTP (1 Ci/mmol) in the absence of RNase H. After incubation for 30 min at 30°C the reaction mixture (60 μl) was chilled and immediately passed through a Sephadex G-50 column as described (3). Fractions (300 μl) excluded from the column were pooled, and RNase H (2 units/ml), DNA polymerase I (35 units/ml), dATP (10 μM), and [α-$^{32}$P]dCTP (10 μM, 100 Ci/mmol) were added. The mixture was divided into two equal portions; to one pNT7-RNA I (2.0 μg/ml) was added (lane b); both were incubated for 20 min at 30°C. The arrow indicates the position of primer RNA to which [α-$^{32}$P]dCMP was added (3). Only nucleic acids labeled with $^{32}$P are seen on the electropherogram.

**Mechanism of Inhibition by RNA I.** Primer formation might be suppressed by inhibition of either initiation or propagation of transcription. Alternatively, because the primer is formed through the action of RNase H on the transcription products, RNA I might inhibit formation of the RNA·DNA hybrid required by RNase H or might inhibit the nuclease itself. The results presented above (Fig. 2) show that the yield of certain transcripts that start at the primer promoter (e.g., RNAs V and VII) is actually increased by the addition of RNA I. This suggests

that RNA I does not interfere with initiation or extension of transcripts. In this section we provide additional evidence to support this conclusion and also show that RNA I does not inhibit RNase H itself. From these findings, we infer that the step affected is the formation of the RNA·DNA hybrid.

To study the effect of RNA I on the earliest events on transcription, we used a two-stage reaction in which transcription was initiated in the absence of UTP. Because the first UMP in transcripts that begin at the primer promoter is the 21st nucleotide, products made in the absence of UTP should contain no more than 20 nucleotides. However, probably because of the presence of a trace amount of UTP in the reaction, most transcripts extended as far as a UMP-rich sequence about 30 nucleotides from the start (Fig. 6*A*, lane a). Synthesis of these oligonucleotides was not affected by the addition of RNA I (Fig. 6*A*, lane b). Upon addition of UTP to the reaction, the oligonucleotides were elongated (Fig. 6*A*, lanes c and e). As before, the ratio of RNA V to RNA VIII was affected by the addition of RNA I but the total yield of elongated molecules (i.e., the sum of RNAs V and VIII) was unaltered (Fig. 6*A*, lanes d and f).

To look for an effect of RNA I on cleavage of the primer precursor or on use of the cleavage product to initiate DNA synthesis, pNT7 DNA was transcribed by RNA polymerase and the reaction mixture was passed through a column of Sephadex G-50 to remove rNTPs and to stop transcription (3). The fraction excluded from the column was incubated with RNase H, DNA polymerase I, dATP, and [$\alpha$-$^{32}$P]dCTP. Analysis of the products (Fig. 6*B*, lane a) showed formation of labeled material resulting from the addition of dCMP to the primer RNA (3). The presence of RNA I in this second-stage reaction did not prevent the addition of dCMP (Fig. 6*B*, lane b). These results indicate that neither processing of the primer-precursor once formed nor use of the processed RNA as primer is affected by RNA I.

Because these results indicate that RNA I does not affect the other steps in primer formation, we conclude that RNA I interferes with the formation of the RNA·DNA hybrid required by RNase H. We believe that the formation of hybrid during transcription somehow arrests synthesis of the primer precursors such as RNAs III and IV. In the absence of hybrid formation, transcription usually continued downstream to form longer products such as RNAs V and VII. Thus, their yield was increased by addition of RNA I. The shorter RNA II molecules probably arose when the progress of RNA polymerase through the origin was prevented by the presence of hybridized precursor. Thus, their yield was decreased by the addition of RNase H (3) or RNA I. This interpretation is supported by results of experiments in which the ratio of RNA polymerase to template was reduced (data not shown) and of others in which initiation occurred in the absence of some rNTPs (Fig. 6*A*, lanes e and f and legend). In both of these cases in which the chance of multiple initiation at the primer promoter was minimal, a reduced yield of RNA II was observed.

Finally, the occurrence of initiation at the primer promoter in the absence of UTP indicates that synthesis of complete molecules of RNA I is not required for initiation of transcription at the primer-promoter. RNA I molecules synthesized under this condition would have been observed in Fig. 6*A* (lanes a and b). It is therefore unlikely that synthesis of the short RNA plays an essential role in activation of the primer-promoter although the region that specifies RNA I lies just beyond the start site for the primer transcripts.

## DISCUSSION

When ColE1, p15A, CloDF13, RSF1030, or their derivatives are used as templates in the *in vitro* system used here, certain transcripts that start 530–590 nucleotides upstream of the origin of DNA replication can be cleaved by RNase H at the origin and used as primers for initiation of DNA replication. In addition to these transcripts, each plasmid directs synthesis of a short RNA (RNA I), about 100 nucleotides long, whose transcription begins 400–480 nucleotides upstream of the origin and terminates close to the site where the primer transcripts start. The results presented here show that the addition of purified RNA I to the reaction inhibits the formation of primer RNA and that this inhibition is observed only if the template for primer synthesis is closely related to the source of the RNA I.

How does RNA I inhibit primer formation? We have shown that the RNA does not inhibit initiation of transcription at the primer-promoter or elongation of the transcripts once initiated. Nor does the RNA prevent the correct processing of primer precursors formed in its absence. From these negative results, we infer that RNA I inhibits the formation of the required substrate for RNase H—namely, a RNA·DNA hybrid between the primer-precursor and the template.

Previous work suggests that hybridization occurs during transcription and that the hybridized portion of the precursor transcript extends from near the origin to the downstream sites where transcription is arrested (3). On the other hand, results described here indicate that the inhibition of hybrid formation probably results from an interaction between RNA I and either the template or transcript in a region well upstream of the origin. The way in which an event at an upstream site affects an event occurring at the origin remains an intriguing puzzle.

The properties of the mechanism described here suggest that RNA I plays an important role in both copy number control and plasmid incompatibility. As will be shown elsewhere, this is confirmed by the isolation of pNT7 mutants that have different copy number and incompatibility as a result of single base changes in the region specifying RNA I. These mutations also alter the inhibitory activity of RNA I and the sensitivity of primer formation to RNA I. Although this mechanism may be entirely responsible for the incompatibility of pNT7, results obtained with mutants of ColE1 derivatives suggest that a region downstream of the ColE1 origin provides a product that limits plasmid copy number (15). Whether or not the product acts in concert with RNA I is as yet unknown.


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