Reconstitution of F Factor DNA Replication in Vitro with Purified Proteins*

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Jacob, Brenner, and Cuzin pioneered the development of the F plasmid as a model system to study replication control, and these investigations led to the development of the “replicon model” (Jacob, F., Brenner, S., and Cuzin, F. (1964) Cold Spring Harbor Symp. Quant. Biol. 28, 329–348). To elucidate further the mechanism of initiation of replication of this plasmid and its control, we have reconstituted its replication in vitro with 21 purified host-encoded proteins and the plasmid-encoded initiator RepE. The replication in vitro was specifically initiated at the F ori (oriV) and required both the bacterial initiator protein DnaA and the plasmid-encoded initiator RepE. The wild type dimeric RepE was inactive in catalyzing replication, whereas a monomeric mutant form called RepE* (R118P) was capable of catalyzing vigorous replication. The replication topology was mostly of the Cairns form, and the fork movement was unidirectional and mostly from right to left. The replication was dependent on the HU protein, and the structurally and functionally related DNA bending protein IHF could not efficiently substitute for HU. The priming was dependent on DnaG primase. Many of the characteristics of the in vitro replication closely mimicked those of in vivo replication. We believe that the in vitro system should be very useful in unraveling the mechanism of replication initiation and its control.

The discovery of the F factor and sexuality in bacteria by Lederberg and Tatum not only opened up the study of bacterial genetics (1, 2) but also provided important genetic tools for gene mapping, strain construction, and generation of merodiploids that proved essential for the discovery of the regulation of gene mapping, strain construction, and generation of merodiploids (1, 2) but also provided important genetic tools for genetic analysis of the F factor, along with that of the phage, used a crude extract of a plasmid containing host cells was initially unsuccessful (3). To elucidate further the mechanism of initiation of replication, we have used the oriV (origin of vegetative replication; called oriF here) of F, the plasmid-encoded RepE initiator protein (8, 9), and multiple purified host-encoded proteins to develop a reconstituted replication system. The system was developed to further delineate the various steps of F replication initiation and its control and, in conjunction with the crystal structure of RepE-iteron DNA complex (10), to attempt to provide a better understanding of replication initiation and its control at high resolution.

In vivo experiments are consistent with a model of regulation of initiation that includes not only positive regulation of replication, as postulated by Jacob et al. (4), by RepE-origin DNA interaction, but also negative regulation caused by the oligomerization of RepE that, in turn, promotes intramolecular coupling between a set of four iterons (19-bp repeated sequences) at the oriF and a second set of five of the same iterons located at a region down stream of the oriF called the incC, which is believed to shut down the oriF (7, 11).

The minimal oriF contains the following sequence elements (Fig. 1A): two tandem dnaA sequences that bind to the host-encoded initiator DnaA; an AT-rich region followed by a consensus 13-mer sequence that is melted in the presence of RepE, DnaA, and HU proteins preparatory to the loading of the DnaB helicase (12); and four 19-bp-long iterons, mentioned above, that bind to the RepE protein. The inverted repeats consisting of iteron sequences that bind to dimeric RepE are located immediately upstream of the open reading frame (ORF)1 of RepE. The binding of dimeric RepE to the sequences autoregulates RepE transcription (13). Following the minimal oriF and the RepE ORF, five iterons that constitute the negative regulatory element called incC are present (Fig. 1A). These iterons also bind to RepE and, presumably, simultaneous binding of the dimeric wild type (WT) RepE protein to the incC and ori iterons causes DNA looping and the consequent turning off of initiation (Fig. 1B). Although more definitive and mechanistic evidence is needed, there are some indirect in vitro and in vivo data suggesting that this looping shuts down initiation from oriF (7).

The wild type RepE protein is dimeric and postulated to be inactive in catalyzing replication initiation. Mutations in the region of the amino acid residues 111–161 cause a high copy number and the concomitant monomerization of the protein (14). The mini F plasmid is not maintained in host cells that are deficient in DnaK, DnaJ, and GrpE proteins. These chaperones are believed to act on dimeric RepE and convert it into active monomers, and the monomer-dimer equilibrium is believed to regulate the frequency of initiation of replication (13, 15).

Previously, an in vitro system for replicating F plasmid that used a crude extract of a plasmid containing host cells was

1 The abbreviations used are: ORF, open reading frame; WT, wild type; SSB, single-stranded DNA-binding protein; dd, dideoxy (prefix); IHF, integration host factor.

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reported (16), and a combination of DnaA, RepE, and HU proteins were shown to induce DNA melting in vitro at the oriF (12). To unravel further the mechanistic steps of replication initiation at the origin of replication and understand its control, mutants in the RepE protein, host replication proteins defective in replication of the plasmid, and a replication system reconstituted from highly purified proteins were needed.

In this report, we describe the development of a reconstituted in vitro replication system consisting of up to 22 purified proteins that catalyzed origin-specific initiation and a complete round of replication of F plasmid DNA in vitro.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—The Escherichia coli strain DH5α (F ’end 1, had R17 (pHT115) gln V44 thi-1 rec A1 gyr A (Nalr) rel A1Δ (Lac ZA-arg F) U189 deo R1 [80 d Lac A (Lac Z) M15] was used for all cloning experiments. The plasmid pORF1, which contained the minimal ori sequences consisting of the dnaA1 and dnaA2 sites, four iterons, and the sequences extending up to the end of RepE ORF, was used as the standard template DNA. A polymerase chain reaction was used to amplify the ori DNA, which was cloned as an EcoRI-HindIII fragment into pUC19. The pUC 18-oriC template contained the minimal oriC region cloned in the pUC18 vector (17). Supercoiled DNA templates were prepared by QIAGEN ethidium bromide equilibrium centrifugation. The primers used for PCR (2.61) contained nine subunits of pol III under the control of a lac promoter, was a gift from Drs. C. McHenry and Arthur Pritchard (University of Colorado Health Sciences Center, Denver, CO). The E. coli strains BL21(DE3) and/or BL21(DE3/plysS) were used for all protein expression work. All clones were sequenced at the DNA sequencing facility of the Biotechnology Resource Laboratory of the Medical University of South Carolina.

Enzymes—Highly purified RNase H and SSB were purchased from a commercial source (USB, Cleveland, OH). Plasmid-encoded RepE WT and RepE* (R118P) initiator proteins (5) were purified from an in vivo vector pTXB1 (New England Biolabs, Boston, MA) containing the RepE ORF fused with in vitro translation at the C terminus, followed by the chitin binding domain. The fusion protein was purified on chitin-agarose affinity column in the absence of reducing agents, incubated at 4 °C for 12 h with 10 mM dithiothreitol followed by 5 h of further incubation at 11 °C to effect complete in vitro cleavage and release of the RepE moiety. The protein was further purified by gel filtration through a Superose 12 column. The host initiator protein DnaA was purified as described (18), DnaB and DnaC under the Tac promoter (19), DnaG (20), HIF (21), and HU (22) were purified as described. GyrA was purified from a pET15b clone, and GyrB was from pTXB1 vector. Details of purification will be described elsewhere. PolIII* was purified from pHO 2.61, and the β-slider clamp was purified from an overproducer plasmid constructed in our laboratory by Dr. I. Patel.

In Vitro Replication—In vitro origin-specific replication took place within a narrow window of protein concentrations as described below. The standard reaction mixture (in 25 μl) contained 40 mM HEPES/KOH (pH 7.6), 10 mM MgCl₂, 5 mM dithiothreitol, 20 mM KCl, 100 μg/ml bovine serum albumin, 10% glycerol, 0.4 μM DNTP, 3000 cpm/pmol [3H]dATP, 5 mM ATP, 500 μM each CTP, UTP, and GTP, 50 mM creatine phosphate, 20 μg/ml creatine kinase type 1 (Sigma), DNA template (200 ng), 1.5 μg of SSB, 10 ng of HU, 50 ng of DnaA, 50 ng of RepE*, 1 unit of RNase H, 540 ng of gyrase AB, 100 ng of DnaB, 50 ng of DnaC, 50 ng of DnaG, and 120 ng of pol III holenzyme. The reaction components were assembled on ice and, after the addition of the initiator proteins, preincubated for 10 min. at 30 °C. DnaB, DnaC, DnaG, the Pol III holenzyme, DNTPs, and NTTPs were then added to the reaction mixture to start the synthesis. The standard reaction was carried out at 37 °C for 30–60 min. The reaction was stopped with the addition of 20 μl of 0.2 M sodium pyrophosphate to each tube and precipitated with an equal volume of 10% chilled trichloroacetic acid. The precipitate was trapped on a GF/C filter, washed with 95% ethanol, air dried, and total nucleotide incorporation was measured in a liquid scintillation counter.

The DNA synthesis was quantified by measuring the total picomoles of acid precipitable radioactivity in the reaction product.

**OriC Replication**—Replication in vitro of oriC (cloned in pUC18) template was carried out as described (17, 24, 25). The oriC replication was used as a system to check for optimizing the stoichiometry of the various components needed for maximal synthesis.

**Formation of a Preinitiation Complex**—The kinetic assay was carried out as mentioned above with minor modifications. Each reaction mixture (175 μl) was assembled in triplicate at 0 °C and preincubated at 30 °C for 30 min. The preincubation mixture contained template DNA, HU, DnaA, and RepE*. The addition of ATP or its omission from the preincubation mix did not show a significant difference in net synthesis. In other reactions, DnaA, HU, and RepE* were omitted one at a time, and the effect of the omissions on net replication were measured. After preincubation, DnaB, DnaC, DnaG, the Pol III holenzyme, dNTPs, and rNTPs were added to initiate replication at 37 °C, and 25 μl from each tube was taken out at the times indicated (under “Results”) to measure total DNA synthesis.

**OriC Identification by Dickoxygen NTP (ddNTP) Incorporation**—The reaction was carried out as described above but with the addition of [α-32P]dCTP, [α-32P]dATP, ddGTP, and ddTTP from 30 to 110 μM. The reaction products were purified as described (23), followed by digestion with EcoRI, XhoI, HindIII, and PvuI and electrophoresis in 1.5% agarose gels. The gels were dried and exposed to a PhosphorImager and/or x-ray films, analyzed with the Imagequant™ software, and plotted.

**Analysis of Replication Intermediates in Neutral Two-Dimensional Gels**—We wished to determine the topology of the replication intermediates (i.e. Cairns form or the θ type versus possible rolling circles or the α type) that were synthesized in the presence of [α-32P]dNTPs by two-dimensional gel analyses. We linearized the product DNA with Scal and resolved the intermediates in two-dimensional Brewer-Fangman gels at neutral pH (26). In some cases, replication was carried out in the presence of various concentrations of ddNTP, and the labeled intermediates were purified, cleaved with Scal, and resolved by two-dimensional gel electrophoresis as described above. All of the samples yielded comparable gel patterns.

**RESULTS**

**Proteins Used in the In Vitro Replication**—The SDS gel profiles of all of the proteins with the exception of RNaseH are
shown in Fig. 2. The proteins appear to be nearly homogeneous with the exception of the pol III* complex, which appears to be ~98% pure. The pol III holoenzyme was reconstituted from the core complex, the γ2τ complex, and the β-sliding clamp as described (27).

Replicon-specific Initiation—While working out conditions for replicon-specific initiation of a chimeric template DNA that had both origins (oriF and ori of pUC19), we discovered that replicon-specific initiation occurred within a narrow window of protein concentrations and reaction conditions that have been described in a preceding section. The chimeric template was used to take advantage of the much higher yield of the template DNA obtainable when the pUC19 ori was used to drive the replication of the chimeric template DNA. To make sure that the replication was F-specific, we attempted to replicate both the chimeric template and pUC19 DNA separately in vitro and observed that, whereas the reconstituted system elicited vigorous replication (up to ~400 pmol in 1 h at 37 °C) of the chimeric template, the pUC19 template did not yield measurable levels of replication under identical conditions (Fig. 3). It should be noted that the system did not include DNA polymerase I, which would have been needed to support replication from the ori of pUC19. The results are consistent with the conclusion that replication initiation from the chimeric template was at least specific to the oriF sequence.

Role of Other Host-encoded Proteins—The effect of systematic omission of each of the protein components, ATP and DNA, on the net replication in vitro is shown in Fig. 4. The net replication in the complete system was relatively robust, yielding ~200 pmol of synthesis at 37 °C in 1 h. Omission of ATP eliminated the synthesis. The synthesis was dependent on SSB, DnaA, RepE* (R118P), HU, DnaB, DnaC, DnaG, gyrase A and B, and pol III holoenzyme. The reaction did not require RNA polymerase but was dependent on DnaG primase, thus indicating that all of the primings were catalyzed by DNA primase. Although omission of RNaseH did not result in a marked reduction in overall synthesis, the enzyme was needed to suppress DnaA-independent synthesis (data not shown).

Dependence on WT and Mutant Forms of RepE—We proceeded to determine the optimal concentration of WT RepE and that of a mutant form, RepE*, needed to catalyze maximal replication. Titration experiments showed that, whereas the mutant form of the protein catalyzed maximal replication (~200 pmol/h) at 50 ng per reaction, the WT protein was relatively inefficient and required 8-fold more protein to yield 100 pmol of reaction product in 1 h (Fig. 5). It should be noted that published work has shown that the WT RepE is dimeric, whereas the mutant form is monomeric (14). It is possible that the lower efficiency and much higher concentrations of the WT protein are needed to catalyze replication of the template reflects residual monomers present in the majority of dimers in the population of WT RepE.

Optimal Concentration of DnaA Needed in Vitro—Previous work from other laboratories as well as our own have shown that plasmid replicons generally depend on dual initiators, namely host-encoded DnaA and plasmid-encoded initiators, for replication in vivo and in vitro (28–30). The same dual requirement for DnaA and RepE was also observed for F replication as reported here. We carried out in vitro replication over a range of concentrations of DnaA and discovered that replication was promoted within a relatively narrow window of DnaA concentration, with the optimum being ~50 ng/reaction. Higher levels of DnaA inhibited the reaction, with significant inhibition observed at double the optimal DnaA concentration (i.e. at 100 pmol).
ng; see Fig. 6). We have observed that a previously reported mutant form of DnaA that fails to bind to ATP (31) was just as efficient in catalyzing replication of mini F in vivo as the wild type DnaA. In vitro, however, the mutant form of DnaA was inert. We suspect that the inactivity is due to the absence of chaperones that may be needed to refold and stabilize the mutant form of DnaA (32). Work is currently in progress to activate the mutant form of DnaA in vitro and to work out its interaction with RepE.

Replication in the Presence of HU Versus IHF—Our previous work has shown that the plasmid R6K needed the DNA bending protein IHF to replicate efficiently, and the substitution of IHF by the structurally and functionally very similar protein HU significantly reduced the efficiency of replication in vitro (27). We wished to examine whether the HU-dependent replication of oriF can be duplicated by replacing HU with the structurally and functionally similar protein IHF (33, 34). We carried out sets of in vitro replication reactions of oriF with a range of concentrations of HU and IHF separately and compared and contrasted the data with similar reactions carried out with an oriC template (17). The results showed that both oriC and oriF DNAs replicated optimally at ~20 ng of HU protein per reaction (Fig. 7A). The oriC template replicated equally well in the presence of either HU or IHF, but, in contrast, IHF could not replace HU for optimal replication of oriF (Fig. 7B). The aforementioned observations are further discussed in a later section. It should be noted that Fig. 7A shows the concentrations of HU and IHF needed to elicit the maximum amount of synthesis; these concentrations were determined by titration experiments such as that shown in Fig. 7A.

Reaction Kinetics—We investigated the extent of synthesis as a function of time and noticed, as in the case of other in vitro replication systems (24, 27, 30), a lag time of ~5 min before a rapid synthesis was established (Fig. 8). We preincubated the template DNA with DnaA, RepE, and HU in the presence or absence of ATP for 10 min at 30 °C to allow the formation of a DNA-protein complex and then added the remainder of the reaction components and continued incubation at 37 °C. We sampled equal aliquots of the reaction mixture as a function of time following the preincubation step. We found, as expected, that the preincubation step eliminated the lag period (Fig. 8). Withholding DnaA, RepE, or HU from the preincubation mixture failed to eliminate the lag time, thus indicating that each of the aforementioned components was needed to form the preinitiation complex. The presence of ATP in the preincubation mixture had no effect on the subsequent elimination of the lag time, suggesting that the formation of the preinitiation complex was not energy dependent.

Origin-specific Initiation—One of the critical questions to be answered was whether replication in vitro started from oriF.
We attempted to answer the question by performing in vitro replication reactions with \([\alpha^{32}P]dATP, [\alpha^{32}P]dCTP, dGTP, and TTP in the presence of different concentrations of unlabeled ddTTP. The rationale was that, at higher ratios of ddNTP/dNTP, the fork extension was more likely to be terminated at higher ratios of ddNTP/dTTP, the 1226-bp band should have had the highest specific activity with a decreasing gradient of radioactivity present in the adjacent fragments in both directions, proportionate to their distance from the ori. An inspection of the autoradiogram (Fig. 9B) and its quantification (Fig. 9C) showed that, with increasing ddTTP (e.g., at 70 \(\mu\)mol ddTTP), the 1226-bp fragment had the highest specific activity, and a detectable label was also present in the 896-bp, 210-bp, and 120-bp fragments. However, the 1670-bp fragment located to the right of the 1226-bp fragment rapidly lost the label with an increasing concentration of ddTTP (Fig. 9B). At 90 \(\mu\)mol ddTTP, the highest level of radioactivity was present in the 1226-bp band, and some label could be detected in the 896- and 210-bp bands. The 120-bp band was not visibly labeled because of its small size. At 110 \(\mu\)mol ddTTP, the 1226-bp band was the only visible band, although a small amount of label could still be measured in the 896-bp bands. The data are most consistent with the interpretation that most of the forks from oriF were moving from right to left, although the possibility that a minor population of forks were moving from left to right could not be eliminated by the experimental data.

**Topology of Replication in Vitro**—We wished to examine the topology of in vitro replication intermediates to determine whether these resembled the Cairns type intermediates reported previously from in vivo experiments (8). We replicated the template plasmid pZorIF (Fig. 3) in vitro with \([\alpha^{32}P]labeled precursors either in the presence or absence of 50 and 90 \(\mu\)mol ddTTP, purified the intermediates, linearized the molecules by cleavage with ScaI, and resolved the reaction products in two-dimensional neutral-neutral gels (26). A representative autoradiogram of a two-dimensional gel is shown in Fig. 10. The gel profiles consistently showed a bubble arc that was consistent with Cairns type of replication topology. In addition, we also observed a prominent X arc that we believed was generated by breakage occurring in the replication bubbles. Normally the single stranded regions present at replication forks cause susceptibility to breakage. However, one must keep in mind that, in the present in vitro system, the Okazaki pieces are not processed and ligated because of the absence of DNA polymerase I and DNA ligase, thereby causing the formation of SS regions throughout a replication bubble and not necessarily just at the forks. We believe that random breakage at the SS DNA regions probably generated the X-shaped intermediates.

Consistent with the relative locations of the ScaI site at which...
the DNA was linearized, with respect to the location of the ori, very few Y-shaped molecules would have been generated. Indeed, shorter exposures of the two-dimensional gels do not show any Y-shaped intermediates (Fig. 10B). Only after very prolonged exposures were faint Y-shaped molecules seen; they are diagrammatically represented as a dotted line in the diagram in Fig. 10A.

The ddNTP incorporation results, taken together with the two-dimensional gel analysis, support the conclusion that the replication \textit{in vitro} was mostly of the Cairns type and that the majority of the forks moved from right to left (heavy arrow in Fig. 9A). As contrasted with the \textit{in vivo} data, the \textit{in vitro} replication of mini F was reported to be bi-directional and of the Cairns type (8). It should be noted that the early Cairns type replication of phage \(\lambda\) \textit{in vivo} is mostly bi-directional, whereas the replication \textit{in vitro} was observed to be unidirectional (35).

**DISCUSSION**

The principal reasons for developing a reconstituted replication system for F factor were as follows: (i) to investigate the mechanism of replication initiation and its control using not only the purified host and plasmid-encoded proteins but also mutant forms of the proteins; and (ii) to achieve the longer term goal to work out, \textit{in vitro}, the mechanism of \textit{incC}-mediated negative control of replication. The availability of the crystal structure of the monomeric RepE-iteron complex (10) provides an opportunity to perform rationally guided site-directed mutagenesis of the RepE initiator. With these goals in mind, in this paper we have reconstituted the replication of the mini F factor template \textit{in vitro} with all purified protein components and were able to achieve ori\(F\)-specific initiation.

It is somewhat interesting that, unlike in the R6K system (27), IHF was less effective than HU in supporting optimal replication despite the fact that HU and IHF have very similar crystal structures (33, 34) and also have some similarity in function, because IHF could support ori\(C\) replication just as efficiently as HU (Fig. 7). Our earlier work has shown that the plasmid R6K, for optimal replication \textit{in vitro}, requires IHF and that this requirement cannot be bypassed by supplying HU in the place of IHF (27). Perhaps this observation can be explained by noting that R6K ori\(C\) includes an \(ihf\) site, whereas the minimal sequence of ori\(F\) does not have a consensus \(ihf\) site (Fig. 1A). Binding of IHF to the cognate site causes a bending of the DNA (36) that promotes more stable binding of DnaA to the ori with a single DnaA box. IHF-promoted DNA bending is also believed to promote physical interaction between the plasmid-encoded initiators of R6K and pSC101 with the DnaA protein, which is needed for origin melting and helicase loading (28, 31). Perhaps the availability of tandem \(dnaA\) boxes at the minimal ori\(F\) generates cooperativity and stable binding of DnaA without the need for IHF-induced DNA bending. Furthermore, the binding of HU to the origin region, although not strictly sequence-specific, also wraps/bends ori DNA and thus creates a "chromatin structure" that promotes protein-protein contact between DnaA and RepE at the ori. Both DnaA and RepE are needed for \(F\) replication, and, in fact, DnaA, HU, and RepE are needed for \(ori\) melting \textit{in vitro} (12). We have observed that both DnaA and DnaB proteins physically interact with RepE (2), and it would not be surprising if these protein-protein interactions were important in helicase loading (31, 37, 38). It would be interesting to map the surfaces of DnaA and DnaB that interact with RepE and attempt to work out the ternary structures of host peptide-RepE-iteron complexes. Such information should be valuable in understanding of replication initiation at high resolutions.

The data derived from the reconstituted system presented here is consistent with previous \textit{in vivo} data supporting the conclusion that wild type dimeric RepE is functionally inert and probably needs chaperones for activation by monomerization (13, 39). We have treated WT RepE with the chaperones DnaJ, DnaK, GrpE, and ATP but have not observed significant activation of the dimeric initiator \textit{in vitro}, suggesting the requirement of additional proteins for the activation process. We are currently investigating this question further.

The reconstituting the reconstituted system appears to be exclusively catalyzed by DnaG primase. Interestingly, DnaG primase physically interacts with the P\(_2\) initiator of R6K (40) and also with RepE of F (2). It would be interesting to determine whether an initiator-primase interaction plays a mechanistic role in primase loading and/or priming. Perhaps the initial recruitment of DnaG not only requires DnaG-DnaB interaction (20, 41) but also RepE-DnaG interaction.

The replication of F occurred within a narrow range of concentrations of DnaA protein, and the excess of it was inhibitory. Perhaps the oligomerization of DnaA at higher concentration was the cause for this inhibition.

Although RNaseH was not strictly needed for initiation, its absence caused significant amounts of a spurious initiation that was independent of RepE and DnaA. It appears that, as in the case of ori\(C\), RNaseH also acts like a specificity factor that promoted ori\(F\)-specific replication of F \textit{in vitro} (24, 42, 43).

In summary, the \textit{in vitro} reconstitution system described in this paper should be very useful in future attempts to unravel the precise roles of various protein-protein interaction in replication initiation in this model system. We hope that one should be able to work out the molecular mechanism of \textit{incC}-mediated negative control of replication \textit{in vitro} by using the system.

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