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Copy-number of broad host-range plasmid R1162 is regulated by a small RNA

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ABSTRACT

We have shown previously [Kim, K. and Meyer, R.J. (1985) J. Mol. Biol. 185,755-767] that copy-number of the broad host-range plasmid R1162 is controlled by the amounts of two proteins, encoded by cotranscribed genes comprising a region of the plasmid called RepI. We have now demonstrated that expression of RepI is negatively regulated by a 75 base RNA that is complementary to a segment of the RepI message. Increased intracellular amounts of RNA molecules that include this segment relieve the inhibition of RepI gene expression, suggesting that the target for regulation is the mRNA itself. A mutation decreasing the amount of the 75 base RNA results in elevated plasmid copy-number. Thus, consistent with our previous observations, regulation of the expression of the RepI genes is a factor in controlling plasmid copy-number.

INTRODUCTION

R1162 is a broad host-range, 8.75 kilobase-pair plasmid that is replicated with a high copy-number in Escherichia coli (1-4). It is identical or very similar to RSF1010 (5), R300B (2), and to several other incompatibility group Q plasmids isolated from numerous species (2,3). The products of three plasmid genes are essential for replication of R1162 DNA (6). Two of the genes are co-transcribed, and together make up a region designated RepI (Fig. 1). Increased transcription of the RepI genes, in the direction right to left in Fig. 1, results in a higher plasmid copy-number (7). An increase in gene dosage does not have the same effect, indicating that expression of RepI is regulated, and that this determines plasmid copy-number (6,7). In this report we describe the molecular mechanism of this regulation. We find that a negative regulatory element is encoded within a 100 base-pair (bp) region at the promoter-proximal end of RepI. A small, 75 base RNA, complementary to RepI mRNA, is transcribed within this region, and is responsible for the regulation of RepI gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The E. coli K-12 strains used in this work are MV10 (thr leu thi lacY supE44 fhuA ΔtrpE5) (8), M182 (ΔlacIPOZY X74 galK galU strA) (9), JM103 (Δ[lac pro] strA thi supE endA sbcB hsdR, F'traD36 proAB lacI^q lacZΔM15) (10), and MC1022 (araD139 Δ[ara leu]7697 lacZΔM15 galU galK strA) (9). We prepared a collection of plasmids containing different cloned fragments of RepI DNA (Fig. 1). A brief summary of how these molecules were constructed is given in Table 1. Bacteria were grown at 37 C in TYE broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl).

Mapping RNA transcripts with S1 endonuclease

Probe DNA was prepared by first constructing derivatives of M13mp9 (12) containing fragments of R1162 DNA. The DNAs of plasmids pUT431, pUT436 or pUT437 (Table 1) were digested with BamHI, and the fragments containing the R1162 DNA cloned into the BamHI site of the phage vector. The inserted fragments consisted of R1162 DNA extending from 2.32 kb to 2.86 kb (pUT431), 2.68 kb (pUT436) or 2.66 kb (pUT437), as well as a 200 base-pair EcoRV-BamHI fragment, derived from pBR322, linked to the variable end. An M13mp9 derivative containing R1162 DNA from 2.57-2.68 kb was similarly prepared by cloning a BamHI fragment present in pUT509 (Table 1). The insert consisted of R1162 DNA with the small EcoRV-BamHI fragment of pBR322 joined to the 2.68 end. The strand specificity of each probe was determined by the orientation of the inserted DNA.

Labelled probe DNA was made by the method of Burke (17): single-stranded phage DNAs of the M13mp9 derivatives were used as templates for the DNA polymerase I-catalyzed synthesis of the radioactively-labelled, complementary strand. The polymerizations were primed with a 17 base oligonucleotide complementary to M13 (New England Biolabs). Each reaction mixture consisted of 50 µg template DNA and 25 ng primer in 120 µl 0.0125 M Tris (pH 7.5), 0.006 M $MgCl_2$, 0.01 M DTT, to which was added 3 µl 100 µM dATP, 10 µl α-[^{35}S]-dATP (500 Ci/mmole, 10 mCi/ml, New England Nuclear), 80 µl of a solution containing dTTP, dCTP, and dGTP (each 37.5 µM) in 0.01 M Tris (pH 7.5), 0.005 M $MgCl_2$, 0.075 M DTT, and 6-7 units Klenow fragment (Bethesda Research Laboratories). The mixture was incubated at 30 C for 20-30 min, and 40 µl of a chase solution (0.25 mM in each of the deoxyribonucleoside triphosphates) was then added. Incubation was continued at 30 C for an additional 30 min. The product was then digested with HindIII endonuclease, denatured with alkali, and applied to an alkaline agarose gel

Table 1. Construction of plasmids containing cloned fragments of RepI DNA.

Plasmid	Construction
pUT128	Replacement of small EcoRI-BamHI fragment of pBR322 with EcoRI-BamHI linker DNA derived from M13mp7 (10).
pUT191	Replacement of small EcoRI-BamHI fragment of pBR322 with an EcoRI-PvuII DNA fragment derived from the plasmid ptacl2 (11) and containing the tac promoter. The fragment was cloned by means of a HincII-BamHI linker derived from M13mp8 (12). (See Ref. 7).
pUT431, pUT434, pUT436, pUT437, pUT452	Replacement of small EcoRI-EcoRV DNA fragment of pBR322 (13) with fragment containing R1162 DNA, extending from 2.32 kb to a variable, second end point. Each of the R1162 DNA-containing fragments consists of the following: EcoRI-BamHI linker fragment from M13mp7 (10); small BamHI-SmaI linker fragment from pMC1403; R1162 DNA from 2.32 kb endpoint to a Bal31-generated blunt end.
pUT509, pUT514	Replacement of small EcoRI-EcoRV fragment of pBR322 with fragment generated by digestion of R1162 DNA with SspI and Bal31 (pUT509) or RsaI and Bal31 (pUT514). The Bal31-generated ends were joined directly to the EcoRV-generated blunt end of pBR322. The SspI or RsaI-generated blunt ends were linked to the EcoRI site of pBR322 by means of the EcoRI-BamHI-SmaI linkers used for pUT434, pUT436, pUT437 and pUT452.
pUT520, pUT521	Insertion of R1162 DNA cloned in pUT509 into pBR322-ptac vector pUT191 (7) at BamHI cloning site.
pUT449, pUT705	Replacement of small HindIII-SalI fragment of pACYC184 (14) with fragment containing R1162 DNA (3.0-2.32 kb) fused to lacZ'YA DNA from pMC1403 (15). The fragment consists of the following components, listed in order from the HindIII to SalI end: HindIII-SalI linker fragment from M13mp9 (12); small SalI - BamHI fragment of pBR322; R1162 DNA (BclI end at 3.0 kb to Bal31-generated end at 2.32 kb [pUT449], or SspI-generated end at 2.57 [pUT705]); pMC1403 DNA (SmaI site to SalI site).
pUT532	Replacement of small EcoRI-SalI fragment of pBR322 with fragment consisting of R1162 DNA (2.68-2.51 kb) fused to lacZ'YA DNA from pMC1403 (15). The fragment consists of the following components, listed in order from the EcoRI to the SalI end: EcoRI-XbaI linker fragment from M13mp18 (16); small NheI-EcoRV pBR322 DNA fragment; R1162 DNA from Bal31-generated end at 2.68 kb to RsaI-generated end at 2.51 kb; pMC1403 DNA (SmaI site to SalI site)

(18). After electrophoresis under alkaline conditions for 4 hr, the labelled probe DNA, an extension of the primer through the R1162 DNA insert to the M13 HindIII site, was subsequently isolated by neutralizing the gel

and continuing electrophoresis of the DNA onto NA45 DEAE cellulose paper (19).

Bacterial RNA was prepared according to the cold-phenol method of Ikemura and Dahlberg (20). Residual DNA was eliminated by passage of the preparation through nitrocellulose filters, as described by Nygaard and Hall (21). The RNA (200 µg) was resuspended with labelled DNA probe (approx. 5 ng, $5x10^5$ cpm) in 10 µl 0.4 M NaCl, 0.04 M 1,4-piperazine-diethanesulfonic acid, 0.001 M EDTA, pH 6.4, and boiled for 3 min. The sample was then held at 65 C for 60 min, and for an additional 120 min at 37 C. Digestion of the nucleic acid was initiated by the addition of 280 units of S1 endonuclease (Bethesda Research Laboratories) in 200 µl of a pH 4.6 solution containing 0.25 M NaCl, 0.03 M sodium acetate, 0.001 M $ZnSO_4$, denatured salmon sperm DNA (20 µg/ml) and 5% (w/v) glycerol. The reaction was terminated after 1 hr at 37 C by the addition of 10 µl 0.25 M EDTA (pH 8.0). The nucleic acid was precipitated with ethanol, resuspended in 15 µl 80% deionized formamide, 0.01 N NaOH, 0.001 M EDTA, 0.025% bromphenol blue, 0.025% xylene cyanol, and then boiled for 3 minutes, chilled, and loaded onto a 33 x 41 x .035 cm, 7% polyacrylamide-urea sequencing gel (22). Protected DNA fragments were visualized by autoradiography of the dried gel with Kodak XRP1 film. The film was exposed for 4-5 days at room temperature.

Measurement of enzyme activities in cell extracts

Activities of β-galactosidase, β-lactamase, and chloramphenicol acetyltransferase were determined in crude cell extracts. Pelleted cells from 5 ml of mid-log phase cultures (approximately $5x10^8$/ml) were resuspended in 5 ml 0.1 M potassium phosphate buffer, pH 7.0, and sonically disrupted by three 15 sec, 80 W pulses applied at 30 sec intervals. After the cell debris was removed by centrifugation, the protein concentration in each supernatant was determined by the method of Bradford (23). Assay of β-galactosidase activity was done essentially as described by Miller (24), except that specific activity is defined here as OD_{420}/min/mg protein. Chloramphenicol acetyltransferase was assayed according to Shaw (25). Specific activity is defined as µmoles product/min/mg protein. The method of Boxer and Everett (26) was used to assay β-lactamase. Specific activity is defined as the decrease in µmoles substrate/min/mg protein.

Region-specific mutagenesis of R1162 DNA

The method for the isolation of mutagenized plasmids is identical in principle to that described by McEachern et al. (27). Approximately 25 µg

PstI-cleaved pUT532 DNA and an equal amount of the same DNA, digested with SmaI and BamHI, were taken up in 50 µl 0.01 M Tris (pH 7.4), 0.1 M NaCl, boiled for three minutes, and then held at room temperature for 45 min. There is a single PstI cleavage site in the vector portion of pUT532 DNA, and single BamHI and SmaI cleavage sites which together bracket the R1162 DNA insert. Thus, among the reannealed molecules will be those having a PstI-generated cut in one strand, and a BamHI + SmaI-generated gap in the other, exposing as a single strand the R1162 portion of the molecule. The DNA sample was precipitated with ethanol, and resuspended in 50 µl 0.01 M Tris, 0.05 M NaCl, 0.05 M EDTA, pH 7.5. Methoxylamine (150 µl of a 1 M solution in 1 M sodium acetate, pH 5.5) was then added to this solution. The mixture was incubated at 50 C and samples taken at 0, 20, 40, 60 and 80 min. The nucleic acid in each sample was immediately precipitated, resuspended in 50 µl 0.006 M Tris (pH7.9), 0.15 M NaCl, 0.008 M $MgCl_2$, and digested with BamHI. This last step reduces background transformation with unmutagenized, PstI-cleaved molecules (27). The DNA was used directly for transformation of *E. coli* MC1022.

Other procedures

Plasmid DNA was isolated by the method of Holmes and Quigley (28) or Marko *et al.* (29). Bacteria were transformed with plasmid DNA according to the procedure of Cohen *et al.* (30). DNA base sequencing by the dideoxy procedure (22,31), and agarose and polyacrylamide gel electrophoresis, were carried out by standard methods. Measurement of plasmid DNA content by densitometry was carried out by scanning photographs of agarose gels with a densitometer (E-C Apparatus Corp.). The ethidium bromide-stained gels were photographed under ultraviolet light using Polaroid type 55 P/N film and a Vivitar Type 25A filter. The amount of DNA applied to the gel was determined by experiment to be within the linear range of response of the film.

RESULTS

The regulatory region for RepI expression is located at the promoter-proximal end of the RepI region.

To locate the RepI regulatory region we first constructed the plasmid pUT705, which contains a *lacZ* gene fragment (beginning at codon 8) (15) fused in phase to codon 8 of the promoter-proximal RepI gene, *repIA* (Figs. 1,2). We then cloned various fragments of RepI DNA into pBR322, which is compatible with pUT705, and tested their effect in *trans* on the expression

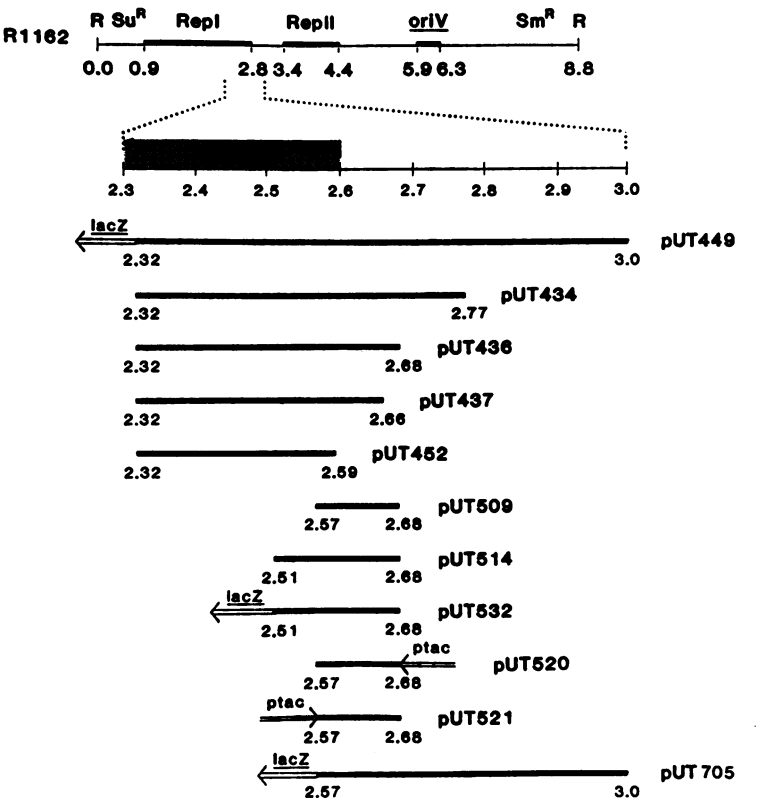

FIG. 1. Map of R1162. Regions required for plasmid replication (RepI, RepII, oriV) are indicated by thicker segments. Su^R and Sm^R mark approximate locations for genes encoding resistance to sulfonamides and streptomycin. Below, an expanded map of part of RepI shows the promoter-proximal region of repIA. Segments of this region cloned in the designated plasmids, and fusions to DNA containing lacZ or the tac promoter, are indicated. The arrows designate the direction of transcription. Map distances are in kilobase-pairs (kb) from the EcoRI cleavage site (R).

of the fused lacZ gene. The activity of β-galactosidase in each strain was expressed relative to that of chloramphenicol acetyltransferase, the product of the constitutive cat (32) gene also encoded by pUT705, to adjust for possible variation in the copy-number of this plasmid. The results of these experiments are given in Table 2. For the family of fragments with one endpoint at 2.32 kb, those having a second endpoint at 2.68 kb or greater (plasmids pUT434, pUT436) inhibited synthesis of β-galactosidase, whereas those with endpoints less than 2.68 kb (plasmids pUT437, pUT452) did not. DNA between 2.51-2.68 kb (plasmid pUT514) also inhibited

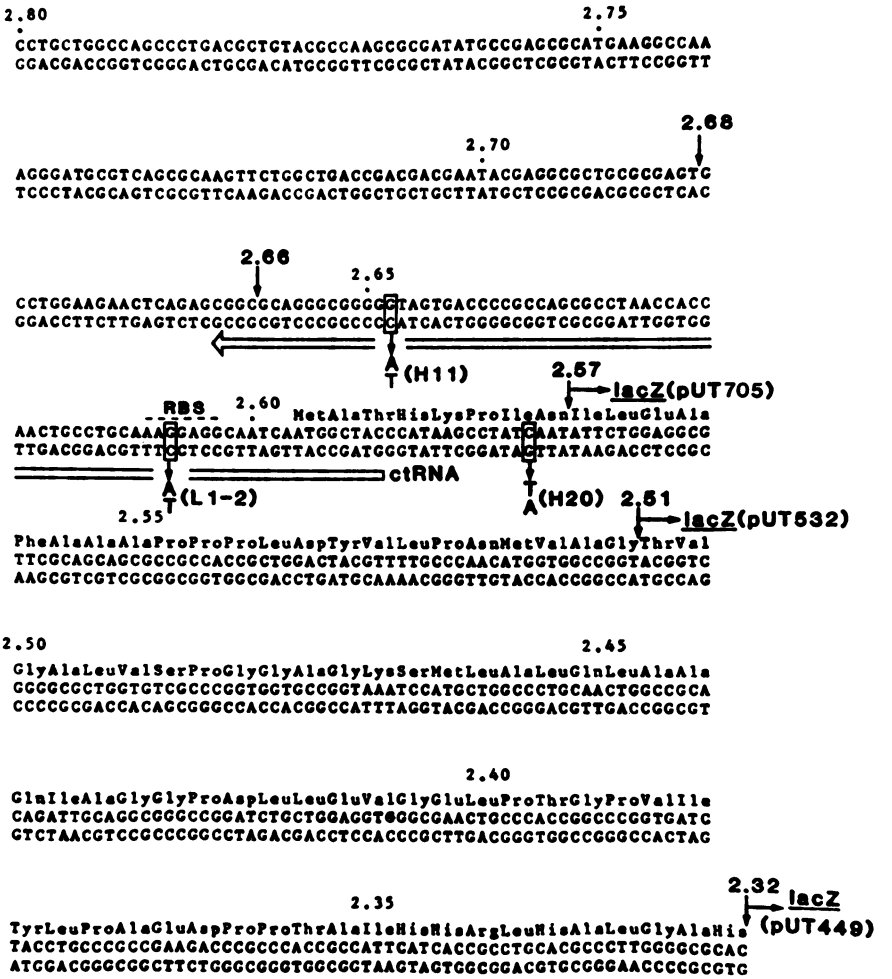

FIG. 2. Nucleotide base sequence of the promoter-proximal region of RepI. Map locations are defined as in Fig. 1. The figure shows the endpoints of various DNA probes referred to in the text, the fusion positions for lacZ fragments in plasmids pUT449, pUT532, and pUT705, the positions of methoxylamine-induced base changes, the translated open reading frame and associated ribosome binding site for the promoter-proximal portion of repIA, and the location of the coding sequence for the regulatory RNA.

synthesis, but a 2.57-2.68 kb fragment was largely ineffective (pUT509). These results indicate that the coding sequence for an element negatively regulating the RepI genes is located within the promotor-proximal region of RepI, with one end mapping between 2.66 and 2.68 and the other between 2.51 and 2.57 kb.

Table 2. Effect of cloned fragments of RepI DNA on expression of RepI-lacZ fusion.

Plasmid:	RepI DNA present:	Enzymatic specific activity in cell extracts: A. β-galactosidase	B. chloramphenicol acetyltransferase	Ratio (A/B):
1. pUT128	---	15.5 (15.2, 15.8)	2.98 (2.81, 3.15)	5.20
2. pUT434	2.32-2.77	2.12 (1.94, 2.30)	3.06 (2.87, 3.24)	0.69
3. pUT436	2.32-2.68	2.06 (2.19, 1.92)	3.16 (3.09, 3.23)	0.65
4. pUT437	2.32-2.66	10.4 (9.99, 10.9)	2.81 (2.35, 3.26)	3.70
5. pUT452	2.32-2.59	14.4 (14.9, 13.8)	3.13 (2.66, 3.59)	4.60
6. pUT509	2.57-2.68	9.45 (9.26, 9.64)	2.95 (2.82, 3.08)	3.20
7. pUT514	2.51-2.68	2.29 (2.39, 2.18)	2.84 (2.87, 2.81)	0.81
8. pUT191	---	1.45 (1.47, 1.43)	2.81 (2.82, 2.79)	0.52
9. pUT509	2.57-2.68	1.29 (1.16, 1.41)	2.48 (2.42, 2.53)	0.52
10. pUT520	2.57-2.68	3.47 (2.94, 3.99)	3.06 (2.69, 3.42)	1.13
11. pUT521	2.57-2.68	0.06 (0.06, 0.05)	2.91 (3.07, 2.74)	0.02
12. pUT191	---	8.72 (8.65, 8.78)	1.36 (1.30, 1.41)	6.41
13. pUT520	2.57-2.68	8.59 (8.59, 8.59)	1.39 (1.40, 1.37)	6.18

Enzyme activities for two independent experiments (in parentheses) and average values are given. Host strains are M182 containing pUT705 (1-7), JM103 containing pUT449 (8-11), and JM103 containing pUT705 (12-13).

A small RNA complementary to RepI message is encoded within the RepI regulatory region.

The small size of the RepI regulatory region, and the lack of open reading frames within it (Fig. 2), indicate that the negative controlling element is probably not a protein repressor. Several unrelated plasmids encode a small RNA complementary to the mRNA of genes essential for replication (33,34). These RNAs are negative regulatory elements and probably control copy-number by hybridizing to message and reducing the rate of translation (33-36). To determine if R1162 encodes a small RNA within the regulatory region, we hybridized unfractionated cellular RNA to radioactively labelled DNA probes consisting of parts of the RepI region. Two sets of probes were used: group I hybridizes to the RepI mRNA, and to other RNA species transcribed from the mRNA coding strand. Group II probes hybridize to RNAs transcribed from the complementary DNA strand. After hybridization, the mixtures were treated with S1 nuclease and applied to a denaturing polyacrylamide gel. The protected DNA fragments, revealed by autoradiography, are shown in Fig. 3.

When a 2.32-2.68(II) probe is hybridized to RNA isolated from MV10(R1162), the major protected species is a small DNA fragment of 75

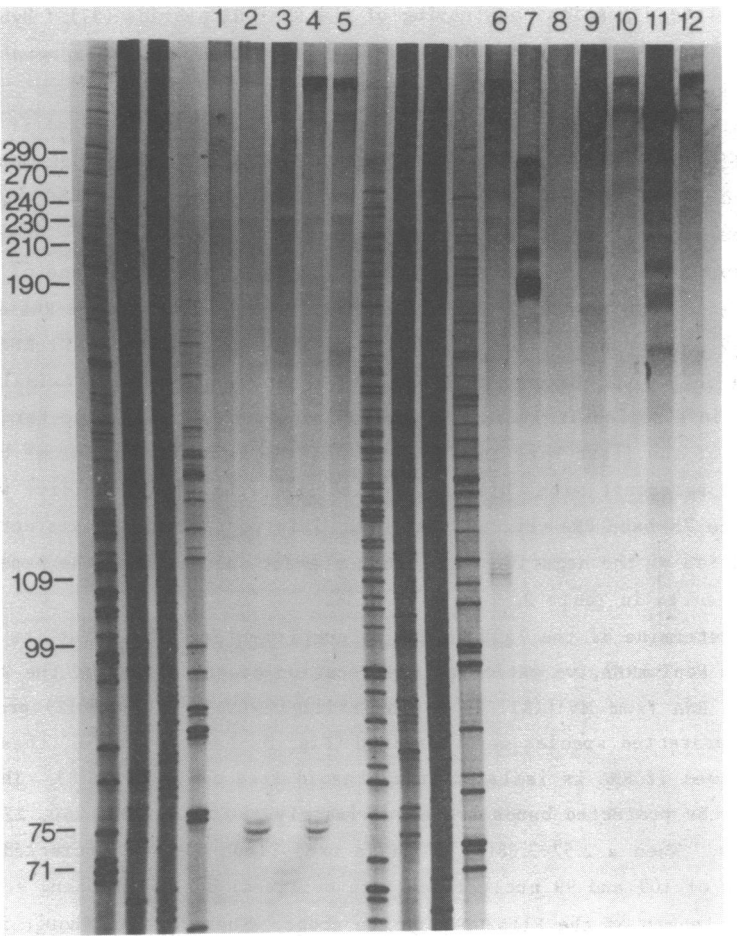

FIG. 3. S1 Mapping of R1162 transcripts with fragments of RepI DNA. Lane 1: MV10 RNA vs. 2.32-2.68(II) probe. Lane 2: MV10(R1162) RNA vs. 2.32-2.68(II) probe. Lane 3: MV10(R1162) RNA vs. 2.32-2.66(II) probe. Lane 4: MV10(R1162) RNA vs. 2.57-2.68(II) probe. Lane 5: MV10(pUT509) RNA vs. 2.57-2.68(II) probe. Lane 6: MV10(R1162) RNA vs. 2.57-2.68(I) probe. Lane 7: MV10(R1162) RNA vs. 2.57-2.86(I) probe. Lane 8: MV10 RNA vs. 2.57-2.86(I) probe. Lane 9: JM103(pUT520) RNA vs. 2.57-2.68(I) probe. Lane 10: JM103(pUT509) RNA vs. 2.57-2.68(I) probe. Lane 11: JM103(pUT521) RNA vs. 2.57-2.68(II) probe. Lane 12: JM103(pUT509) RNA vs. 2.57-2.68(II) probe.

bases (Fig. 3, lane 2). Several other bands, just below or above this one, also appear on the autoradiograph. These additional bands differ in size from the major band in steps of one nucleotide, and may be the consequence

of incomplete digestion, or fraying of the RNA-DNA hybrids (37). Hybridization is to RNA encoded by R1162, because no protection is observed with RNA isolated from plasmid-free cells (Fig. 3, lane 1).

A slightly smaller (71 bases) protected fragment is detected if a 2.32-2.66(II) probe is used (Fig. 3, lane 3). We could therefore map the DNA encoding the 75 base RNA from the exact positions of the 2.66 and 2.68 probe endpoints (Fig. 2), established from DNA base sequencing. From an inspection of the DNA sequences involved, we determined that none of the hybridization can be due to fortuitous homology between plasmid RNA and the non-R1162 portion of the probe. Thus, the DNA encoding the small RNA maps as shown in Fig. 2, between 2.58 and 2.67 kb at the promoter-proximal end of RepI, in a region including the coding sequence for the amino-terminal fragment of the first RepI gene product and the associated ribosome binding site. In agreement with this, a 2.57-2.68(II) probe will hybridize with the entire 75 base RNA (Fig. 3, lane 4). This position is consistent with the location of the negative regulatory element deduced from the experimental results in Table 2.

To determine if the 75 base RNA is complementary throughout its entire length to RepI mRNA, we estimated the location of the 5' end of the RepI message. RNA from MV10(R1162) was hybridized with a 2.57-2.86(I) probe. Several protected species were obtained (Fig. 3, lane 7); again, these are not observed if RNA is isolated from plasmid-free cells (lane 8). The sizes of the protected bands are approximately 190, 210, 230, 240, 270 and 290 bases. When a 2.57-2.68(I) probe is used (lane 6), two protected DNA fragments of 109 and 99 nucleotides, are obtained, the first being equal to the full length of the R1162 DNA in the probe. Therefore, although it is not known which of the hybridizing RNAs correspond to the RepI mRNA *in vivo*, the candidate transcripts must originate either at or to the right of 2.68 (Fig. 1), or at 2.67, 99 base-pairs away from 2.57. In any case the coding sequence for the 75 base RNA would then be fully complementary to message (Fig. 2).

The 75 base RNA is a regulatory element.

We believe that the small RNA is a negative regulator, because transcription through the RepI regulatory region, in the direction required to produce this molecule, is responsible for inhibition of RepI gene expression in *trans*. Two lines of evidence are now described which support this conclusion.

If the small RNA is in fact a negative regulatory element mapping

between 2.58 and 2.67 kb, we would expect plasmids containing the cloned 2.57-2.68 region, like those containing the 2.32-2.68 region, to reduce synthesis of β-galactosidase from replA-lacZ fusions. However, the data in Table 2, lines 3,6, show that this is not the case (compare pUT436 and pUT509). To explain this result, we first examined whether cells containing pUT509, with RepI DNA between 2.57 and 2.68, also contain the 75 base RNA. We observed no protection when RNA isolated from these cells was hybridized with the 2.57-2.68(II) probe (Fig. 3, lanes 5,12). The 5' end of the 75 base RNA coding sequence lies very close to the 2.57 map position (Fig. 2). Therefore we could explain these results if the 2.57-2.68 segment, although containing the complete coding sequence for the small RNA, nevertheless lacked the necessary promoter. To test this possibility, we constructed pUT521 (Fig. 1), which contains the 2.57-2.68 fragment fused to the strong tac promoter (11) at the 2.57 end. When RNA from cells containing this plasmid is hybridized to the 2.57-2.68(II) probe, a large amount of protected DNA is observed (Fig. 3, lane 11). The protected DNA is larger than 75 bases, which we assume is because the RNA is initiated from a novel position, and also because it is not terminated well at the usual location. The RNA made would therefore contain additional sequences homologous to the R1162 portion of the probe, and transcripts extending into the region of pUT521 adjacent to the R1162 insert would also hybridize with the pBR322 portion of the probe.

The plasmid pUT521, but not pUT509, strongly inhibits synthesis of β-galactosidase in cells containing pUT449, which has the lacZ gene fragment fused in phase to replA at 2.32 kb (Table 2, line 11). This result, and the results with the hybridizations, indicate that an intact promoter for transcription of the small RNA is absent from the 2.57-2.68 fragment. Both pUT509 and pUT521 contain the same R1162 sequences, and adjacent pBR322 DNA, but only pUT521 inhibits expression of β-galactosidase and produces a protecting RNA species (Table 2, compare lines 9,11). Thus it is the RNA itself, rather than a site on the DNA, which is responsible for regulation of RepI.

The same conclusion has been reached by examining the properties of mutations which alter the regulation of RepI. These mutations were obtained by first constructing the plasmid pUT532, a derivative of pBR322 containing R1162 DNA spanning the coordinates 2.51-2.68 kb, with the proximal portion of the replA gene fused in phase to the same lacZ fragment present in pUT705 (Figs. 1,2). The lacz gene is expressed at a low level

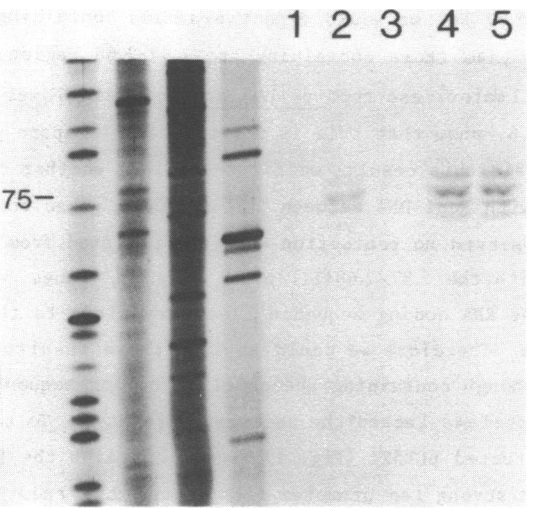

FIG. 4. Detection of regulatory RNA in strains containing pUT532 or mutated plasmid derivatives. Unfractionated RNA from plasmid-containing MC1022 cells were hybridized with a mixture of 2.32-2.68(II) and 2.57-2.68(II) probes prior to S1 digestion and analysis of resistant fraction on polyacrylamide gels. Cells contain plasmid pUT532 with no mutation (lane 2), or with mutations H20 (lane 3), H11 (lane 4), or L1-2 (lane 5). Hybridization to RNA from cells lacking plasmid is shown in lane 1.

in cells containing pUT532. Transcription might originate from within the R1162 DNA, at 2.67 kb (see above), or from within the vector portion of the molecule.

The R1162 DNA in pUT532 was subjected to region-specific mutagenesis with methoxylamine (38, MATERIALS AND METHODS), and then used to transform E. coli strain MC1022 (9). Colonies of transformed cells were screened for changes in the activity of the hybrid β-galactosidase enzyme. Among the mutations affecting expression of the repIA-lacZ fusion product should be those which have altered the activity of the regulatory element.

We obtained two point mutations, designated H11 and H20, which map within R1162 DNA and which result in elevated levels of β-galactosidase activity (Table 3: compare lines 2,3 with line 1). From the results of DNA sequencing, we found that the base change for H11 is at position 2.648, and that for H20 is at 2.576 (Fig. 2). In order to test the effect of these mutations in trans, we first excised the lac DNA portions of pUT532 and the mutated derivatives by digesting the plasmid DNAs with PvuII (39). The resulting plasmids, which retain only 81 base-pairs (codons 9-35) of

Table 3. Effect of mutations in RepI regulatory region on expression of RepI-lacZ fusion.

Enzymatic specific activity in cell extracts:			
Effect of mutation in pUT532:			
Mutation:	A. β-galactosidase:	B. β-lactamase:	Ratio (A/B):
1. none	0.232 (0.196, 0.268)	1.33 (1.08, 1.57)	0.17
2. H11	0.683 (0.582, 0.784)	0.92 (0.84, 0.99)	0.74
3. H20	3.05 (2.76, 3.34)	1.11 (1.00, 1.21)	2.75
4. L1-2	0.059 (0.041, 0.076)	1.15 (0.96, 1.34)	0.05
Effect of mutation in trans:			
Mutation:	A. β-galactosidase:	B. chloramphenicol acetyltransferase:	Ratio (A/B):
5. none	2.98 (2.97, 2.98)	3.22 (2.90, 3.54)	0.93
6. H11	8.46 (8.41, 8.51)	3.47 (3.35, 3.58)	2.44
7. H20	10.5 (10.2, 10.8)	3.29 (3.26, 3.31)	3.19
8. L1-2	14.1 (14.7, 13.5)	3.23 (3.11, 3.34)	4.37

Enzyme activities for two independent experiments (in parentheses) and average values are given. Host strain is M182; for 5-8 cells also contained pUT705. Enzyme activities in cells containing pUT705 and no additional R1162 DNA are shown in Table 2, line 1.

lacZ DNA, were then introduced into cells containing pUT705. Strains containing plasmids with the H11 and H20 mutations showed elevated levels of β-galactosidase activity, relative to the strain containing pUT532 (Table 3, lines 5-7). Thus, both mutations are likely to be affecting the regulation of RepI.

The H11 mutation maps within the coding sequence for the small RNA (Fig. 2). The H20 mutation, however, lies outside this sequence. One way that this mutation could be affecting regulation is by decreasing the amount of 75 base RNA. To test this, we hybridized the RNA from cells containing pUT532 and the mutated derivatives with the 2.57-2.68(II) probe, and again treated the mixture with S1 endonuclease. The results (Fig. 4), show that the 75 base RNA is present both in cells containing pUT532 (lane 2) and also in those containing the derivative with the H11 mutation (lane 4). In contrast, no protecting RNA was observed from cells containing plasmid with the H20 mutation (lane 3). Thus, a mutation decreasing transcription of the 75 base RNA also relieves the inhibition of RepI mRNA synthesis.

Another mutation, L1-2, causes a decrease in β-galactosidase activity

when present in the pUT532 derivative (Table 3, line 4). This mutation lies within the coding sequence for the RepI ribosome binding site (40), and also within the coding sequence for the putative regulatory RNA. In *trans*, the mutation results in an increase in β-galactosidase activity (Table 3, line 8). Cells containing pUT532 with the L1-2 mutation contain approximately normal amounts of the 75 base RNA (Fig. 4, lane 5).

Overproduction of an RNA fragment containing the 5'-end of the RepI mRNA relieves inhibition of RepI gene expression.

The plasmid pUT520 (Fig. 1) contains the *tac* promoter upstream from the 2.68 end of the 2.51-2.68 kb R1162 DNA fragment. When RNA from cells containing this plasmid was hybridized with the 2.57-2.68(I) probe, a substantial amount of labelled DNA was protected from degradation by S1 endonuclease (Fig. 3, lane 9). The size of the protected DNA is larger than the size of the R1162 DNA in the probe, because the probe also includes 200 base-pairs of pBR322 DNA, from the BamHI to the EcoRV cleavage sites (see *MATERIALS AND METHODS*). This DNA also lies between the *tac* promoter and the R1162 DNA in pUT520, and is oriented so that the RNA made will hybridize with the pBR322 DNA in the probe. In contrast, much less protection was afforded by the RNA from cells containing pUT509 (lane 10), which contains the same R1162 DNA and adjacent pBR322 DNA as pUT520, but which lacks the strong exogenous promoter.

The protecting RNA includes the 5' truncated portion of the RepI messenger RNA. When we tested the effect of pUT520 on expression of the *repIA-lacZ* fusion in pUT449, we found that synthesis of β-galactosidase was elevated much above the levels observed when either pUT509, or pUT191, which lacks R1162 DNA, is present instead in the cell (Table 2, lines 8,9,10). In contrast, pUT520 had no effect on the expression of the *repIA-lacz* fusion in pUT705 (Table 2, compare lines 12,13).

A Mutation which affects the RepI regulatory region also affects the copy-number of R1162.

Results presented elsewhere indicate that the levels of the RepI gene products determine the copy-number of R1162 (7). Thus, mutations affecting the regulation of RepI expression should also alter plasmid copy-number. To test this, we introduced the H20 mutation into R1162. This mutation maps within *repIA* (Fig. 2), but the resulting codon change from AUC to AUU does not alter the primary amino acid sequence of the essential gene product. Furthermore, both codons are used in *E. coli* at approximately equal frequency (41). We first constructed a strain containing three plasmids:

R1162, the conjugative plasmid R751, which mobilizes R1162 very efficiently (4), and the pUT532 plasmid derivative with the H20 mutation. Cells were mated with a nalidixic acid-resistant strain, and transconjugants selected for resistance to both this antibiotic and to carbenicillin. Colonies of such cells are very uncommon, because the pUT532 derivative, which encodes resistance to carbenicillin, is not mobilizable. Rare colonies do arise, however, as the result of recombination between the derivative and R1162, due to a region of homology between 2.51 and 2.68 (Fig. 1). The general structure of these recombinants is shown in Fig. 5. The mutation lies within either of two homologous regions of the recombinant molecule, depending on the location of the crossover event. If recombination occurs at a position

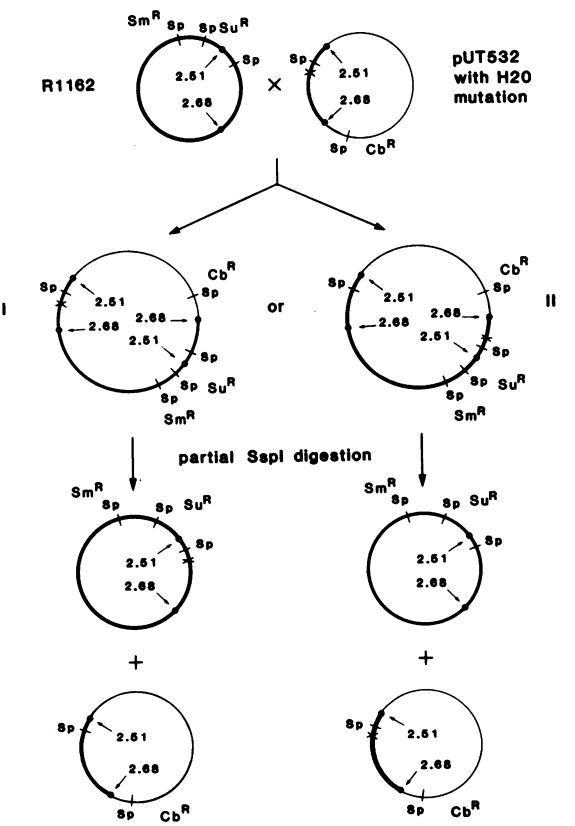

FIG. 5. Method for introduction of H20 mutation into R1162. Thicker portions of circular maps denote R1162 DNA. Sp = SspI cleavage sites; Cb^R, Sm^R and Su^R designate approximate locations of genes encoding resistance to carbenicillin, streptomycin and sulfonamides. Maps are not drawn to scale.

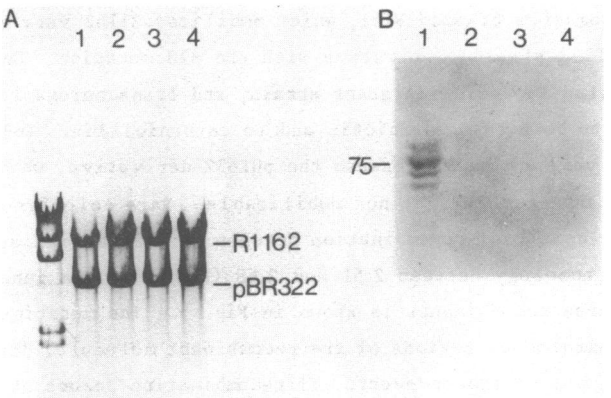

FIG. 6. (A) Agarose gel electrophoresis of EcoRI-cleaved, R1162 DNA with (lanes 2-4) or without (lane 1) the H20 mutation. The host strain is MV10 also containing pBR322 to control for plasmid DNA yields. Marker is HindIII-digested lambda DNA (42). (B) Hybridization of cellular RNA from MV10 containing either R1162 (lane 1), or R1162 with the H20 mutation (lanes 2-4), to a 2.51-2.68(II) probe.

between the site of the mutation and position 2.68, then subsequent partial digestion of the molecule with SspI generates R1162 containing the mutation, and the pUT532 derivative with the wild-type sequence (Fig. 5, left). If the crossover occurs between the site of mutation and position 2.51, then the starting molecules are regenerated by the digestion (Fig. 5, right).

We screened colonies of cells transformed with SspI-digested recombinant DNA for those having a plasmid with the structure of R1162. We then isolated the RNA from three of these strains, each of which had arisen independently, and hybridized it with the 2.51-2.68(II) probe. None of these strains contained a detectable amount of the small RNA (Fig. 6B) indicating that in each case the plasmid had acquired the H20 mutation. When we examined the plasmid DNA content in these cells, we found a small but detectable increase in the amount of plasmid DNA, compared to cells with wild-type R1162. This increase can be visualized directly when large amounts of DNA are applied to an agarose gel (Fig. 6A). However, to estimate the change quantitatively, we measured the amounts of plasmid DNA by densitometric measurement of samples linearized by EcoRI restriction endonuclease and displayed on agarose gels. The DNA was isolated from cells which also contained pBR322, to adjust for variation in plasmid yield. The increase in DNA content for the three derivatives of R1162 containing the H20 mutation, determined from the measurement of the amounts of plasmid DNA from 10 independent cultures, were 32.7$\pm$5.8%, 43.9$\pm$13%, and 35.0$\pm$13.4%.

DISCUSSION

Our results indicate that the small, 75 base RNA negatively regulates expression of the RepI genes. If we assume that target recognition involves complementary base sequences, then it is likely that the target lies on the RepI mRNA, rather than on the corresponding DNA. The plasmids pUT520 and pUT509 contain an identical fragment of R1162 DNA, mapping between 2.68 and 2.57 kb, which includes the base sequences complementary to the 75 base RNA. In pUT520 this DNA is fused to the *tac* promoter, so that large amounts of RNA, containing sequences complementary to the small RNA, are made in the cell. We found that pUT520, but not pUT509, increases in *trans* expression of the *repIA-lacZ* fusion in pUT449 (Table 2). This effect is explained most simply as titration of the negative regulatory element encoded by pUT449. In support of this interpretation, an increase is not observed if pUT705 is present instead of pUT449. Since the fusion in pUT705 is at 2.57 kb, we do not expect cells containing this plasmid to have any 75 base RNA. Finally, both pUT509 and pUT520 are replicated to a similar high copy-number in the cell, and thus would present similar amounts of any DNA target. Therefore, we conclude that the regulatory RNA is titrated by the excess RNA transcribed from the *tac* promoter.

The regulated expression of a gene essential for plasmid replication, involving a small RNA molecule complementary to a portion of the mRNA, is not unique to R1162. Both pT181, from *Staphylococcus aureus* (33), and the enteric R factor R1 (and the closely related R100) (34,36) have this arrangement. In these cases, as well as for R1162, regulation appears to be an intrinsic part of the copy-control mechanism (33-36). Regulation of *repA*, the essential gene for replication of R1, is at the level of translation, and this is probably true as well for pT181. Thus, the mechanism of copy-control is similar for several groups of unrelated plasmids.

In common with many other regulatory RNAs, the small RNA of R1162 has the capacity for substantial secondary structure. One especially stable structure, having two stem-loops with calculated G values of -40 and -17 kcal (43), is shown in Fig. 7. The figure also emphasizes another feature of the R1162 regulatory RNA: the coding sequence overlaps the ribosome binding site (RBS) and the first two codons of the promoter-proximal RepI gene. This differs from the position of the analogous coding sequences in pT181 and R1, where the countertranscript is encoded entirely within a region preceding the structural gene (33,34). The location of the coding sequence for the R1162 regulator might reflect the molecular events taking

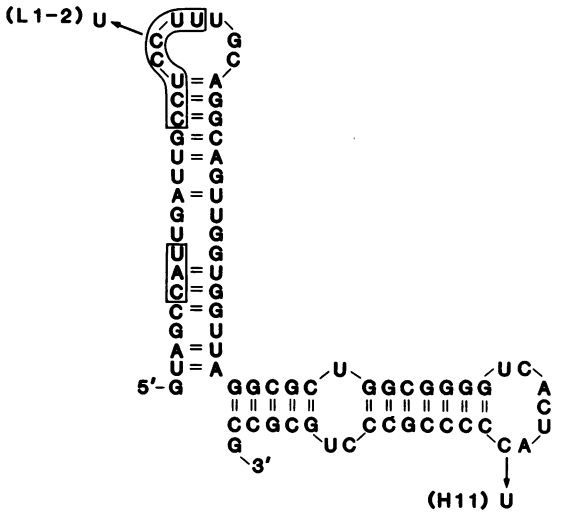

FIG. 7. Base sequence of regulatory RNA, drawn to show possible stable secondary structure. The segment of the sequence complementary to the likely ribosome binding site of RepI mRNA is outlined. The base changes for the L1-2 and H11 mutations are indicated. The boxed triplet CAU is at the position of the initiation codon of the repIA gene.

place in the control of RepI expression. Studies on RNA I, which regulates replication of ColE1-type plasmids by hybridizing with a pre-primer RNA (44), have indicated that initial contact between the two RNA species occurs between the complementary loops at the end of base-paired stems (45). For R1162, the location of the RBS at one loop of the mRNA suggests that ribosomes and the small RNA might compete for binding at this site. The properties of mutation L1-2 (Figs. 2,7) can be described in terms of this idea. When present in pUT532, the mutation affects the RBS but not the homology between the messenger RNA and the inhibitor (although the base sequence is different than for wild-type). Therefore, ribosomes compete less effectively for message, and the gene is poorly expressed. The mutated DNA has the opposite effect in trans, however, because it is no longer perfectly homologous with the wild-type message in the loop segment, and so competes less well with ribosomes in initial binding. This competitive model for regulation may explain why the RBS of the RepI message shows a degree of homology with E. coli 16S ribosomal RNA that is unusual for transcripts of Gram-negative bacteria (46). In addition, the extensive homology may be an advantage for a broad host-range plasmid, because it may serve to insure adequate levels of gene expression in backgrounds where the

plasmid promoters are poorly recognized.

The H20 mutation in pUT532 profoundly decreases the amount of counter-transcript (Fig. 4), but when this mutation is present in R1162 there is only a small increase in plasmid copy-number (Fig. 6). We have previously observed an increase in copy-number if the RepI region is put under the control of a strong exogenous promoter (7). Only a two or three-fold increase in copy-number was observed, although the RepI proteins were increased 50 to 100-fold. Thus, some other component of the plasmid replicative machinery must limit copy-number to a new level when replication is released from RepI control. Preliminary studies indicate that the limitation is not imposed by the third R1162 replication protein, encoded within RepII (unpublished). The limitation may therefore be species-specific, a possibility we will test with several of the many different potential hosts of R1162.

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