

Activities of the RNAI and RNAII Promoters of Plasmid pBR322

S. LIN-CHAO AND H. BREMER*

Biology Programs, The University of Texas at Dallas, Richardson, Texas 75080

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The synthesis rates of the replication control RNAs of plasmid pBR322, RNAI, an inhibitor of replication, and RNAII, the preprimer, have been determined by hybridizing in vivo pulse-labeled RNA to specific, single-stranded DNA probes for RNAI and RNAII. In *Escherichia coli* growing in glycerol minimal medium, RNAI transcripts were made at a rate of one molecule per 30 s per plasmid; RNAII was transcribed fivefold less, at a rate of one molecule per 3 min per plasmid. It is estimated that only 1 in 20 prepriming events leads to replication.

Replication of ColE1-type plasmids, like pBR322 (1), begins with the synthesis and processing of an RNA primer, RNAII, and is negatively controlled by a small, unstable, plasmid-coded RNA molecule, RNAI (3, 5). This control maintains the plasmid concentration at an equilibrium level, which depends on the activities of the RNAI and RNAII promoters, the turnover rate of RNAI, the efficiency of the RNAI inhibition, and the dilution of plasmids and control factors by cell growth (1a, 21, 24). The relative accumulation rates of RNAI and RNAII within the bacteria have been estimated previously from the amounts of specific radioactive probes hybridized to electrophoretically separated bacterial RNA species (Northern blot method), and their turnover has been determined after the addition of rifampin to the culture (6, 10, 11), which stops RNA chain initiation. Those experiments suggested that, in a given growth medium, RNAI and RNAII molecules were synthesized at a ratio of about 3 to 1 (10). Measurements of galactokinase activities expressed from RNAI and RNAII promoters suggested a ratio of 5.5 to 1 (20). Since those experiments did not give absolute rates and are subject to several kinds of systematic error, we have here hybridized RNA pulse-labeled in vivo to nonradioactive probes immobilized on nitrocellulose filters, a method which allows the determination of the absolute rates of synthesis of RNAI and RNAII. Similar methods have been used to measure the complementary transcripts involved in the control of plasmid NR1 (26). The results indicate that, under the conditions used, an RNAII preprimer is synthesized about every 3 min from each plasmid, such that only 1 out of 20 prepriming events leads to replication. The RNAI promoter is five times more active, with two transcripts per minute.

MATERIALS AND METHODS

Strains and conditions of growth. The bacteria, phages, and plasmids used in this work are listed in Table 1. RL331T, transformed with plasmid pBR322, was grown at 37°C in medium C (8) supplemented with 0.2% glycerol and 20 µg of phenylalanine per ml. Growth was followed by measuring the optical density at 460 nm (OD₄₆₀).

For M13 phage work, *Escherichia coli* JM103 was grown in 2×YT medium as described previously (16). Two derivatives of M13mp7 were constructed by inserting an *Fnu*DII fragment containing the replication origin of pBR322 (-568 base pairs [bp] upstream to +13 bp downstream of the

origin) in either orientation into the *Hinc*II site of the replicative form of phage M13mp7. The phage whose plus strand is homologous to the plasmid replication inhibitor, RNAI, was named M13mp7.RI; the phage with the other orientation, whose plus strand is homologous to the replication primer, RNAII, was named M13mp7.RII. The orientation was verified by restriction analysis and by examining the effect of the phage DNA on the hybridization of an in vitro ³²P-labeled fragment of the pBR322 replication origin (the same 581-bp *Fnu*DII fragment as used above) to RNAI and RNAII synthesized in vivo (Northern blot experiment); nonradioactive M13mp7.RI phage DNA blocked hybridization of the radioactive probe to RNAI, whereas M13mp7.RII phage DNA blocked hybridization to RNAII (Fig. 1).

For in vitro transcription, two plasmids were constructed which contain the pBR322 origin region flanked by promoters of bacteriophages T3 and T7. For this purpose, the pBR322 insert of M13mp7.RI (replicative form) was cut out with *Eco*RI enzyme and inserted in both directions into the *Eco*RI site of the pT7/T3-18 vector. The plasmid whose pT7 transcript produces RNAI sequences was named pT7/T3-18.RI, and the plasmid whose pT7 transcript produces RNAII sequences was named pT7/T3-18.RII. These plasmids were used to transform the host strain DH1. The orientation was checked by restriction analysis and by hybridizing the in vitro RNA product obtained with T7 RNA polymerase to M13mp7.RI and M13mp7.RII DNA (data not shown).

In vivo labeling of RNA. Strains RL331T and RL331T (pBR322) were each grown in glycerol minimal medium to an OD₄₆₀ of 0.7, and then 0.5 ml of the culture was added to 5 µl of [5-³H]uridine (specific activity, 22 Ci/mmol) in a 1.5-ml centrifuge tube (Eppendorf). After incubation for 1 min with occasional shaking, 100 µl of ethanol-phenol stopping solution (5% phenol in ethanol) (9) was added to prevent enzymatic degradation of RNA during the following procedures. After centrifugation in a microfuge (Fisher model 235A; 1.5 min at 15,000 rpm), the radioactive supernatant was drained, and remaining drops were removed with tissue paper. The cell pellet was suspended in 75 µl of medium C; then an equal volume of 100°C sodium dodecyl sulfate lysis buffer (0.2 M NaCl, 0.2 M Tris [pH 7.5], 40 mM EDTA, 1% sodium dodecyl sulfate) was added, and the mixture was kept at 100°C for 15 s. This produced a clear, viscous lysate, which was 10-fold diluted with hybridization buffer (100 ml containing 50 ml of formamide, 25 ml of 20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2 ml 50× Denhardt solution [12], 10 ml of 0.5 M NaPO₄ buffer [pH

* Corresponding author.

TABLE 1. Bacteria, bacteriophages, and plasmids

Strain, phage, or plasmid	Description	Reference or source
<i>E. coli</i>		
RL331T	B/rA <i>phe</i> (Am) <i>hsdR hsdM</i> (K-12)	22
JM103	<i>endA hsdR supE sbcB thi-1 rpsL</i> Δ (<i>lac-pro</i>)/F' <i>traD36 proAB</i> ⁺ <i>lacI</i> ^s <i>ZM15</i>	17
DH1	<i>endA1 hsdR17 supE44 thi-1 recA1</i> <i>gyrA96</i>	7
Bacteriophages		
M13mp7		17
M13mp7.RI	581-bp <i>Fnu</i> DII fragment containing the pBR322 replication origin inserted into M13mp7; plus strand homologous to RNAI	This work
M13mp7.RII	Same as M13mp7.RI, but insert in the other direction; plus strand homologous to RNAII	This work
Plasmids		
pBR322		1
pT7/T3-18		BRL ^a
pT7/T3-18.RI	581-bp <i>Fnu</i> DII fragment containing the pBR322 replication origin inserted into pT7/T3-18 such that RNAI is transcribed from pT7	This work
pT7/T3-18.RII	Same as pT7/T3-18.RI, but insert in the other orientation, such that RNAII is transcribed from pT7	This work

^a Bethesda Research Laboratories, Bethesda, Md. (1986).

7.0], 1 ml of 10% sodium dodecyl sulfate, 1 ml 25 mg of calf thymus DNA per ml, 10 ml of H₂O) to a final volume of 1.5 ml and stored at room temperature. A 0.1-ml sample of this diluted lysate contained about 30,000 acid-precipitable cpm.

DNA preparations. Single-stranded M13 phage DNA was prepared as described (16). The yields were about 0.5 mg from 100 ml of culture for M13mp7.RI and consistently only half as much with M13mp7.RII. Supercoiled plasmid DNA or M13 replicative form DNA were prepared by the alkaline lysis method (12) and further purified by isopycnic banding in a CsCl density gradient in the presence of ethidium bromide, followed by butanol extraction, dialysis, and ethanol precipitation.

Binding of single-stranded DNA to nitrocellulose filters. Samples (5 μ l) of M13mp7.RI or M13mp7.RII DNA corresponding to 1.5 μ g of single-stranded DNA were spotted onto 3-mm squares cut out of nitrocellulose membrane filters (Schleicher & Schuell Co.; 0.45- μ m pore size) laid out on a sheet of Parafilm. After drying in air for 1 h, the filters were placed into sterile 50-ml screw-cap tubes and washed twice at room temperature with 50 ml of 6 \times SSC, each time for about 5 min. After most of the liquid was decanted, the filters were removed with fine tweezers, quickly blotted on tissue paper, dried for 1 h in air on a sheet of Parafilm, placed into a sterile glass petri dish with lid, baked for 2 to 3 h at 80°C in a vacuum oven, and stored at room temperature under vacuum.

Hybridization assay. Unless stated otherwise, three marked filters loaded with different single-stranded phage DNAs (M13mp7 as background control, M13mp7.RI, or M13mp7.RII) were placed into a sterile, 0.5-ml Eppendorf

tube containing 0.1 ml of preheated (10 min, 65°C) hybridization buffer. After overnight incubation at 42°C (prehybridization to reduce background), the filters were removed, quickly blotted with tissue paper, and put into a second small tube with 0.1 ml of hybridization buffer containing the lysate of pulse-labeled cells (see above). After further incubation at 42°C with low-speed shaking for 2 days, the filters were transferred into a clean vial with 5 ml of 42°C 2 \times SSC and washed at 42°C for about 3 min. The washing was repeated four times by changing the medium with a transfer pipette; the last time at 65°C. The filters were then transferred to a new vial and rinsed three times with distilled water; adhering liquid was blotted off between rinses. After drying for 1 h at room temperature, each filter was placed into a 0.5-ml Eppendorf tube with 0.4 ml scintillation fluid; radioactivity was counted for 20 min.

Specificity of hybridization reaction. Increasing amounts of the *in vivo* pulse-labeled RNA from strains RL331T and RL331T(pBR322) were hybridized to M13mp7.RII phage DNA. Only radioactive RNA from the plasmid-carrying strain (i.e., RNAI), but not that from the plasmid-free strain, hybridized (data not shown). When the amount of RNA in the hybridization reaction was kept constant while the amount of DNA bound to the filters was varied, maximum hybridization was reached with about 0.8 μ g of DNA on the filter, corresponding to a more than 100-fold molar excess over the number of RNAI molecules present (data not shown). In further experiments (Fig. 2), 1.5 μ g of DNA (3.8×10^{11} M13 DNA molecules), but also more bacterial RNA, was used, such that the molar excess was about the same.

To test whether the phenol in the solution used to stop metabolic activities had made the cells leaky for smaller RNA molecules, the phenol concentration was varied between 0 and 25%. Except in the sample without phenol, which gave 50% lower values, the recovery of hybridizable radioactivity was found to be constant. The concentration of 5% phenol in the stopping solution used in the standard

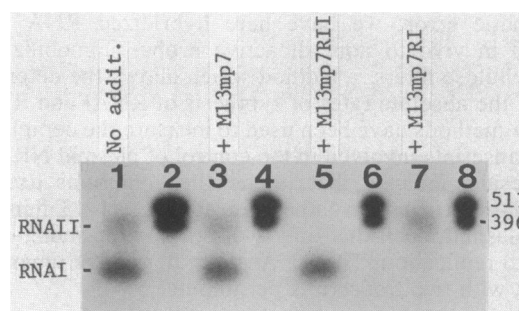


FIG. 1. RNAI and RNAII visualized by Northern blots. RNA of strain RL331T(pBR322) grown in glycerol-minimal medium was subjected to agarose gel electrophoresis in the presence of 7 M urea (odd-numbered lanes); molecular weight standards (*Hin*FI-cleaved pBR322 DNA) were run in parallel (even-numbered lanes). The gel was blotted onto DBM-cellulose and cut into four sections; each section was allowed to hybridize with a 581-bp probe specific for the replication origin and labeled with ³²P by nick translation. The hybridization reactions for lanes 3 to 8 contained, in addition to the probes, 7.5 μ g of single-stranded DNA of the phages M13mp7 (lanes 3 and 4), M13mp7.RII (lanes 5 and 6), or M13mp7.RI (lanes 7 and 8). M13mp7.RI and M13mp7.RII DNAs have inserts from the pBR322 origin region homologous to RNAI and RNAII, respectively. The molecular weight standard contained two fragments (517 and 396 bp) which overlap with the replication origin fragment and are also labeled by the radioactive probes. For details see text and reference 10. For interpretation see Fig. 3.

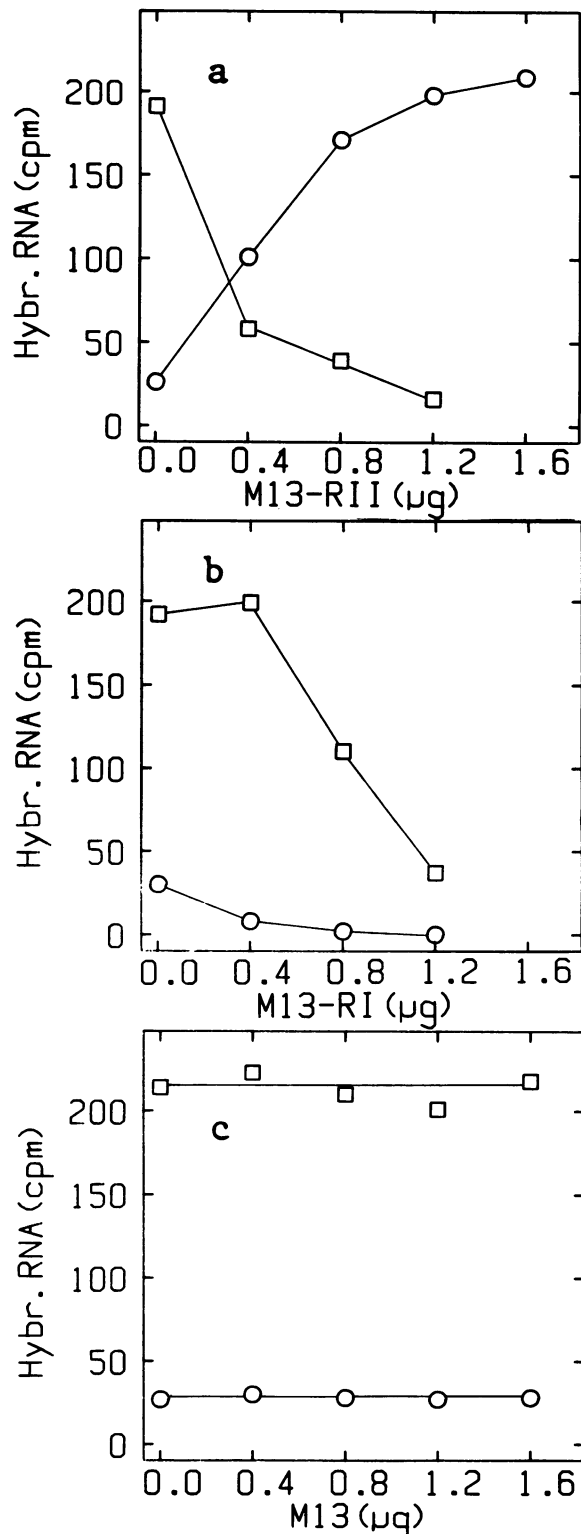


FIG. 2. Hybridization of RNAI and RNAII, pulse-labeled in vivo, to filters loaded with 1.5 μg of single-stranded DNA of phage M13mp7.RI (\circ , RNAII curve), and M13mp7.RII (\square , RNAI curve), as a function of the concentration of M13mp7.RII (a), M13mp7.RI (b), or M13mp7 (c) present during prehybridization. Each hybridization mixture contained three filters loaded with 1.5 μg of one of the same three probes. For prehybridization, the pulse-labeled RNA and various amounts of nonradioactive M13 probes, as indicated,

procedure was therefore considered to be adequate. (If that concentration had caused any loss of RNA, the loss could be expected to be greater at 25% phenol, which was not found.)

In preliminary experiments, nucleic acids in the cell lysate had been phenol extracted and ethanol precipitated. During these procedures, about 30% of acid-precipitable radioactivity was lost. However, the hybridization results did not significantly change when phenol extraction and alcohol precipitation were omitted. It was found best to dilute the whole lysate 10-fold with hybridization buffer as described above; this reduced some scatter in the data, presumably caused by inaccurate sampling due to the high viscosity of the undiluted lysate.

The kinetics of the hybridization reaction were biphasic; a linear increase of the hybridization during the first 10 h to about 75% of the final level was followed by a further 10% increase until 20 h and a further 15% increase during the next 24 h. For these reasons, the hybridization was carried out over 2 days, as described above.

Determination of the hybridization efficiency. DNA of the plasmids pT7/T3-18.RI and pT7/T3-18.RII was linearized by cleavage with *Hind*III enzyme and used as templates for T7 RNA polymerase. The *Hind*III cleavage site is adjacent to the pBR322 origin insert, such that the expected length of the runoff transcript was 623 nucleotides. After electrophoresis and autoradiography, a reaction product of the predicted length was found; with pT7/T3-18.RI, an additional shorter transcript (about 560 nucleotides) was found. The reaction mixture (50 μl) contained 1 μg of DNA, 40 mM Tris hydrochloride (pH 8), 8 mM MgCl_2 , 5 mM dithiothreitol, 2 mM spermidine-(HCl)₃, 400 mM each of the four nucleoside triphosphates, 1 μCi of [α -³²P]UTP, and 1 μl (20 U) of T7 RNA polymerase (15). After 1 h at 37°C, the reaction was stopped with 5 μl of 100 mM EDTA; the reaction mixture was then stored at -20°C. Initially, 1 μl of the reaction mixture contained 32,000 cpm of acid-precipitable RNA.

This RNA was hybridized to M13mp7.RI and M13mp7.RII phage DNA on filters by using the same conditions as for in vivo pulse-labeled RNA. The RNA made in vitro was found to be specific for its probe, and the hybridization efficiency was about 65% (data not shown). The hybridization temperature of 42°C was found to be optimal. The value of 65% could not be increased by increasing the amount of DNA on the filter relative to the amount of RNA. It is not clear what has limited the hybridization under these conditions.

We originally intended to include some [³²P]RNA in each hybridization mixture. This would have meant loading more DNA onto the filters, which were already loaded to a high DNA density, and it would have meant a spillover correction for the ³H radioactivity, which was already quite low. Moreover, the added [³²P]RNA would have interfered with and complicated the interpretation of the prehybridization reaction (see Results), and it would have prohibited the use of a single hybridization mixture for both RNAI and RNAII determinations on different filters. For these reasons, standards to measure the hybridization efficiency were not included in the hybridization mixtures.

Other procedures. All recombinant DNA work was done

were first heated to 100°C and allowed to hybridize for 2 h at 42°C, the three filters with DNA were added, and incubation was continued. The values from the M13mp7 filters (essentially counting background) have been subtracted from the radioactivities on the filters loaded with M13mp7.RI or M13mp7.RII (see the text for experimental details and interpretation).

as described previously (12, 16). The Northern blot experiment (Fig. 1) was done by first separating the RNAs by agarose gel electrophoresis in the presence of 7 M urea and then blotting of the RNA bands to a sheet of activated DBM cellulose, followed by hybridization with a denatured ³²P-labeled *Fnu*DII 581-bp fragment containing the replication origin of pBR322 (see above). The details of this procedure have been described (10), except that in the experiment of Fig. 1 the denatured radioactive probe was prehybridized for 1 h with M13 phage DNA probes, as indicated, before the diazobenzyloxymethyl (DBM)-cellulose sheet with the blotted RNAI and RNAII was added.

RESULTS

The *E. coli* B/r derivative RL331T, transformed with plasmid pBR322, was grown in glycerol-minimal medium and pulse-labeled with [³H]uridine. Previous Northern blot experiments had indicated that bacteria grown in glycerol-minimal medium contained higher levels of RNAI and RNAII than bacteria grown in rich media, such as LB (10). The amounts of labeled plasmid RNAI and RNAII were determined by filter hybridization to specific probes, constructed from the single-stranded DNA phage M13, carrying a 581-nucleotide region homologous to RNAI and RNAII, respectively.

A difficulty in estimating RNAI and RNAII by this method arises from the self-complementarity of these RNAs and from the presence of plasmid DNA with complementary regions in the cell lysate. To dissociate potential hybrids and liberate all RNAI and RNAII, samples of the radioactive lysates were heated to 100°C in the presence of increasing amounts of either one of the single-stranded DNA probes and then incubated for 2 h at 42°C for hybridization in liquid before the filters loaded with either one of the two probes were added.

Without the added probe during prehybridization, about 200 cpm hybridized to the probe for RNAI on the filter, corresponding to 0.8% of the total acid-precipitable radioactivity present, and 30 cpm (0.11% of the total) hybridized to the probe for RNAII (Fig. 2a and b, values at zero on the abscissa). With increasing amounts of phage DNA complementary to RNAI present during prehybridization, hybridization of RNAI to the probe on the filter decreased, as to be expected, and hybridization of RNAII to its filter probe increased until it leveled off slightly above 200 cpm, also corresponding to 0.8% of the total (Fig. 2a). We presume that this additional RNAII was liberated during the prehybridization with RNAII-like DNA.

Increasing amounts of phage DNA complementary to RNAII present during prehybridization did not liberate extra RNAI, such that the maximum was still equal to about 200 cpm or 0.8% of the total (Fig. 2b). This maximum occurred at low concentrations of the prehybridization DNA, whereas higher concentrations reduced hybridization of both RNAI and RNAII to their respective filters. Since the number of RNAI molecules is about 5 times greater than the number of RNAII molecules (see below), it was to be expected that during prehybridization more M13 phage DNA was needed in the experiment of Fig. 2a to saturate all RNAI-like sequences with complementary phage DNA than to saturate all RNAII-like sequences in the experiment of Fig. 2b.

A control experiment in Fig. 2c shows that the presence during prehybridization of M13 phage DNA without inserted pBR322 sequences has no effect on the hybridization of RNAI and RNAII to the filters, indicating that the effects

seen in the experiments of Fig. 2a and b are specific for the pBR322 origin sequences present during prehybridization.

The variability of the hybridization assays, as estimated from the control curves in Fig. 2c, was less than 10%. The variation from culture to culture was about 20% (data not shown).

The results of Fig. 2 thus indicate that for bacteria grown in glycerol minimal medium about equal amounts, corresponding to 0.8% of the total pulse-labeled RNA, hybridized to the probes for RNAI or RNAII. Assuming the hybridization efficiency to be 65% (see Materials and Methods), the synthesis rates of these RNAs correspond to 1.2% of the total rate of RNA synthesis.

DISCUSSION

Prehybridization method. The phenol-ethanol treatment employed here, followed by sodium dodecyl sulfate lysis, stops degradation of RNA instantly, and without further extraction or fractionation no RNA could be lost. This method precluded a DNase treatment of the sample. Since 95% of all RNAII transcription events do not lead to replication and therefore were presumably intercepted by RNAI (see below), most RNAII in the sample was expected to be hybridized to RNAI. Prehybridization with various amounts of single-stranded DNA probes was used to dissociate these hybrids before hybridization to the filter. At the same time, the prehybridization makes DNase treatment unnecessary.

Since RNAI is 108 nucleotides long, whereas the M13 probe contains a stretch of 581 nucleotides with RNAII homology, the M13 DNA with RNAII insert could in principle hybridize to the probe for RNAII on the membrane filter, carrying a radioactive RNAI along with it. In that case, the curve (Fig. 2a) would represent an overestimate of RNAII. For the experiment of Fig. 2b, this ambiguity does not exist, since RNAII molecules hybridizing to the phage DNA cover essentially the whole pBR322 insert in the phage DNA. The results of lane 5 in Fig. 1 suggest, however, that the RNAII hybrids in Fig. 2a are not significantly overestimated for the following reasons.

In the Northern blot experiment of Fig. 1, the radioactive probe was (nick-translated) double-stranded DNA and therefore homologous to both RNAI and RNAII (Fig. 3a). This is the reason why both bands are being labeled in lane 1 and 3 (Fig. 1). The addition of single-stranded M13 DNA homologous to RNAI (Fig. 1, lane 7) should therefore saturate the RNAII band on the DBM-cellulose such that it cannot become labeled. It should also have saturated the labeled probe with RNAII homology and thereby also prevent the labeling of the RNAI band on the DBM-cellulose (Fig. 3b); however, the phage DNA with RNAI homology has only blocked labeling of RNAI on the DBM-cellulose without significantly diminishing labeling of RNAII. This means that the M13 phage DNA with pBR322 insert has efficiently hybridized to the complementary radioactive probe in liquid but was inefficient in hybridizing to the complementary RNA on the DBM-cellulose. The same difference in hybridization efficiencies can be inferred from the results in Fig. 1, lane 5. This difference might have several reasons. For example, since small molecules diffuse faster than larger ones, the small radioactive probes reach their targets with complementary sequences on the DBM-cellulose before the M13 phage DNA; or steric hindrance might be involved, since the region with pBR322 homology is buried in the larger circular phage DNA and would have a lower probability to establish

the proper contact with its target region on the DBM-cellulose in comparison to the small probes. Irrespective of the explanation, the same effects should apply to the prehybridization in Fig. 2. We assume, therefore, that the curve in Fig. 2a (circular symbols) represents pulse-labeled RNAII only.

Relative synthesis rates of RNAI and RNAII. The experiments in Fig. 2a and b suggested that in pBR322-carrying bacteria about equal fractions, 1.2%, of the pulse-label were in RNAI and RNAII. Exogenous [^3H]uridine enters U and C residues in RNA and C residues in DNA; after a 1-min pulse-label, 75% of the nucleoside triphosphate label is in UTP, 25% is in CTP, and a negligible fraction is in dCTP (18). The U and UC content is 31 and 53% for RNAI, 22 and 46% for RNAII (12), and 23 and 47%, respectively, for pulse-labeled *E. coli* RNA (19). Thus, to express the pulse-labeling as a fraction of total RNA synthesis rates, the value for RNAI would have to be slightly reduced, since it contains somewhat more U and C than total RNA. Considering the overall accuracy of the method, this correction has been ignored in the following evaluation.

Northern blots suggest that the major RNAII band contains molecules of 500 to 600 nucleotides, whereas the primary RNAI transcript is 108 nucleotides (25) (a degradation product of 103 nucleotides, RNAI*, arises later due to RNase E [23]). Assuming 108 and 600 nucleotides for the average RNAI and RNAII transcripts, respectively, their nearly equal synthesis rates in terms of amounts would represent a 5:1 molar ratio. Assuming the same average life (10), their steady-state levels should also correspond to a 5:1 ratio. This ratio agrees with estimates from Northern blots, which measure steady-state levels rather than synthesis rates, and which ranged between 2.5:1 and 5:1 in different experiments (10). For Fig. 1 (lanes 1 and 3), a densitometer scan gave a 3.5:1 ratio. Due to the nonlinearity of the autoradiographic process, an underestimate of this ratio by this method would be plausible. Measurements of galactokinase activities expressed from RNAI and RNAII promoters gave a value of 5.5:1 (20; values at 30°C in a wild-type strain, normalized to equal plasmid numbers), again in agreement with the ratio estimated here.

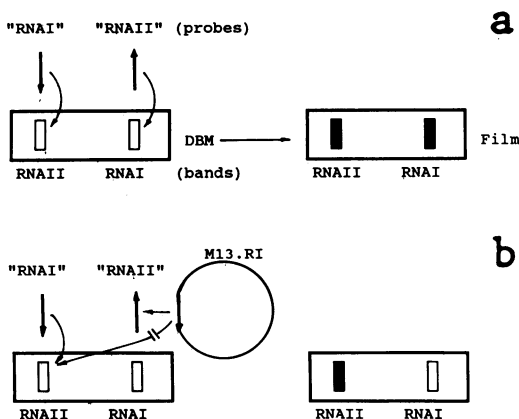


FIG. 3. Interpretation of the Northern blot in Fig. 1. (a) Lane 1: no additions, RNAI and RNAII bands on DBM cellulose are both being labeled by the indicated radioactive probes. (b) Lane 7: the addition of M13mp7.RI traps the probe homologous to RNAII and thereby prevents labeling of the RNAI band, but it does not efficiently hybridize to the RNAII band on the DBM-cellulose and thus permits labeling of that band by the probe homologous to RNAI.

Absolute rates of RNAI and RNAII synthesis. The instantaneous rate of RNA synthesis in *E. coli* B/r has been previously determined as a function of growth rate (4, 18, 22). This was done by measuring the rate of RNA accumulation (stable RNA synthesis) from the UV absorption of RNA hydrolysates and by measuring the rate of mRNA synthesis relative to the rate of stable RNA synthesis by using a hybridization assay for rRNA. Under the conditions used here (doubling time, 68 min), the instantaneous rate of RNA synthesis was equal to 1.1×10^{15} RNA nucleotides per min per OD₄₆₀ unit of cell mass. If 1.2% of this value corresponds to the synthesis rate of RNAI or RNAII and if the average transcript lengths are 600 nucleotides for RNAII and 108 nucleotides for the RNAI, we obtain 1.3×10^{11} RNAI molecules and 2.3×10^{10} RNAII molecules synthesized per min per OD₄₆₀ unit of cell mass.

The copy number of pBR322 plasmids determined under these conditions in unfractionated bacterial lysates (including all supercoiled, open circular, and linearized monomers and dimers) was 6.6×10^{10} plasmids per OD₄₆₀ unit of culture mass (10; unpublished measurements from this laboratory). Dividing the synthesis rates above by this value gives 2 RNAI molecules per min per plasmid and 0.35 RNAII molecules per min per plasmid. Since successful priming occurs, on the average, once per generation time, i.e., once per 68 min, it means that 1 in 22 (0.35×68) prepriming events leads to replication.

With an RNA chain elongation rate of 50 nucleotides per s (2), it would take about 2 s to make an RNAI chain. Thus, with one RNAII transcription every 180 s and one RNAI transcript every 30 s, the probability that transcription in the opposite direction leads to head-on collision of two RNA polymerase molecules is small (2 of 30 for RNAI and 2 of 180 for RNAII transcription).

Assuming the average lifetimes of RNAI and RNAII to be 0.8 min (10), the bacteria would contain, at any instant, 1.6 (2×0.8) RNAI molecules per plasmid and 0.28 (0.35×0.8) RNAII molecules per plasmid. Since it takes about 12 s to synthesize a preprimer, the time period during which RNAI must act to prevent switching and primer processing (13, 14) can only be a few seconds. It seems important, therefore, that the cells contain a sufficient concentration of RNAI molecules. The RNAI-hybridizable material includes molecules that have hybridized to RNAII which are thus functionally inactive.

If the major RNAII transcript seen in Fig. 1 were the 555-nucleotide RNase H processing product, it would mean that about 20% (i.e., somewhat less than 0.28) of the plasmids in the cell have a primer waiting to prime replication. If replication occurred once per 68 min generation time and 20% of the plasmids had an RNA primer waiting for replication to begin, this waiting period would last for about 14 min. Since this is difficult to reconcile with the short lifespan of RNAII, we assume that the major RNAII transcript seen on Northern blots is a longer transcript terminated beyond the replication origin without switching.

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