Reconstitution of R6K DNA Replication in Vitro Using 22 Purified Proteins

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We have reconstituted a multiprotein system consisting of 22 purified proteins that catalyzed the initiation of replication specifically at ori γ of R6K, elongation of the forks, and their termination at specific replication terminators. The initiation was strictly dependent on the plasmid-encoded initiator protein π and on the host-encoded initiator DnaA. The wild type π was almost inert, whereas a mutant form containing 3 amino acid substitutions that tended to monomerize the protein was effective in initiating replication. The replication in vitro was primed by DnaG primase, whereas in a crude extract system that had not been fractionated, it was dependent on RNA polymerase. The DNA-bending protein IHF was needed for optimal replication and its substitution by HU, unlike in the oriC system, was less effective in promoting optimal replication. In contrast, wild type π-mediated replication in vitro requires IHF. Using a template that contained ori γ flanked by two asymmetrically placed Ter sites in the blocking orientation, replication proceeded in the Cairns type mode and generated the expected types of termination products. A majority of the molecules progressed counterclockwise from the ori, in the same direction that has been observed in vivo. Many features of replication in the reconstituted system appeared to mimic those of in vivo replication. The system developed here is an important milestone in continuing biochemical analysis of this interesting replicon.

The plasmid R6K is a fascinating model system to investigate the mechanisms of initiation and termination of DNA replication for the following reasons. First, it has three origins of replication called α, β, and γ (1–7). At a given time and in a given molecule, only one of the three origins (ori) is active. In vivo, ori α and β are almost equally active, whereas γ remains mostly silent (8–10). These observations present an opportunity to uncover the mechanism of origin selection and activation in a multiorigin system. Second, although α and β are the most frequent sites of initiation in vivo, these are not the primary binding sites for the plasmid-encoded π initiator protein. In fact, the single iteron at α and the half iteron at β are incapable of binding to π protein by themselves in the absence of the seven γ iterons present in cis (7, 11, 12). Rather, π binds to the seven iterons at ori γ. Action at a distance (initiation) is potentiated by looping of the π bound to the seven iterons of γ to the single iteron at α or the half iteron at β that are located ~4000 and 1200 bp away, respectively, from γ (8, 13). DNA looping is believed to convey the π protein along with other replisomal proteins to the physically separated α and β origins, thus providing additional opportunity for investigating the mechanism of long range ori activation (7, 11). In addition, R6K has sequence-specific replication termini that invite further investigations into the physiological roles of replication termination (14–16).

In a series of in vitro experiments we have shown that π binds to α and β by DNA looping between γ-α or γ-β sequences (7, 11, 12). Consistent with the biochemical results, both α and β require the presence of γ in cis to be active (8, 9, 13). We have previously reported the isolation of a looping defective mutant form of π protein (P42L) that fails to activate α and β in vivo but derepresses ori γ (7). Thus the π γ sequences are the central elements in the replication initiation from all three origins.

ori γ contains several sequence elements that are essential for its function (see Fig. 1). First, it has seven tandem 21-bp repeated sequences called iterons that bind to π protein (17, 18). The binding of π protein to the iterons causes localized DNA bending (19). There are two binding sites for the host-encoded DnaA initiator protein called dnaA1 and dnaA2 (20–22). Whereas, dnaA1 can be deleted without destroying ori activity, either deletion of dnaA2 or insertion of a 6-bp sequence between the seventh iteron and dnaA2 site causes inactivation of ori γ (5, 6, 20–22). There is an AT-rich region between the dnaA1 site and the iterons and several point mutations in this region inactivate ori γ (6, 23). Embedded in the AT-rich region is a binding site (ithf) for the DNA-bending protein IHF (22, 24). Binding of IHF at the ithf locus causes bending of the DNA (6) that presumably brings the DnaA protein bound to the dnaA1 site into contact with π protein bound to the seven iterons and also with DnaA bound to dnaA2. DNA bending also promotes contact between the π-iteron complex and the AT-rich region by forming a local DNA loop (25). Several mutations at the ithf locus that abolish binding to IHF also cause ori γ inactivation.1 ori γ is maintained at a very low copy number in IHFΔ cells (22), and therefore the DNA-bending protein greatly enhances the efficiency of initiation. In addition to the iterons, π binds to inverted half iterons that form the operator of the ORF2 encoding the protein and auto-regulates π synthesis at the transcriptional level (26, 27).

The π initiator proteins can promote pairing of two sets of iterons in trans as determined by the enhancement of topoisomerase II catalyzed formation of catenated dimers (19). This

1 M. Abhyankar and D. Bastia, manuscript in preparation.

2 The abbreviations used are: ORF, open reading frame; pol, polymerase; DTT, dithiothreitol; WT, wild type; nt, nucleotide(s).
Reconstitution of R6K Replication

in vitro observation was found to be of biological significance in vivo by Helinski and independently by Chattoraj who showed that plasmid-encoded initiator mediated pairing of iteron arrays causes inactivation of the ori pairs (28–32). Presumably, the active ori structure consisting of iteron DNA wrapped around monomeric form of φ is isomerized into paired arrays of iterons held together by dimeric φ. This paired or handcuffed structure apparently shuts down initiation from both of the paired origins (33).

Previous work using crude extract systems had shown the dependence of the plasmid replication on the plasmid-encoded π protein (9, 34, 35). Our previous work, using a partially fractionated crude extract system, had established that both the host-encoded DnaA and the plasmid-encoded π initiators were needed for initiation from ori γ in vitro (36). In vivo work showed that ori α and γ but not ori β require the bacterial initiator DnaA for replication (5, 36). Further advance in the analysis of the mechanism of replication initiation from ori γ and looping-dependent initiation of replication from α and β required the development of a replication system reconstituted from purified proteins. Such a system should not only establish definitively the minimum set of host proteins needed for R6K replication but also should provide an opportunity to dissect the details of the initiation pathway involving dual initiator proteins.

Considering the central role played by the γ sequences in initiation, in this report we have developed a reconstituted system of 21 purified host-encoded proteins and the plasmid-encoded π protein that catalyzed replication initiation specifically from ori γ and produced full-length molecules. The replication was mostly of authentic Cairns type (γ) as revealed by two-dimensional gel electrophoresis of the replication intermediates (37). The replication was dependent on DnaA and on πγ, a mutant form of π that tended to monomerize upon binding to DNA. The wild type dimeric form of π in contrast was mostly inert in promoting initiation. Optimal replication in vitro required the DNA-binding protein IHF and substitution of IHI from HF elicited sub-optimal replication. Many of the characteristics of the replication in vitro in the reconstituted system mimicked that observed in vivo. In this report, we also present the mapping of the start of the leading strand(s) and the direction of replication in vitro.

MATERIALS AND METHODS

Plasmids and Bacterial Strains—The Escherichia coli strain DH5α (F′/end1 hsdR17 (rK mK) rII39011-1 recA1 gyrA (Nal) relA1- (lacYZA-argF) U189 deoR[80 d lac[ΔlacZM15]) was used for all cloning experiments. The plasmid DNA template pMA1 contains the region of R6K starting from the dnaA box and extending into HindIII fragment 15 (2). The DNA prepared by PCR as a BamHI-SphI cassette that lacks the C-terminal 28 amino acids of π and ori β, was cloned between the BamHI-SphI sites of pUC19. Supercollared DNA was prepared by CsCl-ethidium bromide equilibrium centrifugation. PUC19-OriC template contains the minimal oriC region cloned in the pUC19 vector (38). The plasmid pHOC3.2.1 (39) that contains nine subunits of pol III under the transcriptional control of a lac promoter, was a gift from Drs. C. McHenry and Arthur Pritchard (University of Colorado, Health Sciences Center (UCHSC), Denver, CO). The plasmid pMA1-DT contained two TerB sites of E. coli cloned ~350 and ~1200 bp away and on either sides of oriC (see Fig. 9A).

Proteins and Enzymes—SSB and RNaseH were bought from commercial sources (USB, Cleveland, OH). π protein was purified from an intact vector containing the π ORF fused at the sequence corresponding to its C-terminal end to intein followed by the chitin binding domain. The fusion protein was bound to chitin-agarose affinity column in the absence of reducing agents. The column was then infused with 10 mM DTT and incubated at 4 °C for 12 h followed by 4 h of incubation at 16 °C that caused self-cleavage of intein and release of the π moiety. The fusion was constructed in such a way that intein cleavage generated normal WT π or its mutant form π∗ (P42L, P106L, and F107S). The mutant form of the protein has a tendency to monomerize when bound to the iterons. The detailed characterization of the mutant forms and the implication of the data for the mechanism of copy control will be published elsewhere. DnaA was purified as described previously (39). DnaB and DnaC were purified from an overproducer plasmid that was constructed by Dr. N. Dixon (University of Sydney, Australia) that contained the ORF of DnaB and DnaC under the control of a Tac promoter (40). DnaG (41), IHF (42), HU (43), Tus (44) were purified as described. GyrA and GyrB were purified from a His6-tagged GyrA open reading frame cloned into the expression vector pET15b and from an intein vector, respectively. The details of GyrA and GyrB purification will be described elsewhere. DNA polymerase III* was reconstituted from the core complex consisting of the α, δ, ε, and subunits, a gift from Dr. Mike O’Donnell, Rockefeller University (45, 46), and the τ clamp loading complex consisting of the τ, β, δ, ε, and χ subunits (47). We also purified all of the nine subunits of pol III* from an overproducer plasmid pHOC3.2.1 (a gift from Drs. A. Pritchard and Charles McHenry, UCHSC, Denver, CO). τ sliding clamp was purified from an overproducer plasmid, constructed in our laboratory by Dr. I. Patel.

In Vitro Reconstitution of Replication of oriγ—The standard reaction mixture (in 25 μl) contained 40 μM HEPES/KOH (pH 8.0), 50 μg/ml bovine serum albumin, 5 μM DTT, 20 μM/ml creatine kinase (Sigma Chemical Co., St. Louis, MO), 5 μM creatine phosphate, 10% glyceral, 10 mM magnesium acetate, 40 μM of each of the dNTPs, 3000 cpm/pmol of [1H]dATP, 2 mM ATP, 500 μM each of CTP, UTP, and GTP, 1 μg SSB, 120 ng of DnaA, 70 ng of DnaB, 30 ng of DnaC, 30 ng of DnaQ, 150 ng of GyrA, 450 ng of GyrB, 20 ng of IHF, 150 ng of pol III helicoc enzyme, 0.5 unit of NusA, 500 ng of oriγ template, and 200 ng of a mutant form (P42L, P106L, and F107S) of π that tends to form monomers. The reaction components were assembled on ice, and the reaction was carried out at 37 °C for various periods of time, stopped with precipitation with 10% ice-cold trichloroacetic acid containing 10% 2 m sodium pyrophosphate. The precipitate was trapped on GF/C glass fiber filters, dried, and counted with liquid scintillation counter.

oriC Replication—Replication in vitro of the pUC9-OriC template was carried out as described previously (38, 48, 49).

Origin Mapping by DideoxyNTP Incorporation—The reactions were carried out as described above but with [α-32P]dATP and [α-32P]dTTP and 0, 40, 60, and 80 μM dATP. The reaction products were purified as described (44) and analyzed by agarose gel electrophoresis after cleavage with ScaI and HindIII.

Replication Intermediates in Neutral Two-dimensional Gels—To investigate the mode of replication, we analyzed the replication intermediates that were synthesized by incorporation of [α-32P]dNTPs cleaved with ScaI and resolved in a neutral two-dimensional Brewer-Fangman gels at neutral pH (37).

In Vitro Termination of Replication—We wished to obtain independent evidence for origin-specific initiation and on the topology of replication by interposing two TerB sites, in the blocking orientation with respect to the ori on either sides of ori γ, resulting in the plasmid pMA1-DT (Fig. 9A). The pMA1-DT template was replicated in vitro by incorporating α-32P-labeled precursors, and the intermediates were cleaved and resolved in two-dimensional gels at neutral pH (37).

Primer Extension—Replication intermediates were generated as described above from the template pMA1-DT in the presence of either 32H-labeled or unlabeled precursors. The primers RGPX1 (5′-CTGCTATGTTCCCTTCTTTCTGACT) and GPX2 (5′-CGATCGGTTTGGGCTGA) were 5′-end labeled with 32P and separately hybridized to the pool of replication products and subjected to linear, single primer PCR. The products were run in a DNA sequencing gel along with markers generated by dideoxy sequencing reactions made from the same primers. The extension products should end at the 5′-end of the templates and the resolution of the products in DNA sequencing gels provided precise locations of the start points of the leading strands.

RESULTS

Protein Components Required for in Vitro Replication—

Twenty-two purified proteins (counting the heterodimeric IHF as one protein) were used in the reconstitution experiments. The proteins were checked for purity by being resolved in a 12% SDS-polyacrylamide gel are shown in Fig. 2. RNaseH was used as a specificity factor to suppress DnaA-independent synthesis (48). Several pol III* preparations were used that were purified in our laboratory using pHOC3.2.1 (Fig. 2). We have also used highly purified pol III* reconstituted from the individual subunits (gift from Dr. Mike O’Donnell, Rockefeller University) to confirm the results derived from our reconstituted preparations of pol III.

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Using the ensemble of purified proteins we first replicated bacterial oriC plasmid as a test for the activity of many of the proteins used in the system. The oriC replication also provided us with the means, not only to work out the optimal amount of each protein in the reaction mixture but also to compare and contrast its properties with the replication characteristics of the R6K oriC/H9253 system (38, 48). The template pMA1 (Fig. 3A) contained both the oriC and the ori of the vector pUC19. To make sure that the initiation of replication was not nonspecific, we attempted to replicate both pUC19 and separately pMA1 in vitro and observed that the pUC19 template was inert, whereas the pMA1 template yielded from 150 to 200 pmol of the product after ~60 min of replication at 37°C (Fig. 3B).

The effects of omission of the various proteins on oriC replication are shown in Fig. 4. Omission of ATP or the other components of the multiprotein system drastically reduced net synthesis. Although omission of RNaseH caused a modest re-

Monomeric but Not the Wild Type Dimeric Form of π Was Active in Catalyzing Initiation—Unlike the crude extract system in which wild type (WT) π was active in initiating replication (35, 36), the initiator was almost inert in the purified reconstituted system (Fig. 5). Addition of π* (P42L, P106L, and F107S) was essential for promoting vigorous DNA replication (Fig. 5). The WT π was dimeric by gel filtration analysis even in the presence of 3 M guanidine chloride, whereas the π* form under identical conditions was monomeric (data not shown). In the absence of guanidium, both types of proteins behaved as dimers. We have observed that, upon binding to an iteron, the π* almost completely dissociates into monomers. Therefore, it appears likely that some types of chaperones (the identities of which are not known at this time) monomerize and activate WT π in vivo. Determination of the identities of the chaperones is one of our future goals.

Requirement for the DNA-bending Protein IHF—As noted above, the minimal sequence of ori γ contains an ihf site that is embedded in the AT-rich region located between the dnaA1 box and the seven tandem iterons. Replication programmed by WT π requires the bending of DNA by IHF binding to the ihf site (22, 24). In vivo, ori γ tagged with a kanamycin resistance marker can replicate in an IHFΔ strain of E. coli with a very low copy number and form minute colonies. When ampicillin was used as a selection marker, no colonies were recovered on the plates (22). We wished to investigate whether the replication in vitro was IHF-dependent, or could it be functionally replaced, as in the oriC system, by supplying HU protein

3 M. Abhyankar, R. Sharma, J. Reddy, S. Zaman, and D. Bastia, manuscript in preparation.
We carried out replication of oriC and ori/H9253 in vitro with IHF and separately with HU. The results showed that HU could not, as contrasted with the oriC system, fully substitute for IHF in supporting optimal replication of ori/H9253 (up to 180 pmol). As expected, IHF and HU were fully interchangeable for oriC replication (Fig. 6). Why does ori/H9253 replicate at all in the absence of IHF in vitro, whereas in vivo its replication by WT/H9266 requires IHF? This point is discussed in a later section.

Kinetics of Replication Suggested the Formation of a Pre-initiation Complex—As observed in other in vitro systems (48, 51, 52) replication of ori γ in vitro also showed a lag period of ~5 min at 37 °C. The lag period was presumably caused due to the initial time needed to form a preinitiation complex. To determine the proteins needed to form such a preinitiation complex, the template DNA was preincubated with γ, DnaA, IHF, and ATP and that without the preincubation step. Data shown are averages of three independent experiments.

Origin and Direction of Replication—We wished to determine whether the topology of in vitro replication closely mimicked that of the in vivo replication. It was necessary to deter-
mine if initiation occurred specifically at the known location of ori γ and whether the forks showed similar directionality of movement after emanating from the ori. Previous in vivo work (13, 18) had shown that ori γ is located at the junction of the 1.2-kb Scal-HindIII and 0.9-kb HindIII fragment (Fig. 8), and the forks proceed mostly in the right to left (counterclockwise) direction. We replicated the pMA1 template (Figs. 3 and 8) in vitro using increasing molar ratios of ddTTP/dTTP (in the presence of [α-32P]dCTP and [α-32P]dATP) to try to limit replication to the region of the ori. The reaction products were purified and cleaved with Scal and HindIII and resolved in an agarose gel. The radioactivity in each band was quantified and
plotted as a percentage of total counts present in all of the bands using a phosphorimaging device. The data qualitatively and quantitatively showed that the 1.2-kb band had the highest specific activity at 60–80 μM ddTTP (Fig. 8). The data were consistent with initiation occurring mostly from the indicated location of ori γ, and many if not all of the forks were moving from right to left. The highest specific activity of the 1.2-kb band and only background levels of activity of the 0.9-kb, adjacent fragment supported the latter conclusion. Both should have had high specific activity in the higher ddNTP/ddTP ratios if replication had proceeded with equal frequency in either direction. It should be noted that both labeled ddTTP and ddATP were incorporated so that base composition bias would not be a factor in the calculation of the specific activities.

The Template Replicates in the Cairns Type Mode—Early electron microscope analysis of in vivo replication of the plasmid had shown that (i) it replicated in a Cairns type (θ) mode and (ii) the replication was sequentially bidirectional, i.e. the fork progressed from the ori to the Ter site in a unidirectional mode and after the fork was arrested at the Ter, the ori fired in the opposite direction, and the fork went around the circle to meet the stalled fork at the Ter site (1). To investigate further the topology of fork movement in vitro, we constructed plasmid pMA1-DT that had two TerB sites of ori and (ii) the replication was sequentially bidirectional, i.e. the replication bubble could fire unidirectionally to the left (counterclockwise) and get arrested at Ter1 generating a bubble containing a 350-nt long leading strand, generating the Ter1 spot (Fig. 9C). The spot should be located close to the monomer position in the two-dimensional gel pattern (Fig. 9, A and B). Second, a minority of molecules could initiate a leading strand moving clockwise that would stall at Ter2, generating the expected Ter2a spot. Third, fork stalling at Ter1 could induce loading of a second helicase that would cause the second fork to proceed to Ter2, generating a bubble containing a 1200-nt long leading strand located in trans with respect to the 350-nt long first leading strand (Ter2b spot; see Fig. 9, B and C). The Ter2b spot should be more intense than the Ter2A spot because of sequential bidirectional replication in a majority of the molecules. Of course, just from the two-dimensional data, we could not distinguish between simultaneous bidirectional movements form sequential movement of the two forks.

The pMA1-DT template (Fig. 9A) was replicated in vitro with and without the addition of Tus protein at 1.5-fold molar excess over DNA, and the reaction products were linearized by cutting with ScaI and resolved in two-dimensional Brewer–Fangman gels (37). In the absence of Tus protein, monomer length, finished molecules were seen (Fig. 9, C and D). In addition, a clear bubble arc, a double YX arc, and a pattern consistent with a bubble to Y transition were observed in the autoradiograms (Fig. 9C). The template replicated in the presence of Tus protein showed the three expected Ter spots called Ter1, Ter2A, and Ter2B (the spots are designated in italics) on the bubble arc, with traces of Ter2 spots also being present on residual Y arc, that was presumably caused by breakage of some molecules at the replication fork (Fig. 9E). The Ter1 and Ter2b were the major spots, and as expected, the bubble arc between the Ter1 and Ter2 spots showed enhancement of intensity over the remainder of the arc. We note that Ter1 spot was less intense by a factor of ~3, because it contains a smaller labeled leading strand (~350 nt) than Ter2a (which has a ~1200-nt long leading strand). There was no detectable monomer spot, because the double Ter barriers apparently did not allow many forks from completing replication of the full-length template (Fig. 9E). We made sure of the identification of the landmarks by mixing some reaction products from the intermediates generated with Tus and that without the protein (Fig. 9, D and E), cleaved the DNA with ScaI, and resolved it in two-dimensional gels. The results confirmed our interpretation of the locations of the Ter spots to be on the bubble arc (Fig. 9F). The Ter2 spot looks elongated and bilobed, because of the presence of Ter2A and Ter2B spots close to each other.

Our interpretation of the patterns (see Fig 9, A and B) is as follows. Many of the molecules fired leftward, and the leading strand got arrested at Ter1. The first arrest caused the other leading strand to proceed to the right, resulting in molecules arrested at Ter2 (sequentially bidirectional). A significant minority of the molecules fired left to right and got arrested at Ter2 generating the Ter2a spot. The loops generated by the latter molecule should be shorter by ~350 bp from the first type of loops that extends from Ter1 to Ter2 generating the Ter2a spot in two-dimensional gels (Fig. 9F). The location of the Ter spots on the bubble arc and the higher intensity of the bubble arc between the Ter spots are consistent with the formation of a θ-shaped early intermediate.

As noted above, although the two-dimensional gel data could not exclude simultaneous bidirectional movement of the forks as contrasted with sequential fork movement, first counter-clockwise to Ter1 and then proceeding clockwise to Ter2 in the majority of the molecules, taken together with the dideoxy mapping data (Fig. 8), the results were consistent with sequential bidirectional fork movement. Although the classic method of monitoring replication by electron microscopy might be visually pleasing, possible bias caused by selection of molecules that have clear interpretable structures, from many molecules that might not have spread well enough to decipher, could introduce bias in the interpretation. Such bias is greatly reduced in the two-dimensional gel data that represent the entire population of molecules. One potential problem could arise if the two-dimensional gel patterns become too complex causing possible uncertainties in interpretation.

Start Points of Leading Strand Synthesis—We attempted to map the sites of initiation of the leading strand by hybridizing 5′-end labeled primers RGPX1 and GPX2 (see “Material and Methods”) to the reaction products and performing primer extension by linear PCR. The products were resolved in denaturing DNA-sequencing gels with the corresponding dideoxy sequence ladder used as markers (Fig. 10, A and B). The autoradiogram obtained with the primer GPX1 showed that there were multiple points of initiation for the leading strand, located just beyond the dnaA1 box (Figs. 1 and 10B). The major start sites are shown as arrows, and several minor starts are depicted as dots in Fig. 10A. The data from the other primer GPX2 are summarized in Fig. 10B. All show multiple initiation sites located in a limited region located just beyond dnaA1. The data are consistent with a model suggesting helicase loading at the AT-rich region and expansion of the unwound region to the sequences just beyond the dnaA1 site followed by primase loading.

DISCUSSION

The R6K template DNA with all three origins of replication α, β, and γ was previously replicated in crude extracts, and the replication initiated from all three origins of replication with approximately equal frequencies was determined by electron microscopy (9). The replication was dependent on the endoge-
nous $\pi$ protein and was rifampicin-sensitive, thus indicating a role of RNA polymerase in origin activation and/or primer formation (34, 35). Using a partially fractionated extract from a DnaAΔ strain of *E. coli*, we discovered that ori $\gamma$ replication was not only rifampicin-resistant but was stimulated by the RNA polymerase inhibitor suggesting that (i) the priming of replication was probably catalyzed by DnaG primase and (ii) transcription interfered with efficient replication. The results reported here unequivocally show that DnaG primase catalyzed the priming of both leading and lagging strand initiation.

Previous work with the oriC system has shown that either a combination of RNA polymerase and primase or just primase is capable of priming leading and lagging strand synthesis depending on the HU/DNA molar ratios (53, 54).

A second significant point to be unequivocally resolved here is that WT, dimeric forms of $\pi$ were inert and that $\pi^\ast$, which forms monomers upon binding to DNA, was the active form as has been observed in plasmid P1 (55, 56) and in RK2 (57). This question could not have been settled definitively using a crude extract system, which presumably contains chaperons that would monomerize dimeric WT $\pi$. Furthermore, at this time the identities of the chaperons needed for R6K replication remain unknown.

**In vivo** studies have shown that the DNA-bending protein IHF is needed for optimal replication from ori $\gamma$ and from another plasmid ori (22, 24, 58). The data reported here show that the plasmid DNA replicated better with IHF than in the presence of HU and no IHF. It should be pointed out that the $\pi^\ast$ is a looping defective high copy number triple mutant form, and we have shown previously that some of the high copy mutants can bypass the need for IHF (59). The suboptimal replication observed with HU but in the absence of IHF per-
Reconstitution of R6K Replication

45483

haps can be explained on the basis of a partial bypass for IHF requirement. In pSC101, two types of mutations cause bypass of IHF requirement: a missense mutation in the third codon of the plasmid initiator called RepA and mutation in the host-encoded Top1 locus (60, 61). IHF is believed to promote interaction between DnaA and π and between both proteins and the AT-rich region, which is melted in the presence of the three proteins (25). It is conceivable that altered torsional stress caused by alteration in the topoisomerase I concentration in the cell milieu generates alternative conformations that allow helicase loading and replication initiation without IHF. The preinitiation complex consisting of ori γ, π, DnaA, and IHF in which the DNA bending by IHF brings the two DnaA molecules bound to the two dnaA sites into contact with each other and with π bound iterons, and the contact of the protein complex to the AT-rich region probably causes initial melting of the AT-rich region (25) (see Fig. 1B). As described below, we believe that this melting step precedes loading of DnaB helicase at the ori.

The results reported here confirm the observation made with a partially fractionated crude extract replication system that dual initiators DnaA and π are needed for R6K ori γ replication (36). We have shown previously that π physically interacts with DnaA and a mutant form of π, which shows reduced interaction with DnaA, also fails to unwind properly the γ ori (25). We have also reported that the RepA protein of pSC101 interacts with DnaA and DnaB and that a mutant form of RepA, which fails to interact with DnaB, also fails to load DnaB helicase to the plasmid ori (40), despite the known ability of DnaA to load DnaB at ori C (62). Interestingly, ori β, as contrasted with ori α and γ, does not require DnaA for replication (5). Using the reconstituted system, we hope to dissect the mechanism of helicase loading at ori γ, α, and β further in the future.

During the course of this work we detected some DnaA-independent replication that was suppressed by the addition of RNaseH. This observation was also made in the oriC system where RNaseH was identified as a specificity factor (48). Recently, we have reconstituted replication of F factor in vitro and have also observed extensive origin-independent replication that is suppressed by RNaseH. Presumably, RNaseH removes RNA from RNA-DNA hybrids trapped in the DNA that primes replication forks to move in the direction unobstructed by a Tus-Ter complex. In bacteriophage λ, transcription in the vicinity of the ori seems to be responsible for bidirectional fork movement (50). Unraveling the molecular basis of directionality of fork movement in R6K would require further work, using this reconstituted system.

Although the double Ter-Tus experiment reported here provided strong evidence for Cairns type replication initiated from the ori γ, the possibility of other forms (e.g., the σ form) arising later during replication cannot be eliminated at this time because of the complexity of the gel patterns that showed Ys- and X-shaped intermediates.

In conclusion, we have developed a reconstituted system with purified proteins that catalyzes authentic ori specific initiation, elongation, and specific replication termination at Ter sites. We believe that the system is an important landmark in our continuing endeavor toward further mechanistic analysis of initiation and termination of replication in this interesting model system.

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In conclusion, we have developed a reconstituted system with purified proteins that catalyzes authentic ori specific initiation, elongation, and specific replication termination at Ter sites. We believe that the system is an important landmark in our continuing endeavor toward further mechanistic analysis of initiation and termination of replication in this interesting model system.

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The results reported here confirm the observation made with a partially fractionated crude extract replication system that dual initiators DnaA and π are needed for R6K ori γ replication (36). Presumably, RNaseH removes RNA from RNA-DNA hybrids trapped in the DNA that primes replication forks to move in the direction unobstructed by a Tus-Ter complex. In bacteriophage λ, transcription in the vicinity of the ori seems to be responsible for bidirectional fork movement (50). Unraveling the molecular basis of directionality of fork movement in R6K would require further work, using this reconstituted system.

Although the double Ter-Tus experiment reported here provided strong evidence for Cairns type replication initiated from the ori γ, the possibility of other forms (e.g., the σ form) arising later during replication cannot be eliminated at this time because of the complexity of the gel patterns that showed Ys- and X-shaped intermediates.

In conclusion, we have developed a reconstituted system

4 S. Zzaman and D. Bastia, manuscript in preparation.
Reconstitution of R6K Replication

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