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Quantitation of ColE1-encoded replication elements

(plasmid copy number/RNA I/RNA II/Rom protein)

MICHAEL BRENNER† AND JUN-ICHI TOMIZAWA‡

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Replication of the *Escherichia coli* plasmid ColE1 initiates from an RNA primer. This primer is formed by a ColE1 RNA II molecule that remains hybridized to its DNA template in the origin region after transcription. Continued hybridization is inhibited by prior binding to RNA II of another ColE1 transcript, RNA I; and this interaction is regulated by the plasmid-encoded Rom protein. To understand the quantitative aspects of regulation of ColE1 synthesis, we have measured the levels of RNA I, RNA II, and Rom protein *in vivo*, as well as the half-lives of the RNAs. The intracellular concentrations of RNA I, RNA II, and Rom protein were found to be about 1 μM, 7 nM, and 1 μM, respectively; and the RNAs had half-lives of about 2 min. A simple model derived from these results indicates that the plasmid copy number is little affected by the rate of RNA II synthesis but is strongly dependent on that of RNA I.**

Replication of the *Escherichia coli* plasmid ColE1 is controlled by the regulated production of an RNA primer of DNA synthesis, whose transcription is initiated 555 base pairs (bp) upstream from the replication origin. When transcription of this RNA (RNA II) reaches the origin region, the RNA may commence forming a persistent hybrid with the template DNA. The primer for DNA synthesis is formed by cleavage of the hybridized RNA at the origin region by RNase H (1). Another RNA, RNA I, negatively regulates primer formation (2, 3). This 108-nucleotide RNA is specified by the same DNA region coding for the 5′ end of RNA II but by the complementary strand (see Fig. 1) (4). Binding of RNA I to RNA II inhibits primer formation by preventing RNA II from assuming a conformation that is required for its persistent hybridization with the origin DNA (5). The interaction of RNA I and RNA II is in turn modulated by the Rom protein, a small plasmid-encoded protein whose structural gene starts about 600 bp downstream of the replication origin (6–9).

Although the regulation of ColE1 replication is now understood qualitatively, little is known about its quantitative aspects. No data are available concerning the intracellular levels of RNA I and RNA II, and only a rough estimate has been made of the level of the Rom protein (9). Herein we report the *in vivo* levels of RNA I, RNA II, and Rom protein and provide estimates of the half-lives of the RNAs. The results are discussed in terms of a simple model relating the cellular plasmid level to the concentrations of these components and their rates of interaction.

MATERIALS AND METHODS

The source of materials and details of methods are given in ref. 6, except as described below.

Bacterial Strains and Plasmids. Both strain N100 (*rpsL galK2 recA3*) (10) and strain CSR603 (*recA1 uvrA6 phr-1*) (11) are derivatives of *E. coli* K-12, and strain SA791 is a derivative of strain C600 (12) selected for thymine auxotrophy by Sankar Adhya (National Cancer Institute, Bethesda, MD). Bacteria were grown at 37°C in LB medium (13) or in a minimal Mops medium (14), supplemented with ampicillin (30 μg/ml) when plasmids were resident. For growth of strain SA791, thymidine was added to each medium to 10 μg/ml, and the minimal medium was further supplemented with 0.4% glucose, threonine at 40 μg/ml, leucine at 40 μg/ml, and thiamine at 1 μg/ml. These supplemented media are referred to as LBThy and MMGS, respectively. Plasmids previously described include pNT205 (15), which contains the replication region and *rom* gene of ColE1 and the *bla* gene of pBR322; pNT211 (15), a tetracycline-resistant derivative of pNT205; pNT220 (6), which contains the *E. coli recA* terminator region inserted 128 bp downstream of the replication origin of pNT205; pNTMB1 (6), which contains another copy of the *recA* terminator inserted within the *rom* gene of pNT220; and pGEMB1 (6), which contains the ColE1 *Pvu* II fragment (see Fig. 1, probe C) inserted into the *Sma* I site of pGEM-1 (Promega). Plasmid pCer was constructed for this study by inserting the *cer* region of ColE1 into pNT205 at the *Taq* I site 128 bp downstream of the replication origin. The *cer* region, which facilitates maintenance of plasmids as monomers (16), was obtained on a 377-bp *Hpa* II fragment spanning bp 3687–4063 of ColE1 (17) and was inserted so that the 3687-bp end was closer to the replication origin. Plasmid pΔP4 was obtained from pNT205 by deleting the *Taq* I–*Tth* III fragment located between 128 and 298 bp downstream of the replication origin. Both restriction sites were filled-in during the construction (this plasmid was made for reasons incidental to the present study). Plasmid pPRB3 was constructed by inserting the *Fnu*DII–*Hae* III fragment spanning the RNA I region of ColE1 (see Fig. 1) into the *Sma* I site of pGEM-1 in the orientation that placed the upstream end of the fragment nearer to the T7 promoter.

Synthesis of Probes and Internal Standards. ^{32}P-labeled RNA probes were synthesized from pPRB3 and pGEMB1 by using SP6 or T7 RNA polymerase as described (6). The primary ^{3}H-labeled internal standard was made similarly (6); it contains the *rom* sense strand from the *Pvu* II–*Mnl* I fragment (see Fig. 1, Int. Std.) flanked by short pGEM-1 sequences. [^{3}H]RNA I and [^{3}H]RNA II were synthesized from pNTMB1 using the procedure described for *in vitro* transcription of ColE1 plasmids (6), except that ATP was at 100 μM, GTP was at 200 μM, CTP was at 200 μM, and [^{3}H]UTP (4 Ci/mmol; 1 Ci = 37 GBq) was at 50 μM. The reaction products were purified by gel electrophoresis (6), and the positions of the 108-base RNA I species and the ≈770 nucleotide RNA II species (terminating at the *recA* transcription terminator present downstream of the replication origin in pNTMB1) were identified by autoradiography of ^{32}P-labeled reaction products in flanking lanes.

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†Present address: Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892.
‡Present address: National Institute of Genetics, Mishima, Shizuoka-ken 411, Japan.

Probe Protection Experiments. The amounts of RNA transcripts were quantitated by their ability to hybridize to ^{32}P-labeled riboprobes and thus protect them from degradation by RNases. Procedures used were as described (6) with the following modifications: to detect rom mRNA, hybridization mixtures contained ≈40 μg of RNA and probe C [80,000 cpm (about 1 ng)], to detect RNA I, they contained 4 μg of RNA and probe A (80,000 cpm), and to detect RNA II, they contained 20 μg of RNA and probe B (350,000 cpm). RNases used per reaction mixture were 150 ng of RNase A and 20 units of RNase T1 for rom mRNA, 30 ng of RNase A and 5 units of RNase T1 for RNA II, and 36 units of RNase T1 for RNA I. For the latter reaction, NaCl was added to 200 mM. Control experiments showed these conditions to yield quantitative values for each RNA that were proportional to the amount of RNA added.

To correct for the efficiency of RNA detection, a known amount of a ^{3}H-labeled internal standard that contained a partial transcript of the *rom* region (see above) was added at the beginning of many of the cell RNA preparations (for details, see ref. 6). To determine if the recoveries obtained with the *rom* internal standard were appropriate for RNA I and RNA II, ^{3}H-labeled authentic transcripts of each of these two RNAs were prepared by *in vitro* transcription (see above) and then used as internal standards for the preparation of RNA from cells lacking a plasmid. Subsequent assay of the preparations for the added transcripts yielded a recovery of 14% for the RNA I sample and of 15% for the RNA II sample, values quite similar to the average of 13% recovery observed for the *rom* internal standard.

Rom Radioimmunoassays. Antibody was prepared by injecting purified Rom protein both intradermally and into the footpads of rabbits. The initial inoculation contained 200 μg of Rom protein in complete Freund's adjuvant, and this was followed by four booster injections of 100 μg of Rom protein in incomplete adjuvant. Levels of Rom antibody reached a plateau after the third booster injection, and the animals were bled-out after the last booster injection.

To obtain labeled antigen, CSR603(pNT211) cells were labeled with [^{35}S]methionine by the maxicell method (11). The labeled cells were sonicated, and the Rom protein partially purified by DEAE-Sephadex chromatography (9) of the supernatent obtained by centrifugation at 30,000 rpm for 30 min in a Beckman SW 50.1 rotor. The column was assayed by immunoprecipitation as described below, and the peak fractions were used without further processing. About 65% of the radioactivity in the peak fractions was precipitable by Rom antibody.

The cell extracts used for the Rom protein measurements were prepared by growing N100 containing the indicated plasmids in LB to an OD_{550} value of 0.75, harvesting by centrifugation, washing twice with 30 ml of 50 mM Tris·HCl (pH 7.4), and resuspending in 2 ml of Rom sonication buffer (9). Cells were broken by sonication and centrifuged at 30,000 rpm for 30 min in a Beckman SW 50.1 rotor, and the supernatant was used for the radioimmunoassays.

Binding for the radioimmunoassays was conducted in a final volume of 500 μl containing 50 mM Tris·HCl (pH 7.4), 250 μg of bovine serum albumin, 0.5 μl of rabbit serum, 1000 cpm (30 ng) of ^{35}S-labeled Rom, and test samples as indicated. The mixtures were incubated for 3 hr on ice, and then 50 μl containing 1.25 mg of *Staphylococcus aureus* cells (Sigma; catalogue number P-9151) in 50 mM Tris·HCl (pH 7.4) was added and the tubes were rotated gently at 4°C for 1.5 hr. Samples were then centrifuged for 5 min in a microcentrifuge, the pellet was suspended in 400 μl of 50 mM Tris·HCl (pH 7.4) containing 2% (wt/vol) SDS and boiled for 2 min to dissolve the cells, and the solution was transferred to 4 ml of liquid scintillation fluid and its radioactivity was determined. The amount of immune serum used was sufficient to precipitate about 75% as much ^{35}S-labeled Rom as was precipitated by a saturating level of antibody. Radioactivity precipitated by the same dilution of preimmune serum, which was about 5% of the total ^{35}S-labeled Rom cpm, was subtracted from values obtained.

The Rom protein used as a standard for the assays was purified in this laboratory by Tapan Som (9); its concentration was determined by nitrogen analysis.

RNA Stability Measurements. SA791(pCer) was grown in MMGS medium to an OD_{550} value of 1.0 and rifampicin (Calbiochem) was added to 100 μg/ml from a stock of 10 mg/ml in 50% (vol/vol) dimethyl sulfoxide. Samples of 5 ml were taken for RNA preparation immediately before and at intervals after the addition of rifampicin.

Intracellular Volumes, Cell Numbers, and Plasmid Numbers. Quantitative data for RNA I, RNA II, and Rom protein levels were initially obtained per OD unit of culture. They were converted to numbers of molecules per cell and intracellular concentrations by using factors obtained as follows. Intracellular volumes were determined by the difference in volumes occupied by [^{3}H]water and [^{14}C]sucrose using the centrifugation method of Stock *et al.* (18). For SA791(pCer) we obtained intracellular values per ml of cultures grown to an OD_{550} value of 1.0 in LBThy and MMGS media of 0.17 μl and 0.30 μl, respectively. The number of cells per ml at an OD_{550} value of 1.0 in these two media was found by plating to be 3.7×10^{8} and 4.8×10^{8}, respectively.

We assumed the number of pCer plasmids per cell to be the same as its closely related parent, pNT205. This plasmid is present at about 10 copies per chromosome in cells growing in LB (15) and, since such cells average about 1.5 chromosomes (19), about 15 plasmids are present per cell. To determine if the plasmid level changes when cells are grown in MMGS, alkaline minipreps were made from SA791(pCer) grown to an OD_{550} value of 1.0 in LBThy and MMGS media, and the plasmid yields were compared by photography of the ethidium-stained plasmid after electrophoresis in an agarose gel (15). No significant difference in plasmid content per cell was found.

RESULTS

Quantitation of RNA I and RNA II. The *in vivo* concentrations of RNA I and RNA II were determined by quantitative probe protection experiments. To detect each RNA, we used a riboprobe made by *in vitro* transcription from a DNA fragment that overlaps the 5′ end of the RNAs (Fig. 1) so that only *in vivo* transcripts initiated from the correct promoter would be quantitated. Briefly, the labeled riboprobe specific for each RNA was hybridized to cellular RNA, the hybridization mixtures were treated with RNases specific for single-stranded molecules (RNase A and/or RNase T1), and the protected fragment(s) of probe were resolved on a denaturing polyacrylamide gel. The radiolabeled band of the correct size was then excised, its radioactivity was determined, and the cpm observed was used to calculate the number of protected molecules based on the specific activity of the probe. These values were then adjusted for the efficiency of detection by using the level of detection of an internal standard RNA that had been added at the beginning of the RNA preparations.

Typical probe protection patterns for RNA I and RNA II are shown in Fig. 2. The size of each major protected species (bands A and B), estimated from a series of RNA standards electrophoresed in parallel (lanes 1 and 4) matched closely the size expected. In addition, the RNA I probe also produced a somewhat smaller protected band whose size is that predicted for RNA I_{-5}, a degradation product of RNA I lacking the first five 5′ nucleotides (20, 21). It was present at 5–10% of the level of intact RNA I.

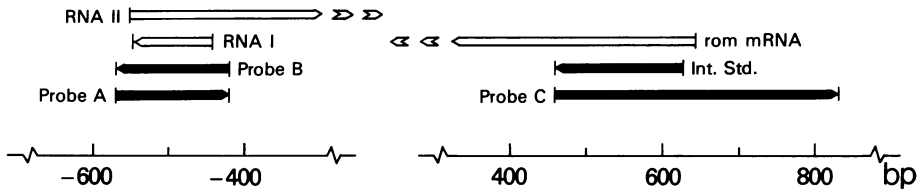

FIG. 1. Map of transcripts, probes, and relevant regions of ColE1. Positions are numbered relative to the origin of DNA replication, negative numbers being upstream and positive numbers being downstream. The start points of transcripts for RNA II (bp −555) (1), RNA I (bp −445) (4), and rom mRNA (bp 641) (6) are shown. Most RNA I transcripts terminate near bp −552 as indicated (4); RNA II (1) and rom mRNA (6) terminate at multiple sites, all of which are beyond the broken ends of the open bars representing the transcripts. Solid bars indicate the positions of probes and of the internal standard (Int. Std.) used in this study. Probes A and B were made by transcribing an *Fnu*DII–*Hae* III fragment (bp −570 to −421) in the downstream or upstream direction, respectively. Probe C was derived from a *Pvu* II–*Pvu* II fragment (bp 457–827), and the Int. Std. was from a *Pvu* II–*Mnl* I fragment (bp 457–624).

Numerical data from a series of quantitative probe protection experiments are presented in Table 1. For these experiments we used SA791 cells growing in the minimal medium MMGS, in which they have about an 80-min generation time. Intact RNA I was found to be present at about 400 molecules per cell, and RNA II at about 3 molecules per cell. These values correspond to intracellular concentrations of about 1 μM and 7 nM, respectively.

RNA Half-Lives. The half-lives of RNA I, RNA II, and Rom mRNA were estimated from the rate of decrease of each RNA after the addition of rifampicin to SA791(pCer) growing in MMGS medium (Fig. 3). The initial rate of decrease for each RNA corresponded to a half-life of about 2 min, a value typical for unstable RNA in *E. coli* (22). While the levels of RNA I and rom mRNA continued to fall for at least 8 min after rifampicin addition, that of RNA II stopped decreasing after about 5 min. One possible explanation of this difference is that those RNA II molecules complexed with RNA I are more stable.

Rom Protein Level. A competitive radioimmunoassay was used to quantitate the amount of Rom protein in cell extracts. The antibody used was raised in rabbits to highly purified Rom protein, and the competing labeled tracer was obtained by partial purification of Rom protein from [^{35}S]methionine-labeled maxicells. Fig. 4*A* shows a calibration curve for the assay in which purified Rom protein was used as the competitor for antibody binding. The assay is seen to be linear over a wide range of Rom levels and capable of detecting less than 10 ng of the protein. Fig. 4*B* presents assay data for an extract made from cells carrying pNT220, which has an intact *rom* gene, and for one made from cells carrying pNTMB1, a derivative of pNT220 that has a disrupted *rom* gene. The former extract competed with the labeled antigen in proportion to the amount of extract added, whereas the extract made from cells with a disrupted *rom* gene produced only a low level of inhibition that did not change with the amount of extract added. These results show the assay to be specific for the Rom protein. In this particular case, the concentration of Rom protein in the extract is seen to be about 100 ng/ml, which corresponded to about 10 ng of Rom per mg of soluble protein and to a cellular concentration of about 1 μM of the 7.2-kDa monomer. Essentially the same concentration was obtained for an extract made from cells containing pNT205, the parent of pNT220, which also has an intact *rom* gene (range, 0.9–1.9 μM for six assays).

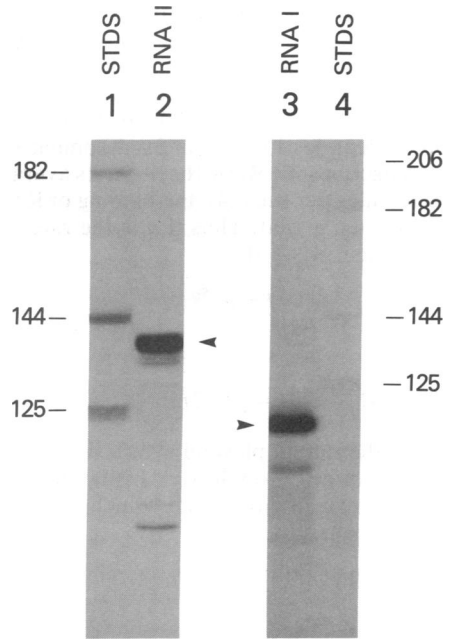

FIG. 2. Riboprobe fragments protected by RNA I and RNA II. Presented is a composite of probe protection assays for RNA II (lanes 1 and 2) and RNA I (lanes 3 and 4). Size standards (in nucleotides) are present in lanes 1 and 4, and the protected riboprobe fragments are in lanes 2 and 3. RNA preparations were made from cells containing the *rom*$^+$ plasmids pNT205 (lane 2) or pCer (lane 3). Arrowheads point to the bands having the gel position predicted for fragments protected by correctly initiated transcripts (Fig. 1).

DISCUSSION

Plasmids must replicate at an average rate of once per cell generation to ensure their continued propagation. Slower replication rates would lead to their being lost by dilution, whereas faster rates would cause them to accumulate to a lethal level. Stabilization of plasmid levels is believed to be primarily controlled by negative feedback of plasmid replication by RNA I (2), whose concentration presumably varies with that of the plasmid.

The quantitative data presented in this paper indicate the importance of negative control by RNA I. We found that cells carrying a rom$^+$ plasmid and growing in minimal medium with a generation time of 80 min have about three copies of RNA

Table 1. Numbers of RNA I and RNA II molecules per cell

RNA preparation	Molecules, no. per cell	
	RNA I	RNA II
1	499 ± 36 (3)	2.3 (1)
2	365 ± 89 (6)	1.9 ± 0.2 (3)
3	382 ± 56 (3)	2.8 ± 1.0 (6)
4	333 ± 49 (8)	3.7 ± 1.0 (6)

RNA preparations 1 and 2 were made from SA791(pCer), and preparations 3 and 4 were from SA791(pΔP4). Both of these plasmids are *rom*$^+$. As pΔP4 differs from pCer only by deletion of a small region downstream of the replication origin, we consider the different values obtained for the two plasmids indicative of the variability inherent in the measurements. Values are presented as mean ± SEM, and the numbers in parentheses are the number of times each preparation was assayed.

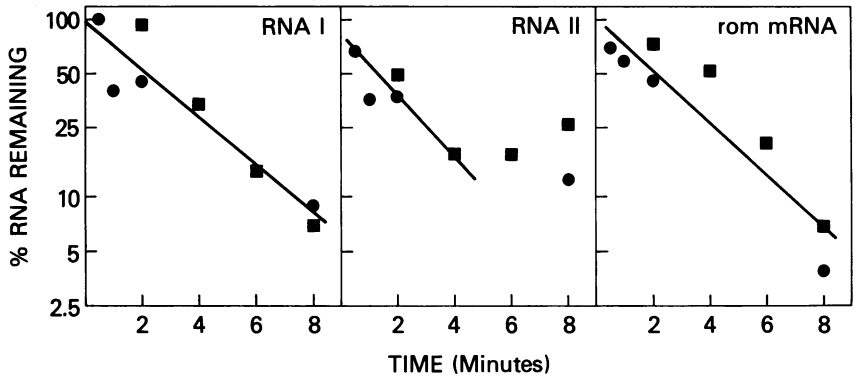

FIG. 3. Half-lives of RNA I, RNA II, and rom mRNA. Percentages were determined of each RNA remaining after addition of rifampicin to SA791(pCer) growing in MMGS medium. Circles (●) and squares (■) represent data from two experiments. A least-squares fit to the data yielded half-lives of 2.0, 2.2, and 1.7 min for rom mRNA, RNA I, and RNA II, respectively.

II per cell and that these transcripts have a half-life of about 2 min. Assuming that the level of RNA II is at a steady state, the rate of RNA II synthesis per cell is thus $3[(\ln 2)/2]$ min^{-1}. Since each cell has about 15 plasmids under these conditions, this corresponds to an RNA II synthesis rate per plasmid of $(\ln 2)/10$ min^{-1}, so that in the 80-min cell generation time each plasmid synthesizes about six RNA II transcripts. To maintain a constant plasmid concentration, on average only one of these transcripts must form a functional primer. If all the RNA II transcripts were able to prime DNA replication, the above rate of RNA II synthesis would result in the initial 15 plasmids increasing to about 4000 in a single 80-min cell generation. Runaway synthesis apparently does occur for some plasmids bearing mutated RNA I regions (9).

We have found the half-life of RNA I also to be about 2 min. This rapid turnover means that the concentration of RNA I quickly adjusts to changes in conditions. Together with the excess capacity for primer formation just described, this provides for a rapid and sensitive control of plasmid replication rate.

By making a few assumptions, it is possible to derive an equation that permits the *in vivo* rate of interaction between RNA I and RNA II to be estimated. One assumption is that the rate of plasmid synthesis R_p is equal to the rate of primer formation, which in turn is the product of the rate of RNA II initiation per plasmid, R_{II}, and the fraction of RNA II molecules, f, that escapes binding by RNA I:

$$R_p = R_{II} f.$$

This assumption is a simplification in that some RNA II transcripts uncomplexed with RNA I may not form primers; for example, *in vitro* about a third of the uncomplexed RNA II molecules fail to remain hybridized to the origin region (5). Although the fraction of free RNA II transcripts failing to form primer *in vivo* is not known, unless it is quite high it would have little effect on the calculations that follow.

Above we deduced that $R_{II} = (\ln 2)/10$ min^{-1}. To estimate f, we note that for RNA I to prevent primer formation, it must complex with the elongating RNA II when it is between about 110 and 360 nucleotides long (23). By assuming that RNA polymerase transcribes the RNA II gene at its average rate of about 50 nucleotides per sec (24), the binding of RNA I must occur within a 5-sec period. Thus if q is the rate of binding between RNA I and RNA II

$$f = e^{-5q}$$

and

$$R_p = R_{II} f = [(\ln 2)/10] e^{-5q}.$$

At steady state the rate of plasmid synthesis R_p must be the same as the rate of cell growth R_g. This latter is simply $(\ln 2)/g$, where g is the generation time, which is 80 min for our conditions. Consequently,

$$[(\ln 2)/10] e^{-5q} = (\ln 2)/80, \qquad [1]$$

which yields $q = 0.42$ sec^{-1}.

The second-order rate constant k for the interaction of RNA I and RNA II can be derived from the estimated *in vivo* binding rate q by assuming that the latter rate is directly proportional to the RNA I concentration [I]; i.e., $q = k$[I]. With the RNA I concentration at the estimated level of 1 μM, this corresponds to a second-order reaction rate constant of 4.2×10^5 M^{-1}·sec^{-1}. This estimated value is in reasonable agreement with the rate constant of about 2.7×10^6

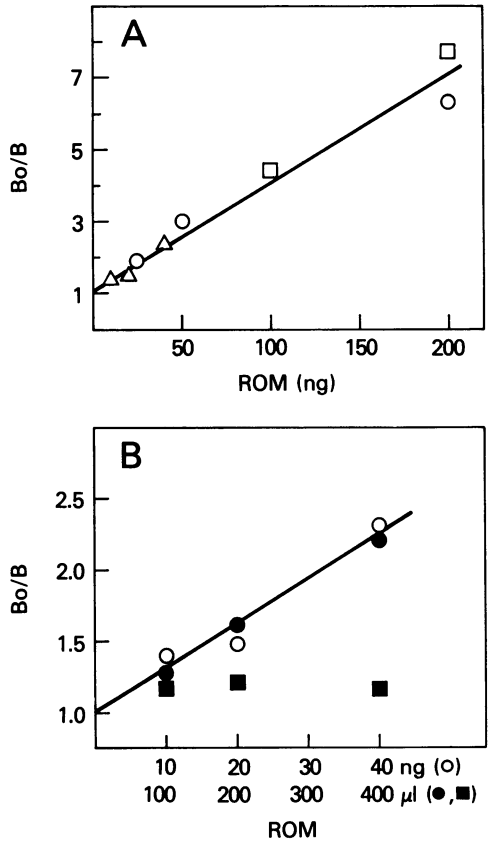

FIG. 4. Competitive radioimmunoassay for Rom protein. (*A*) Data are plotted as the ratio of ^{35}S-labeled Rom precipitated in the absence of added unlabeled Rom protein (B_o) to that precipitated in its presence (B). If the antibody binds with essentially a single affinity and is nearly saturated by the labeled tracer alone, this plot should yield a straight line. Different symbols represent data from independent experiments. (*B*) Data are plotted as in *A*, except unlabeled competitor is either purified Rom protein (○) or extract prepared from cells containing a plasmid with an intact *rom* gene (pNT220) (●) or a disrupted *rom* gene (pNTMB1) (■).

$M^{-1}\cdot sec^{-1}$ obtained *in vitro* in the presence of excess Rom protein. This value was calculated from the average reaction rate at 25°C of RNA I with RNA II species having lengths between 110 and 360 nucleotides (23) corrected for the observation that the reaction proceeds about 1.7 times faster at 37°C (J.T., unpublished experiments).

An equation analogous to Eq. 1 can be derived to predict the effects of changes in the rate of RNA I or RNA II synthesis on plasmid concentration by making the additional assumption that the RNA I concentration [I] is directly proportional to the number of plasmids per cell P; i.e., [I] = $(R_I/d)P$ where R_I is the rate of RNA I synthesis per plasmid and d is the first-order rate constant for RNA I degradation. Then

$$R_p = R_{II}f = R_{II}e^{-5k(R_I/d)P} = (\ln 2)/g.$$

Solving for the number of plasmids per cell,

$$P = (d/5kR_I)\ln(R_{II}g/\ln 2). \quad [2]$$

It is seen that the plasmid level changes as a ln function of the per plasmid rate of RNA II synthesis (R_{II}) but changes inversely with the per plasmid rate of RNA I synthesis (R_I). For example, a doubling of the plasmid number would be achieved either by squaring the value of the term $R_{II}g/\ln 2$ or by decreasing the rate of RNA I synthesis by half. For cells growing in minimal media studied here, where we found $R_{II} = (\ln 2)/10$ and $g = 80$, so that $R_{II}g/\ln 2 = 8$, this means that an 8-fold increase in RNA II synthesis is required to produce the same increase in the number of plasmids per cell that would be produced by a 2 times reduction in RNA I synthesis. To produce a further 2-fold increase in plasmid number would require either an additional 64-fold increase in RNA II synthesis or an additional 2 times decrease in RNA I synthesis. Thus a consequence of the excess capacity for RNA II production is that plasmid levels are relatively insensitive to changes in the rate of RNA II synthesis. This conclusion further supports the proposal that RNA I is the primary regulator of plasmid copy number.

Eq. 2 also predicts that conditions that alter the rate constant for inhibitor complex formation (k) should proportionately affect the plasmid level. It has been shown (9) that changes in k produced by base substitutions in RNA I and RNA II indeed produce proportional changes in plasmid copy number.

Another factor affecting the rate of inhibitory complex formation is the presence of the Rom protein. By using a specific competitive radioimmunoassay, we have determined that the intracellular concentration of Rom protein monomer is about 1 μM. Previously an estimate of 5 μM (9) had been made by measuring the amount of Rom protein purified from a given quantity of cells and adjusting the yield for recovery of labeled Rom protein added as an internal standard. As it was assumed that the added tracer (a crude extract of labeled maxicells) contained no radiolabeled components other than Rom and that the final product was completely pure, the value obtained was an upper level estimate. Consequently, we do not consider this previous estimate to be inconsistent with the lower value obtained here by a more direct procedure.

Several *in vitro* measurements have been made of the dependence of complex formation on Rom concentration, but their interpretation is unclear. About 2 μM Rom was found to be required for half-maximal stimulation of formation of a stable complex (C^s of refs. 9 and 25); whereas only 0.1 μM Rom was found to be required to convert half of an earlier precursor complex (C^{m*} to C^{m**} of ref. 25) to a Rom-bound form. Although appearing to give discrepant results, these two observations cannot be readily compared; one measured a composite rate whereas the other measured a binding constant. They differ as well in the product formed and the concentrations and types of substrates used.

In vitro studies using maximally effective amounts of Rom yield an average increase of 2- to 3-fold in the rate of formation of a stable complex between RNA I and RNA II molecules between 110 and 360 nucleotides long (23). *In vivo*, Rom reduces copy number 2- to 3-fold, suggesting that the 1 μM concentration of Rom that we observe *in vivo* is maximally effective.

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