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The level of the pUB110 replication initiator protein is autoregulated, which provides an additional control for plasmid copy number

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ABSTRACT

Plasmids control their copy number by limiting the amount of the initiator for DNA replication. The plasmid pUB110 initiator protein is termed RepU. Expression of the pUB110 *rep*U gene is controlled by two antisense RNAs that interfere with *rep*U mRNA translation. Genetic evidence suggests that Rep protein levels may be regulated by additional uncharacterized mechanisms. The *rep*U gene product was radiolabeled and purified by monitoring the radioactive label. RepU overproduction was performed in cells containing the plasmid leading strand replication origin (*dso*), to allow for a putative inactivation of RepU. Polypeptides with apparent molecular masses of 42 (RepU*) and 39 (RepU) kDa were purified, both having the N-terminal sequence expected for the *rep*U gene. The RepU/RepU* protein mixture bound specifically to *dso*. At low protein concentrations, about six RepU/RepU* protomers bound to the *dso* region. At higher concentrations, an extended nucleoprotein complex was formed. The promoter for the *rep*U gene was localized downstream of the *dso* region. The results suggest that the extended RepU/RepU*–*dso* DNA complex interferes with *rep*U promoter utilization. This provides an additional copy number control by limiting RepU concentration. Our results suggest that during replication the RepU protein might be converted into an inactive RepU–RepU* hetero-oligomer, further limiting the amount of RepU protein available for replication initiation.

INTRODUCTION

The genomic organization, protein sequence conservation and replication mode of small, high copy number plasmids of Gram-positive bacteria led to their classification into four distinct families (reviewed in 1–3). Plasmids pT181, pMV158, pIM13 and pUB110 are representatives of each family. All these plasmids, which replicate via a rolling circle mechanism like the single-stranded *Escherichia coli* bacteriophages, carry genetic information for the protein that initiates and terminates DNA replication (Rep) and for their own replication control (2–4). For such plasmids, initiation is the rate-limiting step in replication and it is controlled by regulating expression of the Rep protein (reviewed in 3,4). To avoid re-utilization of the rate limiting initiator protein, plasmids of the pT181 family, and perhaps all the rolling circle replicating plasmids, use the strategy of inactivating the initiator protein (RepC in pT181) during the replication process by the addition of an oligodeoxynucleotide, giving rise to a new form, termed RepC* (5,6). The active (RepC) and the inactive (RepC*) proteins, which are present in similar relative amounts in cells, form an inactive RepC–RepC* heterodimer in solution (7).

Rolling circle replicating plasmids control the rate of synthesis of the initiator protein by using different strategies. In the case of plasmids of the pT181 family, antisense RNAs (ctRNA) transcribed from the *cop* region block expression of the plasmid-encoded initiator protein (RepC). The ctRNAs induce premature termination (attenuation) of *rep*C mRNA by promoting the formation of a termination-causing hairpin just upstream of the initiation codon of the *rep*C gene (8). The copy number of plasmids of the pMV158 family is regulated by two replication control functions acting as inhibitors of expression of the initiator protein (RepB). One of these products is a small, ctRNA molecule that interferes with *rep*B translation, whereas the other is the CopG protein (formerly RepA). The CopG protein specifically represses transcription of the promoter from which the *rep*B gene is transcribed (9). The copy number control mechanism of plasmids classified within the pIM13 family is poorly understood. In the case of plasmids of the pUB110 family, expression of the *rep* gene (*rep*H and *rep*U in the case of pC194 and pUB110, respectively) is controlled by two small unstable ctRNA molecules transcribed from the major incompatibility (*inc*A) region (10,11). In both plasmids the ctRNAs (*inc*A function) sequester the ribosomal binding site of the *rep* gene by base pairing, thus interfering with *rep* mRNA translation (reviewed in 1). Genetic evidence suggested that the level of the Rep protein may be regulated by an additional unknown mechanism. When a pUB110 DNA fragment containing the leading strand replication origin (double strand origin, *dso*) and the first codons of the *rep*U gene (see Fig. 1) was fused to a reporter gene, production *in trans* of the RepU protein inhibited expression of the reporter (11). The

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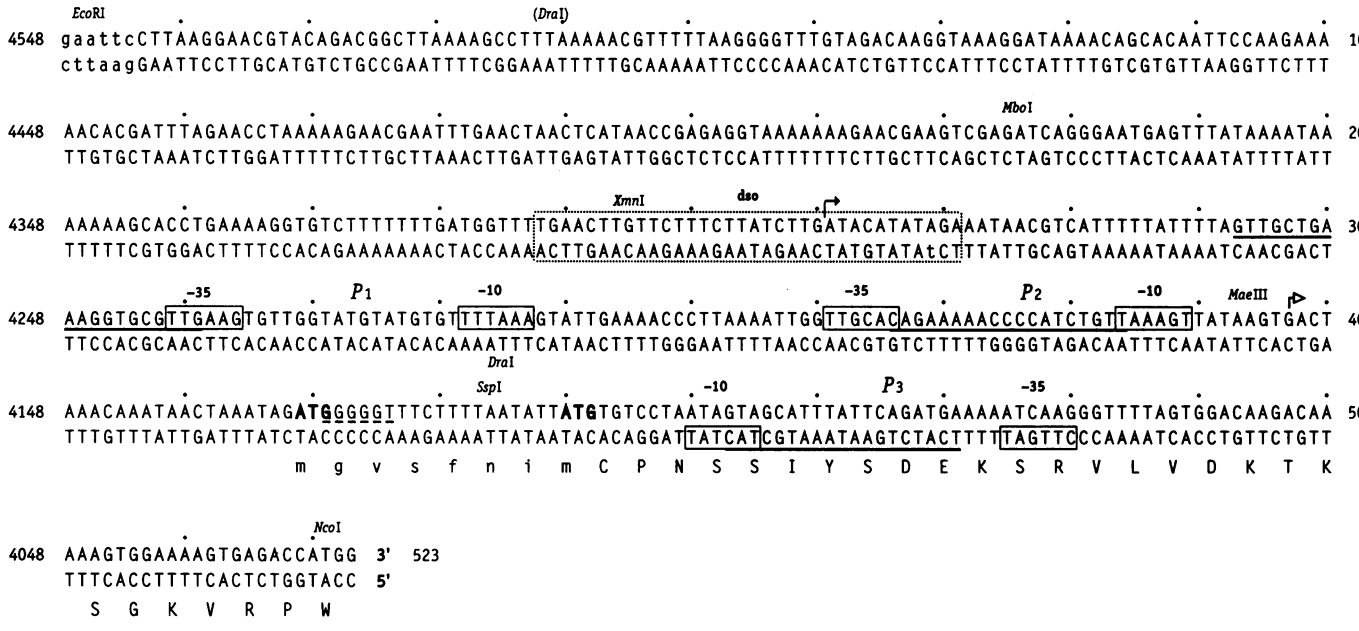

Figure 1. Scheme of the pUB110 *dso* region. The 523 bp *Eco*RI–*Nco*I DNA fragment of pUB110 is shown. The minimal pUB110 *dso* site (boxed in dotted lines), the computer predicted –10 and –35 consensus sequences of promoters *P*1, *P*2 and *P*3 (boxed in straight lines) and the 5′-end of the *rep*U gene are indicated. The numbers on the left-hand side denote pUB110 coordinates of the capital letters. Lower case letters denote pUB110 coodinates 1–6. The numbers on the right-hand side denote the nucleotide position. The ribosomal binding site (underlined with dashed lines) and the initiator codons (in bold) are indicated. The amino acid sequence of the RepU protein is denoted in capital letters and those residues predicted from the nucleotide sequence absent in the purified protein are indicated in lower case letters. The transcription start site of the *P*2 promoter is indicated by an open arrow. The filled arrow shows the position at which the RepU protein introduces the nick that serves as the replication initiation site. Relevant restriction sites are shown; the *Dra*I site in parentheses was not used in the cloning procedures.

same results were observed when a pC194 DNA fragment containing the *dso* region and the 5′-end of the *rep* H gene was fused to a reporter gene (10). In principle, the RepU or RepH proteins could interfere with translation by binding to the *rep* mRNA or could interfere with transcription of the *rep* gene by binding to its own promoter. An additional control of plasmid copy number could be exerted by inactivating the RepU protein in a way analogous to that described for the initiator protein of plasmids of the pT181 family.

We have previously shown that purified pUB110 initiator protein (RepU) specifically binds to a double-stranded (ds) or single-stranded (ss) DNA segment containing the *dso* region, but does not bind to the *rep*U mRNA (11; our unpublished results). To test whether an inactive derivative of the RepU protein (RepU*) accumulates and to learn if the RepU protein could control its own synthesis, we have overexpressed and purified the *rep*U gene product under conditions in which we could detect accumulation of the putative RepU*. The promoter from which the *rep*U gene is transcribed has been localized and the effect of purified RepU protein on the promoter activity has been analyzed *in vitro*. The results presented suggest that a RepU–RepU* hetero-oligomer, which is inactive as an initiator of pUB110 replication, can form a large nucleoprotein structure at the *dso* region that interferes with expression of the *rep*U promoter.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strains used were: BL21DE3 *hsd*S20 *gal* [T7RNApol] (12) and JM103 [Δ(*lac pro*)] *thi str*A *sup*E *end*A *sbc*B *hsd*R F′*tra*D36 *pro*AB *lac*I^q ΔM15 (13).

Plasmids pBT88 and pHV1 (11), pUB110 (14), pBT338 (15) and pUC18 (13) have been previously described. Plasmid pBT360 contains the 523 bp *Nco*I–*Eco*RI DNA fragment from pUB110 (coordinates 4031–4548 plus coordinates 1–6; Fig. 1) cloned into *Hin*dIII/*Eco*RI-cleaved pUC18. Plasmid pBT361 contains the 278 bp *Xmn*I–*Nco*I DNA segment from pUB110 (coordinates 4031–4309; Fig. 1) cloned into *Hin*dII-cleaved pUC18. Plasmid pBT362 contains the 159 bp *Dra*I–*Mbo*I DNA segment from pUB110 (coordinates 4221–4380; Fig. 1) cloned into *Bam*HI/*Hin*dII-cleaved pUC18. Plasmid pBT363 contains the 190 bp *Dra*I–*Nco*I DNA segment from pUB110 (coordinates 4031–4221; Fig. 1) cloned into *Hin*dII-cleaved pUC18.

DNA preparation and manipulation

Covalently closed circular plasmid DNA was purified by using the SDS lysis method (16), followed by purification on a cesium chloride–ethidium bromide gradient.

End-labeling of purified DNA fragments was performed by filling in the 3′-recessive ends with the large fragment of DNA polymerase I.

Purification of the *rep*U gene product

For purification of the RepU/RepU* proteins, we have expressed the *rep*U gene from a vector that also contains the plasmid replication origin (*dso*). A culture of *E.coli* strain BL21(DE3) containing the pBT88-borne pUB110 minimal replicon and the pHV1-borne *lac*I^q gene was grown in L medium and the T7 promoter was induced as previously described (11). The predicted molecular mass of the RepU protein is 39 kDa. Proteins with apparent masses of 42 and 39 kDa, as determined by SDS gel

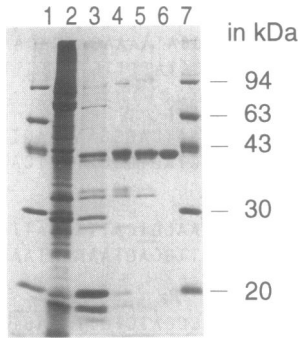

Figure 2. Purification of the RepU/RepU* protein mixture. Coomassie blue stained 15% SDS–PAGE. The purification procedure is described in the text. Lanes 1 and 7, molecular weight standards; lane 2, Fraction I (cell lysate after PEI and ammonium sulfate precipitation); lane 3, Fraction II (after S-Sepharose); lane 4, Fraction III (after phosphocellulose); lane 5, Fraction IV (after heparin–Sepharose); lane 6, Fraction V (after phenyl-Sepharose).

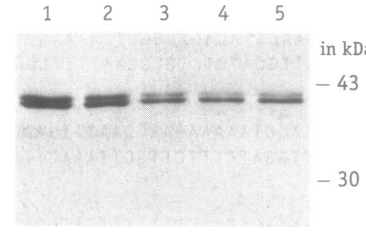

Figure 3. Fractions enriched in either the RepU or the RepU* proteins. The RepU/RepU* proteins of Fraction IV (see Fig. 2) were loaded on a S-Sepharose column. Fractions containing different relative amounts of RepU and RepU* can be obtained by a NaCl step gradient. A Coomassie blue stained SDS–PAGE is shown.

electrophoresis, were observed. Since both the 42 and the 39 kDa proteins share the same N-terminal sequence (see below), they were termed RepU* and RepU respectively.

The cells were harvested by centrifugation at 4°C and mixed with a similar cell lysate containing the RepU (39 kDa) and RepU* (42 kDa) proteins labeled with [^{35}S]methionine (see 11). The cell paste (~30 g wet weight) was resuspended in 150 ml of buffer A [50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol (DTT), 5% glycerol] containing 500 mM NaCl. Lysozyme was added to a final concentration of 200 μg/ml, followed by incubation on water–ice for 30 min. The cells were lysed by grinding with alumina (2 g/g wet weight pellet). The extract was centrifuged at 3000 r.p.m. (GSA Sorval rotor) for 10 min to remove the alumina. The supernatant was then centrifuged at 12 000 r.p.m. (GSA Sorval rotor) for 60 min to separate the soluble proteins from the cell debris. The supernatant was saved and the pellet re-extracted.

Chromosomal DNA was removed by polyethylenimine (PEI) precipitation. PEI (10%, pH 7.5) was slowly added with constant stirring to a final concentration of 0.25%, when the OD_{260} was 120. The solution was centrifuged at 12 000 r.p.m. (GSA Sorval rotor) for 30 min. The pellet was discarded and the proteins of the supernatant precipitated twice (12 000 r.p.m. for 30 min, GSA Sorval rotor) with 70% ammonium sulfate (Fraction I).

Fraction I (Fig. 2, lane 2) was dialyzed against buffer A containing 200 mM NaCl and loaded on a S-Sepharose column equilibrated in the same buffer. The RepU/RepU* proteins eluted at ~260 mM NaCl (Fraction II; Fig. 2, lane 3).

Fraction II was loaded on a phosphocellulose column equilibrated in buffer A containing 260 mM NaCl. The RepU/RepU* proteins eluted at ~360 mM NaCl (Fraction III; see Fig. 2, lane 4).

Fraction III was loaded on a heparin–Sepharose column equilibrated with buffer A containing 360 mM NaCl. The RepU/RepU* proteins eluted at ~450 mM NaCl (Fraction IV; Fig. 2, lane 5). Under these conditions we can obtain fractions enriched either in the 42 or in the 39 kDa proteins (see below).

Fraction IV was dialyzed against buffer A containing 1 M ammonium sulfate and loaded on a phenyl–Sepharose column equilibrated in the same buffer. The RepU/RepU* proteins eluted at ~850 mM ammonium sulfate (Fraction V; see Fig. 2, lane 6).

The purified RepU/RepU* proteins of Fraction IV were precipitated by the addition of solid ammonium sulfate (70% final concentration). The pellet was resuspended in buffer A and dialyzed against buffer A containing either 600 or 200 mM NaCl. The purified RepU/RepU* proteins in buffer A containing 600 mM NaCl, which coincides with the radioactive material (data not shown), were diluted with glycerol (50% final concentration) and stored at –20°C.

The RepU/RepU* protein mixture containing 200 mM NaCl was loaded on a 1 ml S-Sepharose column equilibrated in the same buffer. Fractions containing different relative amounts of the 42 (RepU*) and 39 kDa (RepU) proteins were obtained by a NaCl step gradient (see Fig. 3). The fractions were pooled, glycerol was added to 50% (final concentration) and they were stored at –20°C. When not specified otherwise, we used a RepU/RepU* mixture with equivalent amounts of both proteins.

Molecular mass determination

The native molecular mass of the RepU/RepU* fraction enriched in RepU* was determined by gel filtration FPLC using a Superose 12 column (Pharmacia, Sweden). Chromatography was carried out in buffer A containing 250 mM NaCl at 4°C with a flow rate of 0.5 ml/min. Aliquots (40 μg) of RepU/RepU* were applied to the column. A standard curve of K_{av} versus $\log_{10}$ relative mobility was determined as recommended by Pharmacia. Protein standards were obtained from Pharmacia (vitamin B12, 1.35 kDa; myoglobulin, 17 kDa; ovalbumin, 44 kDa; aldolase, 158 kDa; thyroglobulin, 670 kDa).

Filter binding assay

The formation of RepU/RepU*–DNA complexes was measured as described (11). Standard reaction mixtures (25 μl) contained 4.5×10^{-10} M pUB110 523 bp *Nco*I–*Eco*RI ^{32}P-end-labeled DNA fragment (see Fig. 1) containing the *dso* region (which was obtained from plasmid pBT360 as a 543 bp *Eco*RI–*Hin*dIII DNA segment) and increasing concentrations (6.4×10^{-10} to 2×10^{-7} M) of the RepU/RepU* protein mixture in buffer B (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$). The reactions were incubated for 10 min at room temperature and filtered through KOH-treated nitrocellulose filters. Filters were dried and the amount of radioactivity bound to the nitrocellulose was determined by scintillation counting. The DNA retained on the filters was corrected for retention of radiolabeled DNA in the absence of the proteins. The specific activity of the labeled DNA was measured as TCA-precipitable material. All reactions were performed in duplicate. The apparent equilibrium binding constant was determined as described (17).

DNase I footprint assay

DNase I cleavage reactions were performed essentially as described (15). Reaction mixtures contained 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, ^{32}P-end-labeled 159 bp *Mbo*I–*Dra*I DNA fragment from pUB110 containing the *dso* region, obtained either as a 189 bp *Eco*RI–*Pst*I DNA segment (to footprint the top strand, 6.4×10^{-10} M final concentration) or as a 183 bp *Sma*I–*Hin*dIII DNA fragment (to footprint the bottom strand, 1.3×10^{-9} M final concentration), and different concentrations of RepU/RepU* (3.2, 1.6, 0.8, 0.4, 0.2 and 0.1×10^{-8} M) in a final volume of 25 µl. Poly(dI–dC) (2 µg) was added as non-specific competitor DNA. The mixtures were equilibrated at 37°C for 20 min, after which DNase I was added and the solution was incubated for 2 min. The reactions were terminated by the addition of 20 mM EDTA. The DNA was precipitated with ethanol, resuspended in denaturing formamide loading buffer and separated by denaturing PAGE as described (16). Autoradiographs of the dried gels were subsequently taken.

Transcription assays

Transcription reactions contained 25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 12 mM NaCl, 100 mM KCl, 4% glycerol, 200 µM ATP, CTP, GTP and UTP, 2–4 nM adequate template DNA (see below), increasing concentrations of the RepU/RepU* protein mixture (10^{-8}–18×10^{-8} M) and 35 nM *Bacillus subtilis* σ^A RNA polymerase. The total reaction volume was 25 µl. In the control reactions where no *rep*U gene product was included, the same volume of protein storage buffer was added to keep constant ionic conditions. The DNA templates used were either a 529 bp *Eco*RI–*Pst*I DNA fragment from plasmid pBT360, containing the pUB110 origin of replication (*dso*), or *Hin*dIII-cleaved plasmid pBT338, which contains a DNA fragment that includes the promoter that drives expression of the *orf*α-gene β of the broad host range Gram-positive plasmid pSM19035, unrelated to pUB110 (15). This DNA fragment was used as a control for repression specificity of the RepU protein, since RepU should not interfere with expression of the unrelated promoter. Reaction mixtures were incubated for 5 min at 37°C prior to the addition of RNA polymerase to allow the RepU/RepU* protein mixture to bind to DNA. After the addition of RNA polymerase the incubation was continued for 10 min. The reaction was stopped by the addition of 1 µl of 0.5 M EDTA, 1 µg of carrier tRNA, 3 µl of 3 M potassium acetate and 70 µl of ethanol. RNAs were precipitated and analyzed by primer extension as indicated below.

Primer extension of RNAs

The RNA was resuspended in a solution containing 40 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl, 10 U RNasin ribonuclease inhibitor and 2–4 pmol (a large excess) of oligonucleotides designed to hybridize downstream from the transcription start sites of the analyzed promoters (5′-TCATCTGAATAAATGCTAC-3′ for *rep*U transcripts and 5′-GCATATTCTTGCTCATTA-3′ for transcripts originated at the *orf*α-gene β promoter used as a control). The mixture was heated for 5 min at 70°C and then allowed to cool to 20°C. The solutions were then put on ice for 10 min and the RNA was precipitated by addition of 4 µl 3 M potassium acetate and 3 vol ethanol. The RNA was resuspended in 5 µl water and the primer was extended in a solution containing 50 mM Tris–HCl, pH 7.5, 40 mM KCl, 7 mM $MgAc_2$, 2 mM DTT, 200 µM each dNTP (except dATP, which was 100 µM), 2 µCi [α-^{32}P]dATP (400 Ci/mmol), 10 U RNasin and 5 U AMV reverse transcriptase, in a total volume of 10 µl. The reaction mixture was incubated at 42°C for 60 min and stopped by addition of 0.5 µl 0.5 M EDTA and 30 µl 10 mM Tris–HCl, pH 7.5, 1 mM EDTA. The non-incorporated labeled nucleotide was removed by gel filtration and the eluted cDNA precipitated and analyzed by electrophoresis in 6% urea–polyacrylamide gels.

Other methods

The N-terminal amino acid sequence of the RepU and RepU* proteins was determined by Volker Kruft (Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Berlin, Germany) with automated Edman degradation in a pulsed liquid phase sequencer (model 477A; Applied Biosystems). The concentration of DNA was determined using molar extinction coefficients of 6500/M/cm at 260 nm. The protein concentration was determined by using the molar extinction coefficient of 63800/M/cm at 280 nm.

RESULTS AND DISCUSSION

Purification and physical properties of the pUB110 RepU/RepU* proteins

Plasmid pBT88 contains the *rep*U gene under the control of a T7 promoter and the double strand origin (*dso*) (11). The *rep*U gene product, deduced from the nucleotide sequence, consists of a 327 amino acid long polypeptide corresponding to a molecular mass of 39 023 kDa. When expression of the *rep*U gene from plasmid pBT88 was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (see 18) and the product was specifically labeled with [^{35}S]methionine, polypeptides that migrate in SDS–PAGE with apparent molecular masses of ~42 and 39 kDa were detected. We tentatively termed the 42 kDa protein, which is only detected when the *dso* is present in the background, RepU* (see below).

The RepU/RepU* protein mixture was purified by conventional column chromatography by monitoring radioactively labeled proteins, using a protocol slightly different from that reported earlier (11). Figure 2 shows progressive purification of the RepU/RepU* polypeptides. After the last purification step the RepU/RepU* polypeptides were >95% pure, as judged by SDS–PAGE (Fig. 2, lane 6; data not shown). Fractions enriched in either the 42 kDa (RepU*) or the 39 kDa (RepU) protein were also obtained (see Fig. 3).

Genetic and nucleotide sequence analysis suggested that translation of the *rep*U gene started at the second Met of the open reading frame (see 11 and citations therein). Identification of the final products as the RepU and RepU* proteins was confirmed by sequencing the N-terminus of the purified polypeptides. The sequence of the first 15 N-terminal residues of purified RepU and RepU* revealed that both proteins have the same N-terminal sequence. The N-terminal sequence is in perfect agreement with the nucleotide sequence of the *rep*U gene (see Fig. 1) except that the Met residue was absent in both the 42 and the 39 kDa proteins. For the time being there is no evidence as to whether removal of the Met residue also occurs in the Gram-positive hosts. However, a RepU protein lacking the first Met residue is able to initiate pUB110 DNA replication in an *E.coli* cell-free extract (11).

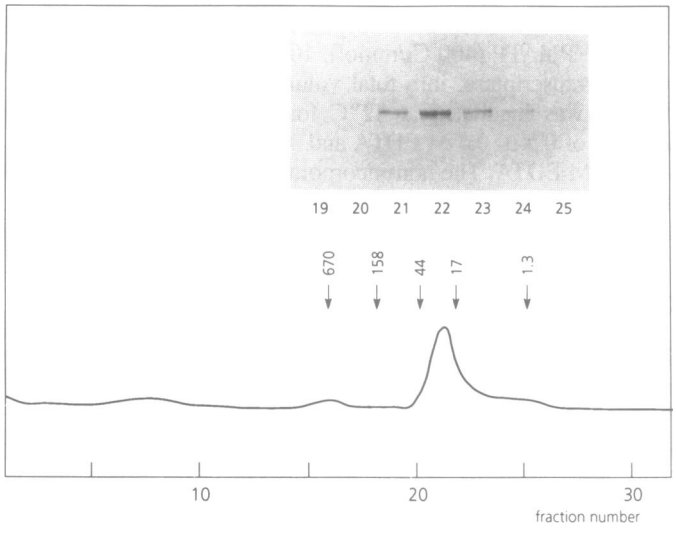

Figure 4. Native molecular mass of the RepU and RepU* proteins. The behavior of a RepU/RepU* protein fraction enriched in the repU* protein was analyzed by gel filtration FPLC using a Superose 12 column. The elution profile is shown, together with the composition of the fractions containing the RepU/RepU* protein mixture. The vertical arrows indicate the positions at which proteins of known molecular masses (in kDa) elute.

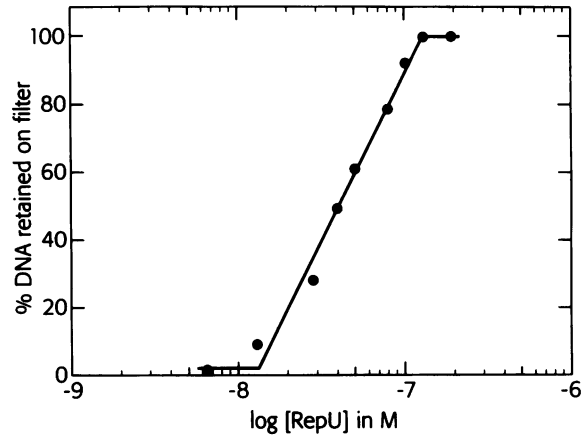

Figure 5. Binding of the RepU/RepU* protein mixture to a DNA fragment containing the *dso* region. Binding reactions contained a 523 bp end-labeled *Nco*I–*Eco*RI DNA fragment from pUB110 containing the *dso* region (see Materials and Methods) and increasing concentrations of the RepU/RepU* protein mixture (6.4×10^{-10} to 2×10^{-7} M). The binding mixtures were filtered through KOH-treated nitrocellulose filters and the radioactivity retained on the filters determined by scintillation counting. The values obtained were corrected for the amount of DNA retained in the absence of protein.

RepU and RepU* are monomers in solution

The molecular weight of the native RepU/RepU* mixture was determined by gel filtration using a protein fraction enriched in RepU*. Gel filtration chromatography was carried out by FPLC using a Superose 12 column. Under the conditions used (50 mM Tris–HCl, pH 7.5, 1 mM DTT, 5% glycerol, 250 mM NaCl) both the RepU* and the RepU proteins eluted as having an estimated M_r of ~40 000 (see Fig. 4), which is in good agreement with a RepU* (RepU) protomer. Unlike the initiator protein of plasmids of the pT181 family, which behave as dimers (see 7,19), RepU* and RepU are probably monomers in solution.

RepU and the RepU/RepU* mixture are biochemically distinct proteins

Initiation of plasmid rolling circle replication is a complex process which requires high precision protein–protein and protein–DNA interactions. A single-stranded cleavage at the *dso* site is a prerequisite for the initiation of plasmid replication. The initiator protein is a specific DNA binding protein with an associated nicking and closing activity (reviewed in 2,3). In the absence of Mg^{2+} the RepU protein binds to a dsDNA segment containing the *dso* sequence, whereas in the presence of the metal ion RepU drives leading strand DNA synthesis when an *E.coli* cell-free extract is used and has a nicking and closing activity at pH 9 (11,20). The RepU/RepU* protein mixture can also bind to a dsDNA fragment (see Fig. 5), provided that the *dso* sequence is present, although with ~3-fold lower efficiency than the RepU protein. The RepU/RepU* protein mixture, however, is inactive as a nicking and closing enzyme (topoisomerase activity) and as a replication initiator (20; data not shown).

On the basis of the biochemical similarities between the initiator proteins (RepC/RepC*) of plasmid pT181 (see 5–7) and the initiator of pUB110 (RepU/RepU* protein mixture), we can assume that: (i) RepU* contains a covalently bound single-stranded oligonucleotide; (ii) RepU* protein is generated during plasmid replication. The latter assumption is in good agreement with our previous results, because when the *rep*U gene product was overexpressed from a vector which lacks the pUB110 *dso*, only the 39 kDa protein was observed (11; I. E. Maciag and J. C. Alonso, unpublished results). Unlike the RepC/RepC* proteins, which are dimers, RepU and RepU* are monomers in solution. It is likely that a protein–protein interaction could occur at the *dso* region.

The RepU/RepU* protein mixture forms a specific complex with the *dso* region

To quantify the affinity of the RepU/RepU* protein mixture for the leading strand replication origin (*dso*) of pUB110 and to determine the stoichiometry of the protein–*dso* DNA complex, specific complexes were formed using the 523 bp *Eco*RI–*Nco*I *dso* DNA fragment (4.5×10^{-10} M) and increasing concentrations of the RepU/RepU* protein mixture. In the presence of 100 mM NaCl, RepU/RepU* is able to bind linear dsDNA containing the pUB110 *dso* region to nitrocellulose membrane filters (see Fig. 5). However, under the same conditions the RepU/RepU* protein mixture did not form complexes that can be retained on nitrocellulose filters with the 190 bp *Dra*I–*Nco*I DNA fragment (see Fig. 1), a subfragment of the above DNA segment that contains the *rep*U promoter region (see below) but does not include the *dso* region (data not shown).

The rate of RepU/RepU*–(*Eco*RI–*Nco*I) *dso* DNA complex formation was determined as a function of protein concentration. The apparent equilibrium constant (K_{app}), which in this case is equal to the protein concentration midpoint, is $\sim 3 \times 10^{-9}$ M at pH 7.5 and 20°C (Fig. 5). From this result we can conclude that about six RepU/RepU* protomers are required to reach such an equilibrium.

When a fraction enriched in the RepU protein was used (see Fig. 3) and the RepU–(*Eco*RI–*Nco*I) *dso* DNA complex trapped by nitrocellulose membrane filters, the K_{app} was $\sim 1 \times 10^{-9}$ M (data not shown). This is in good agreement with our previous results, in which at protein concentration midpoint about two

RepU protomers bind to the 523 bp (*Eco*RI–*Nco*I) *dso* DNA fragment (I. E. Maciag and J. C. Alonso, unpublished results).

The RepU/RepU* protein mixture binds to the *dso* region, forming an extended nucleoprotein complex

Genetic evidence suggests that the pUB110 *dso* region lies within a 34 bp DNA segment just upstream of the *rep*U gene (21; Fig. 1). The DNase I cleavage pattern from RepU/RepU*–*dso* DNA complexes revealed that at low protein concentrations RepU/RepU* protein bound to a defined domain of the *dso* region, but at higher protein concentrations the footprint also covered upstream and downstream sequences (see Fig. 6). In the presence of 4×10^{-9} M RepU/RepU* mixture and 6.4×10^{-10} M end-labeled 189 bp *Mbo*I–*Dra*I *dso* DNA, discrete protected sites start to became apparent on the top strand within and downstream of the *dso* region (see Fig. 6A). Similarly, in the presence of 8×10^{-9} M RepU/RepU* mixture and 1.3×10^{-9} M end-labeled 183 bp *Mbo*I–*Dra*I *dso* DNA discrete sites protected from DNase I attack appeared on the bottom strand (see Fig. 6B). Under these experimental conditions there are ~6 RepU/RepU* protomers per *dso* DNA molecule. At higher protein concentrations (1.6×10^{-8} M or 3.2×10^{-8} M) the protected segments differed in number and length and a set of hypersensitive sites was detected with a periodicity of ~34 ± 1 bp (see Fig. 6A and B). Furthermore, at the highest RepU/RepU* protein concentration assayed protected sequences were detected along most of the DNA fragment used.

The protected sites, which become evident when six RepU/RepU* protomers per *dso* DNA molecule are present, are located upstream of the *dso* region, within it and downstream of it. This extended protection suggests that the RepU–RepU* complex could form a nucleoprotein structure covering a substantial length of the DNA, which may even include the region in which putative promoters for the *rep*U gene are located. If this is true, the RepU protein could repress transcription of its own gene. This possibility was therefore analyzed.

The RepU/RepU* protein mixture represses its own synthesis

A computer analysis of the DNA region upstream of the *rep*U gene suggested the presence of two promoter-like sequences, named *P*1 and *P*2, from which this gene could be expressed (11; Fig. 1). To localize the promoter(s) responsible for expression of the *rep*U gene, an *in vitro* transcription reaction with purified *B.subtilis* σ^A RNA polymerase was performed. The DNA used as template was a 529 bp *Eco*RI–*Pst*I DNA fragment from plasmid pBT360, containing the pUB110 origin of replication (*dso*) and the first 28 codons of the *rep*U gene. The start site(s) of the transcript(s) running through the *rep*U gene was determined by primer extension, using as primer an oligonucleotide hybridizing to DNA residues 14–32 of the *rep*U gene (see Materials and Methods). A single band was obtained, indicating that the repU gene is expressed from a single promoter (Fig. 7A, lane 1). The same result was obtained when supercoiled plasmid pBT360 was used as template, ruling out the possibility of the presence of any additional promoter requiring DNA supercoiling for activity (not shown). The start site of the transcript was 75 nt upstream of the first nucleotide of the *rep*U gene. This start site is 8 nt downstream the –10 box of the computer-predicted *P*2 promoter.

Since DNase I footprinting assays had indicated that the RepU/RepU* protein mixture bound at the *dso* region forms a nucleoprotein complex that could extend towards the *rep*U gene,

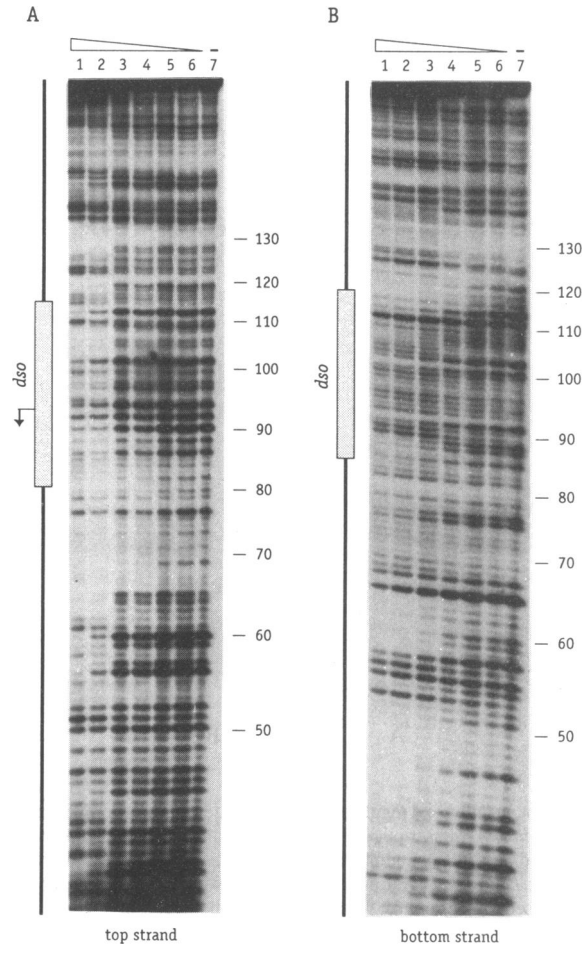

Figure 6. DNase I footprinting of binding of the RepU/RepU* protein mixture to the *dso* region. A 189 bp *Hin*dIII–*Sma*I (**A**, top strand) or a 183 bp *Pst*I–*Eco*RI (**B**, bottom strand) DNA fragment from plasmid pBT362 containing the 159 bp *Mbo*I–*Dra*I pUB110 DNA segment (Fig. 1) were end-labeled at the *Hin*dIII or *Eco*RI restriction site, incubated with different concentrations of RepU/RepU* protein mixture and partially digested with DNase I. The partially digested complexes were separated on 6% denaturing PAGE and the cleavage pattern visualized by autoradiography. The arrow at the *dso* region (shadowed box) indicates the position at which the RepU protein nicks the DNA to start replication, as well as the direction in which replication progresses. The concentrations of the RepU/RepU* protein mixture present in the binding reactions were 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2×10^{-8} M.

we investigated whether these proteins could repress expression of the *rep*U (*P*2) promoter. To test this possibility, we followed by *in vitro* transcription assays the expression of the *rep*U promoter in the presence of increasing amounts of the RepU/RepU* proteins. As shown in Fig. 7A, the RepU/RepU* protein mixture could repress transcription from its own promoter at concentrations of 6×10^{-8} M or higher; these are in the same range as those at which the RepU protein binds to the *dso* region, forming an extended complex (DNase I footprints are done under lower ionic strength conditions than transcription assays and therefore the protein binds to DNA with higher apparent affinity; see also Fig. 5). RepU/RepU* concentrations equal or higher than those that repress the *rep*U promoter did not affect expression of an unrelated promoter (Fig. 7B), thereby ruling out the possibility that the repression effect on the *rep*U promoter could be due to non-specific binding of the protein to DNA or to the presence of RNases in the RepU/RepU* protein mixture.

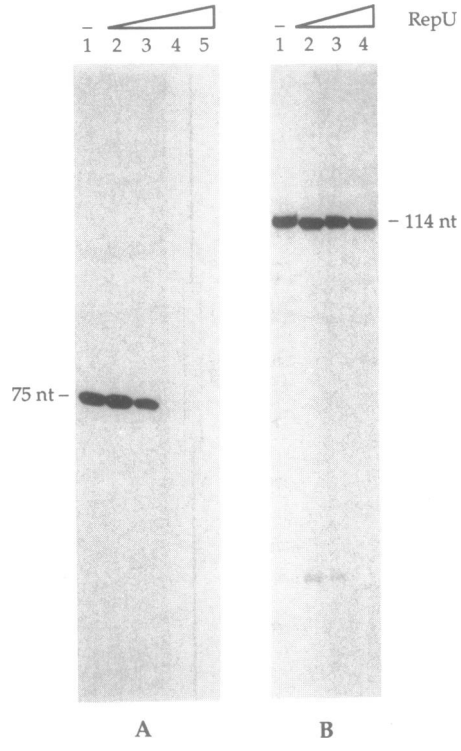

Figure 7. Localization and regulation of the *rep*U gene promoter. The templates used for the *in vitro* transcription reactions were either a 529 bp DNA fragment from plasmid pBT360, containing the pUB110 *dso* region and part of the *rep*U gene (**A**) or *Hin*dIII-cleaved plasmid pBT388, which contains an unrelated promoter used as a control when analyzing the repression effect of the RepU/RepU* protein mixture (**B**). Transcription reactions were performed either in the absence or presence of the RepU/RepU* protein mixture [1×10^{-8}, 3×10^{-8}, 6×10^{-8} and 18×10^{-8} M in (A) and 3×10^{-8}, 6×10^{-8} and 18×10^{-8} M in (B)]. The transcripts produced were detected by primer extension. The size of the cDNAs obtained are indicated.

The filter binding assays described above indicated that the RepU/RepU* protein mixture can bind to a DNA fragment containing the *dso* region, but not to the 190 bp *Dra*I–*Nco*I DNA fragment located immediately downstream of the *dso* site, which contains the *rep*U promoter (see Fig. 1). Considering the DNase I footprinting and transcription results, it is likely that repression of the *P*2 promoter is exerted by the initial binding of the RepU/RepU* protein to the *dso* region, which would then form a nucleoprotein structure that extends towards the *P*2 promoter, therefore inhibiting binding of the RNA polymerase to it. This implies that there is no need for a specific RepU/RepU* protein binding site at the *rep*U promoter and explains why the RepU/RepU* proteins repress the *rep*U promoter but do not bind to it in DNA fragments devoid of the *dso* region, which is located immediately upstream of the promoter. Hence, the *dso* region would act as a nucleation site to which the RepU protein binds with high affinity, while the extended RepU/RepU*–DNA complex would be formed by polymerization of further protein protomers on the DNA. Polymerization would be highly dependent on the cooperative binding effect which is apparent from the DNase I footprinting results. This model for RepU/RepU* binding explains the genetic data showing that expression of the *rep*U gene fused to a reporter gene was inhibited by production of the RepU protein *in trans* when the complete *dso* region was present (*Eco*RI–*Nco*I fragment; see Fig. 1), though repression was not observed when the DNA fragment cloned upstream of the reporter gene was the 190 bp *Dra*I–*Nco*I fragment, which still contains the *P*2 promoter but lacks the *dso* site to which the RepU protein initially binds (see Fig. 1; 11; our unpublished results).

The RepU/RepU* binding mode to *dso* DNA offers an elegant and sensitive repression mechanism to finely control the amount of RepU protein in the cell. Since the cooperative polymerization event is very sensitive to the concentration of the RepU protein, small increases in intracellular RepU concentrations could quickly cover the promoter region and inhibit further RepU synthesis.

The control of the amount of RepU protein available for replication initiation would therefore be exerted in three ways: (i) by limiting translation of the *rep*U gene; (ii) by regulating expression of the *rep*U gene by formation of a nucleoprotein complex that represses the *rep*U promoter; (iii) by inactivating the RepU molecules that have already participated in a replication event by a covalent modification (generation of the RepU* protein), which, by analogy with other high copy rolling circle replicating plasmids, could be the incorporation of a short oligonucleotide into it.

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