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The involvement of host replication proteins and of specific origin sequences in the *in vitro* replication of miniplasmid R1 DNA

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ABSTRACT

The in vitro replication of R1 miniplasmid promoted by purified preparations of the plasmid encoded RepA protein in cell extracts of E.coli is resistant to rifampicin and can be completely inhibited by antibodies against DnaG, the primase of the cell, as well as by antibodies against proteins DnaB and SSB. R1 replication is abolished in extracts deficient in the DnaA protein. This deficiency is efficiently complemented by purified preparations of the DnaA protein. The in vitro replication of plasmid R1 is also abolished in DnaC deficient extracts and by a 10 bp deletion (nucleotides 1463-1472) within the minimal origin region. These data indicate the requirement of the DnaA, DnaB, DnaC, DnaG and SSB replication proteins of the host, as well as of specific oriR1 sequences for the RepA dependent replication of plasmid R1. The implications of these results for the initiation of R1 replication are discussed.

INTRODUCTION

The in vivo replication of the antibiotic resistance factor R1 requires synthesis of a plasmid encoded protein, called RepA (1-3), that promotes replication from a unique or preferential origin (4-8); the requirement of RepA protein for in vivo replication of R1 is clearly demonstrated by the recent isolation of R1 thermosensitive replication mutants specifying a thermosensitive RepA protein (Ortega et al, to be published). R1 replication, which proceeds from the origin via Cairns type replicative intermediates, is unidirectional. The synthesis of RepA protein is regulated negatively by the products of the copy number control genes copA and copB (9). The cop genes, the repA gene and the origin of replication are clustered in a 2.5 kb region that constitutes the minimal unit of replication (10) which has been completely sequenced (11,12).

Replication of plasmid R1 analyzed in an *in vitro* system reveals the same requirements as *in vivo*: (i) dependency on RepA protein (13-15), (ii) initiation proceeds from the *in vivo* origin in the same unidirectional way by a Cairns type mechanism (16). Using this system, a 188 bp region of the origin required for RepA dependent initiation has been defined (15). It contains, at coordinates 1425-1438, a consensus binding site for the DnaA protein of the host (17) and at coordinates 1461-1477, a sequence with residual homology to the phage ØX174 DNA region required for the ATPase activity of the primosomal protein n' (18).

R1 *in vitro* replication requires, in addition to protein RepA, host replication factors; the involvement of DNA polymerase III and DNA gyrase in this replication is inferred by the specific inhibitory effects of arabinosylcytosine triphosphate and of novobiocin respectively (13,16). R1 *in vitro* replication can occur efficiently in the absence of transcription by host RNA polymerase (14) or in extracts deficient in DNA polymerase I (14, 15).

Using an R1 replication assay in which purified fractions of RepA protein are supplied to extracts of *E.coli*, we have further extended the analysis and found that the replication proteins of the host, DnaA, DnaB, DnaC, DnaG and SSB, are required at an early stage for R1 *in vitro* replication. In addition it is shown that the n' site-like sequence of the origin, although not acting as a primosome assembling site, is required for replication. The implications of these results for initiation of R1 replication are discussed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E.coli* K12 strains, C600 (19) and the replication mutants BT1000 (*polA*1; 20), PC-22 (*polA*1, *dnaC*2; 21) and WM433 (*dnaA*204; W.Messer) were used to prepare replication extracts. Strain BMH71-18 and the corresponding repair deficient mutant BMH71-18 *mut*λ (22) were used in site directed mutagenesis. Strain N99(λ*c*I857)/pSO1 (14) was applied for overproduction of RepA protein. pSO3 is a M13mp8 (23) based recombinant that contains the 1.04 kb *Sau3A ori*R1 fragment inserted in

the BamHI site of the vector (Ortega et al, unpublished results) and pSO3O, an oriR1 mutant isolated from pSO3 by site directed mutagenesis. The mutation removes 10 bp of the origin region between nucleotides 1462-1473 (this work). The R1 copy mutant miniplasmids pKN177 (24) and pKN4O2 (3), the ColE1 plasmid (25) and the replicative forms or single-stranded forms of the pSO3 and pSO3O recombinants or the M13mp8 vector were used as substrates in the in vitro replication assays.

Enzymes, Chemicals and Radiochemicals. T4 DNA ligase, the large fragment of the DNA polymerase I and the T4 polynucleotide kinase were from Anglia Biotechnology. Restriction enzymes were obtained from Amersham. Compounds used for synthesizing oligonucleotides were from Cruachem (Livingston, Scotland) and were used by S.O. during an EMBO course on site directed mutagenesis at the EMBL (Heidelberg). Antibodies against E.coli proteins DnaB, DnaG and SSB were prepared according to the method of Stöffler and Wittmann (26). The preparation of R1 RepA protein is described below. A sample of a partially purified DnaA protein was a kind gift from W.Messer's laboratory. Sources for enzymes and chemicals utilized in the in vitro replication assays have been described (13,14). The radiochemicals [^{3}H]-dTTP (43 Ci/mmol, 1 mCi/ml) and [γ-^{32}P]-ATP (> 5000 Ci/mmol, 1 mCi/ml) were obtained from Amersham.

General Methods. For large scale preparations of plasmid DNA, cleared cell lysates were prepared (27) and subsequently the plasmid DNA was separated from chromosomal DNA by isopycnic centrifugation in CsCl-ethidium bromide gradients in vertical rotors (28). Single-stranded forms of M13mp8 and of the pSO3 recombinant were prepared according to the Amersham protocols (M13 cloning and sequencing handbook). Electrophoresis of proteins in polyacrylamide gels was done according to Laemmli (29). Protein concentrations were determined as described by Bradford (3O). Restriction enzyme analysis and electrophoresis in agarose gels were according to Maniatis et al (31). For transformation, competent cells were prepared by the $CaCl_2$ method (32). For the isolation of the pSO3O mutant a manually synthesized (33) oligonucleotide (GACATGAACTT ATGGGGA) was used. The mutant was initially identified by plaque

hybridization (34) using as a probe the oligonucleotide labelled with ^{32}P at its 5' end. Subsequently the 10 bp deletion was verified by DNA sequencing.

Partial Purification of the RepA Protein. Cells of N99(λcI857) containing the recombinant plasmid pSO1 were grown at 30°C in 750 ml of LB supplemented with 50 µg/ml of ampicillin. At A_{450} = 1.0 the same volume of LB prewarmed to 65°C was added to the culture over a period of 15-20 seconds with continuous shaking. The culture was quickly transferred to 42°C and incubated at this temperature for 15-20 minutes. Cells were harvested by centrifugation at 0°C, shock frozen and stored at -80°C. Approximately 1 gram of wet cells were thawed at 4°C and ground with 2 grams of alumina during 15 min at 4°C. The lysate was diluted with 4 ml of buffer A (50 mM Tris-HCl pH 7.5, 0.3 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM p-aminobenzamidine and 5% ethylenglycol) and centrifugated in a SS34 rotor at 11,000 rpm for 10 min at 0°C. The supernatant was transferred to a new tube and centrifugated again in the same rotor at 15,000 rpm for 30 min at 0°C. The sediment of the second centrifugation step was resuspended in 16 ml of buffer A supplemented with 1% of Triton X-100 and 0.5% of the zwittergent detergent 3-12 (Calbiochem). The suspension was shaken gently for 1 hour at 4°C and centrifugated at 15,000 rpm for 15 min. Then the pellet was washed twice with 16 ml of buffer-2 (50 mM Hepes-KOH, pH 8.0, 100 mM KCl, 1 mM dithiothreitol and 1mM p-aminobenzamidine). Finally, the pellet was resuspended in 0.5 - 1 ml of buffer-2, divided in small aliquots, frozen in liquid nitrogen and stored at -80°C. A yield of 0.4 mg of RepA protein with a purity higher than 80% was obtained from 1.5 grams of induced cells in a few hours.

In vitro Replication Assays. To assay for R1 replication, approximately 80 µg/ml of the RepA preparations and 30 µg/ml of the miniplasmid DNA were present in a total volume of 25 µl of standard replication assays (13). After assembling the mixtures at 0°C for 10 min, the samples were incubated at 30°C for 60 min. Extract type II (35) of the E.coli strains was used throughout the analysis presented here; extract was added to concentrations of 5-7 mg/ml of protein. Increasing the amounts of extract above this level was found to be inhibitory in some of the assays (see

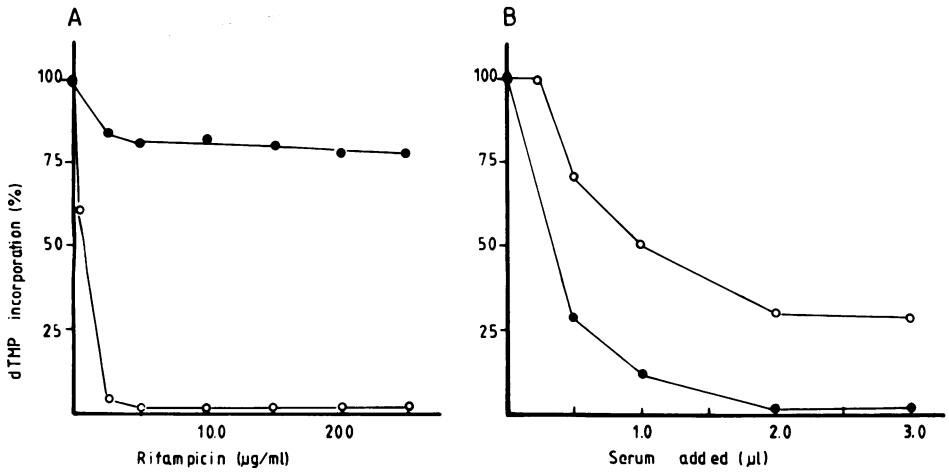

Figure 1. Effects of rifampicin and DnaG antibodies on the replication of R1 and ColE1 DNA. DNA synthesis was analyzed as described in Materials and Methods using fraction II extracts of strain C600 at a final protein concentration of 7 mg/ml. For testing replication of R1, DNA of the R1 miniplasmid pKN177 at a final concentration of 30 µg/ml and RepA preparations at a final concentration of 80 µg/ml were added to the assay. Replication of ColE1 was tested under the same conditions except that RepA was omitted and 30 µg/ml of ColE1 DNA was added as the DNA substrate. 100% dTMP incorporation corresponds to 120,000 cpm (pKN177 replication, 25 µl assay) or 70,000 cpm (ColE1 replication, 25 µl assay). Panel A: effects of increasing amounts of rifampicin on R1 (o) or ColE1 replication (●); panel B: effects of DnaG antibodies on R1 (o) or ColE1 replication (●). In the experiments with DnaG antibodies the preimmune serum gave a maximum inhibition of 15%.

Results, Fig. 4). Replication of ColE1 was assayed under similar conditions, except that RepA was omitted from the reactions and ColE1 was added as the DNA substrate to a concentration of 30 µg/ml.

RESULTS

Role of DnaG and oriR1 Sequences in Replication of R1.

Using an *in vitro* system in which replication of plasmid R1 was triggered by RepA protein synthesized *in situ*, we have previously established that initiation of R1 replication is basically independent of transcription by host RNA polymerase (14). The assay system used in the study presented here relies on functional par-

tially purified preparations of R1 RepA protein being supplied to type II extracts of E.coli. Replication of R1 in this system, as in the previous one, can occur in the absence of transcription by host RNA polymerase; this is shown by the rifampicin resistant synthesis of R1 miniplasmids (Fig.1A). Inhibition by the antibiotic at a concentration of 25 µg/ml to about 25% indicates an auxiliary but not essential role of transcription by RNA polymerase in R1 replication. The replication of plasmid ColE1 in these assays is completely abolished at rifampicin concentrations as low as 0.01 µg/ml. It is well established (36) that this is due to the inhibition of the synthesis of the transcript that primes initiation of ColE1 replication.

Next we compared the replication of R1 and ColE1 DNA in the presence of increasing amounts of antibodies directed against the DnaG protein. Synthesis of both plasmids is inhibited to different extents (Fig.1B) at saturating concentrations of the antibodies, inhibition of R1 replication is complete while ColE1 is still synthesized to about 30% of the untreated control. The complete inhibition of R1 replication by DnaG antibodies and the rifampicin resistant replication of this plasmid strongly suggests that DnaG generates primers for initiation of R1 replication. DnaG is required for lagging strand synthesis but not for leading strand synthesis of ColE1 (37); therefore the residual synthesis of ColE1 observed in the presence of DnaG antibodies is most probably due to leading strand synthesis.

In order to evaluate whether DnaG is acting as part of a primosome complex (38) in the initiation events that trigger replication of R1, a M13mp8-ori R1 recombinant, pSO3, was used to prepare a single-stranded circular oriR1 template (lagging strand). This substrate was subsequently tested for complementary strand synthesis in the presence of rifampicin; replication of the M13mp8 SS vector was analyzed as a control. A similar approach has previously been used to analyze primosome assembling sites on ColE1 strands (39,40). The single-stranded template pSO3 does not promote rifampicin resistant replication of the leading strand of R1, neither in the presence of RepA protein nor in its absence, suggesting that priming of the leading strand synthesis by DnaG in the R1 system does not occur via the primosome com-

Table 1 *In vitro* replication of M13mp8-*ori*R1 recombinants

DNA substrate	Rifampicin added (μg/ml)	dTMP incorporated (pmol) addition of RepA +	-
pSO3 (RF)	—	120.0	1.2
pSO30 (RF)	—	1.3	1.2
pSO3 (SS)	—	17.0	16.3
pSO3 (SS)	25.0	3.0	3.2
M13mp8 (SS)	—	16.8	18.4
M13mp8 (SS)	25.0	3.0	2.8

DNA synthesis was carried out at 30°C for 60 min in standard replication assays as described in Materials and Methods; fraction II extracts of strain C600 were used at a final protein concentration of 7 mg/ml; RepA protein preparations were supplied to the assays at a final concentration of 80 μg/ml, and the double- (RF) and single-stranded (SS) DNA templates at final concentrations of 30 μg/ml and 4 μg/ml respectively.

plex (Table 1). Furthermore, inspection of the sequences of the minimal origin of R1 indicated that the sequence between coordinates 1461-1477 resembles a truncated n' recognition site. To test for the involvement of this sequence in initiation of R1 replication, site-directed mutagenesis, using a synthetic oligonucleotide, was used to introduce a 10 bp deletion in the pSO3 recombinant removing part of this site (nucleotides between coordinates 1463-1474). The deletion derivative (pSO30) is not further replicated (Table 1) indicating that the sequence deleted, although not acting as an n' recognition site, is needed for RepA dependent initiation of R1 replication.

Host Replication Proteins DnaB, DnaC and SSB are Required for the in vitro Replication of R1.

The roles of proteins DnaB and SSB were evaluated by testing the response of increasing amounts of specific antibodies directed against these proteins on R1 replication; these effects were compared with those found in ColE1 replication. The data indicate that both antibodies inhibited completely the replication of R1 miniplasmid (Fig. 2A and 2B), suggesting an involvement of DnaB and SSB in early stages of R1 replication. ColE1 replication was also clearly inhibited by the DnaB antibodies for a residual syn-

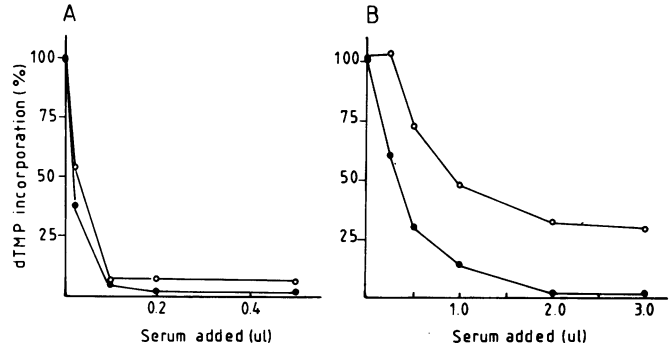

Figure 2. Effects of DnaB and SSB antibodies on the replication of R1 and ColE1 DNA. The assays of R1 or ColE1 replication were carried out under similar conditions to the one described in Fig.1. The effect of DnaB antibodies on R1 (●) or ColE1 (o) replication is shown in panel A and the effect of SSB antibodies on R1 (●) or ColE1 (o) replication in panel B. Preimmune sera inhibited replication to a maximum of 25%. 100% dTMP incorporation corresponds to 120,000 cpm for R1 replication, and 70,000 cpm for ColE1 replication.

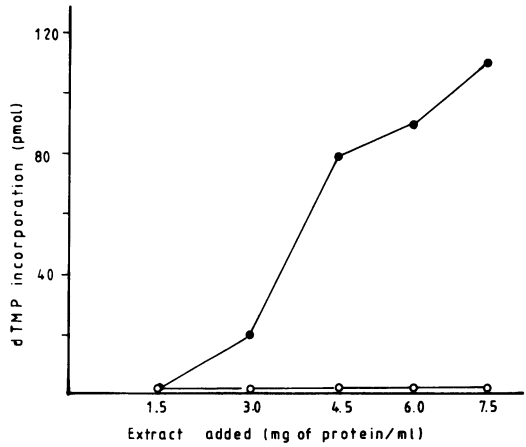

Figure 3. Replication of R1 DNA in dnaC$^+$ and dnaC mutant extracts. Standard replication assays were run at 30°C for 120 min using increasing amounts of fraction II extracts of strains BT1000 (polI,dnaC$^+$) or PC-22 (polI,dnaC) supplemented with 30 µg/ml of pKN177 DNA and 80 µg/ml of RepA preparations. dTMP incorporated in the assays using BT1000 (●) or PC-22 (o) extracts is shown.

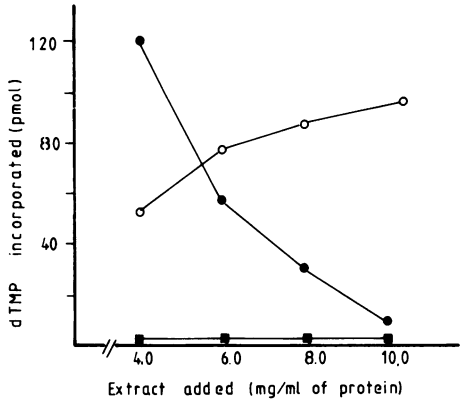

Figure 4. Replication of R1 and ColE1 DNA in extracts deficient in DnaA protein. Standard replication assays were run at 30°C for 2 hours using increasing amounts of fraction II extracts of strain WM433 (dnaA204). To test for R1 replication, the assays were supplemented with 30 µg/ml of R1 miniplasmid pKN402 and 80 µg/ml of RepA preparations; dTMP incorporation (pmols) in the presence (●) or absence (■) of a DnaA preparation (final concentration 270 µg/ml) is shown. ColE1 DNA synthesized in assays supplemented with 30 µg/ml of ColE1 DNA (o) is also shown).

thesis of 5% (Fig.2A); this observation has previously been reported and interpreted as the consequence of the involvement of DnaB in the initiation of lagging strand synthesis of ColE1 as well as in the separation of the DNA strands at the replication fork (37). The SSB antibodies inhibited replication of ColE1 to a maximum of 70% of the control. Residual synthesis of 30% probably reflects a requirement of SSB for lagging but not for leading strand synthesis of ColE1 (41). Note that the amounts of residual ColE1 synthesis in the presence of saturating concentrations of antibodies against the proteins DnaG and SSB are similar.

The role of DnaC in R1 replication was evaluated by comparison of the RepA dependent replication of plasmid R1 in extracts prepared either from strain BT1000 (polA1,dnaC$^+$) or from strain PC-22 (polA1,dnaC2). As previously reported (14), R1 is efficiently replicated in extracts of BT1000 but not in an extract of PC-22 (Fig.3), indicating that DnaC is required for *in vitro* replication of plasmid R1.

DnaA Protein is Required for in vitro Replication of Plasmid R1. Replication of R1 miniplasmid pKN402 and of ColE1 was tested in

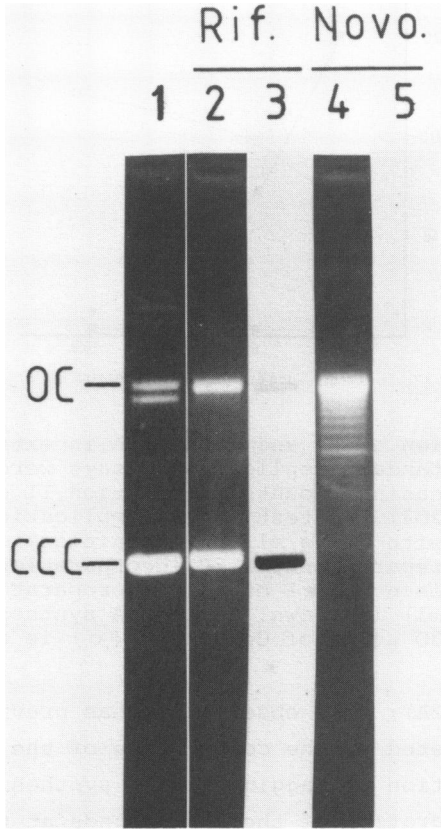

Figure 5. R1 replication products synthesized in DnaA extracts. Standard replication assays of R1 were run using fraction II extracts of strain WM433 supplemented with pKN402 DNA (30 µg/ml), RepA (80 µg/ml), and DnaA (270 µg/ml). After 2 hours of incubation at 30°C, the assays were cooled in ice and supplemented with 50 mM EDTA. The volume of the sample was increased to 100 µl with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Following phenol extraction, ether extraction and ethanol precipitation, samples were resuspended in 20 µl of TE buffer and 10 µl aliquots were run on a 0.8% agarose gel at 2.5 V/cm. At the end of the run, the gel was stained with 1 µg/ml of ethidium bromide, photographed under short wave ultraviolet illumination, and finally treated with Amplify (Amersham) for 25 min and dried. Replication products were identified by autoradiography. Lane 1 shows a sample of the DNA used in the assays, lanes 2 and 3 respectively show the ethidium bromide stain and the autoradiogram of the assays run in the presence of rifampicin (25 µg/ml). Lanes 3 and 4 show the ethidium bromide stain and the autoradiogram of the assays run in the presence of novobiocin (25 µg/ml). Notice that the dimeric supercoiled form of the DNA added to the reaction is also used as substrate for the reaction.

extracts prepared from the dnaA204 strain WM433. It was found that ColE1 synthesis can be sustained efficiently, whereas replication of R1 miniplasmids was totally abolished. Furthermore, at low extract concentrations, the RepA dependent replication of the R1 miniplasmid is efficiently complemented by purified fractions of the DnaA protein (Fig.4). The R1 replication measured in complementation assays is origin specific, as indicated by the efficient replication of pSO3 but not of pSO30 double-stranded DNA (not shown). DnaA dependent replication is also resistant to rifampicin and sensitive to novobiocin (Fig.5). In addition, the products synthesized in the presence of rifampicin are mainly found at the supercoiled position (monomers), indicating that rounds of replication are efficiently terminated and that daughter molecules are matured into monomeric supercoiled DNA (see also Fig.5, lane 3). To summarize, replication of R1 analyzed in complementation assays in DnaA deficient extracts has the same characteristics as the one previously reported in DnaA wild-type extracts (13-15). It is concluded that the failure of DnaA deficient extracts to support R1 replication is due to the requirement of DnaA protein for R1 replication.

DISCUSSION

Using an R1 *in vitro* replication assay in which purified preparations of the replication protein RepA are supplied to fraction II type extracts of *E.coli*, we have shown that the replication proteins of the host, DnaA, DnaB, DnaC, DnaG and SSB, are required for the *in vitro* replication of R1 miniplasmids at an early stage. The analysis is based on the utilization of antibodies against specific replication proteins as well as on assays in cell extracts deficient in host replication functions.

The requirement for DnaA protein is clearly shown by the inability of extracts prepared from the dnaA204 replication mutant WM433 to support *in vitro* replication of miniplasmid R1 and by the efficient complementation of this deficiency by partially purified DnaA protein. Furthermore, the replication found under complementation conditions is indistinguishable from that observed in extracts of $DnaA^+$ strains in terms of origin utilization, RepA dependence, resistance to rifampicin, sensitivity to

novobiocin and products synthesized. These data makes it highly improbable that the DnaA dependence observed *in vitro* was artefactual. However R100.1, a plasmid closely related to R1 replicates *in vivo* in the apparent absence of an active DnaA protein (42) and this plasmid as well as the R1 plasmid have been shown to integratively suppress *dnaA* deficient strains (24,43,44). Leakiness, partial suppression or reversion of the *dnaA* mutations are possible alternatives to explain this apparent conflict; other alternatives include the possibility that R1 and R100.1 encode a DnaA-like protein in a portion of the molecule absent from the minireplicon or that the suppressing plasmids contain a second, normally cryptic, DnaA independent replicon. An obvious target for DnaA protein is a DnaA binding sequence (DnaA box) located between coordinates 1425-1438 within the minimal origin of R1. The involvement of the DnaA box in R1 replication is currently being investigated by site directed mutagenesis.

In addition to the requirement for RepA and DnaA, *in vitro* replication of R1 requires at an early stage proteins DnaB, DnaC and SSB as well as DNA gyrase, a pattern that closely resembles the requirements needed for initiation in the *oriC* system (45,46). In this system, the DnaA protein has been proposed to be guiding proteins DnaB and DnaC to the origin region to form a prepriming complex with additional proteins, including DNA gyrase and SSB. In the R1 system, the initiation mechanism could be a quite similar one; the role of RepA protein could be its necessity for assembling the prepriming complex at the origin of replication, possibly in collaboration with the DnaA protein of the host. Obviously a rigorous comparison between the two systems awaits the reconstitution of the R1 system from purified components. Double-stranded DNA is required for the action of RepA, as shown by the fact that leading strand synthesis is not initiated on the single-stranded template provided by the pSO3 recombinant. Finally, it is important to mention in this context that the 10 bp deletion within the minimal origin region, separated by only 20 bp from the DnaA binding site, prevents replication of R1. DnaG dependent and *E.coli* RNA polymerase independent replication of R1 indicates that DnaG protein generates the primer triggering initiation of R1 replication. Similar results have been reported for

the oriC in vitro system (45,46). Unspecific initiation of R1 replication from transcripts synthesized by RNA polymerase could be prevented by RNAse H, as indicated by the in vitro data reported by Hillenbrand and Staudenbauer (47).

Since pSC101 — like R1 — requires for in vivo maintenance a plasmid encoded factor (48) and the DnaA protein of the host (42), it is noteworthy that the pSC101 replicon appears to be highly unstable in a dnaG ts background at intermediate temperatures (49). This observation suggests that in analogy to oriC and R1, pSC101 also requires DnaG protein as the primer generating enzyme.

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