Roles of a 106-bp origin enhancer and *Escherichia coli* DnaA protein in replication of plasmid R6K

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Received November 4, 1991; Revised and Accepted January 23, 1992

**ABSTRACT**

A dnaA 'null' strain could not support replication of intact plasmid R6K or derivatives containing combinations of its three replication origins (α, γ, β). DnaA binds *in vitro* to sites in two functionally distinct segments of the central γ origin. The 277-bp core segment is common to all three origins and contains DnaA box 2, which cannot be deleted without preventing replication. Immediately to the left of the core lies the 106-bp origin enhancer, which contains DnaA box 1. When the origin enhancer is deleted, the core alone can still initiate replication if levels of wt π protein are decreased or if copy-up π mutant proteins are provided *in trans*. DnaA does not effect expression of R6K replication initiator protein π, although several DnaA boxes were identified in the coding segment of the plr gene, which encodes π. Together these data suggest that a single DnaA box, 2, is sufficient for initiation from the γ origin and might be sufficient and required for the activity of the α and β origins as well. Implications of the DnaA protein binding to two domains of the γ origin and the role of the 106-bp origin enhancer in replication are discussed.

**INTRODUCTION**

DnaA protein facilitates bidirectional replication from the chromosomal origin oriC *in vivo* (1) and *in vitro* (2–4). Four highly conserved DnaA boxes (5'-TTAT^γ^-CA^γ^-A) are present in oriC of *E. coli* and the chromosomal origins of five other gram-negative bacteria (5). In the current model of the initiation of replication at oriC, DnaA protein binds to these sites (6) and successively melts three 13-mers located in an AT-rich segment (7). It is unclear to what extent, if any, a direct interaction of DnaA protein with the AT-rich oriC segment (8) contributes to the opening of the DNA duplex. Following this step, the DnaB-DnaC complex is guided into the melted region (9, 10) to allow a priming event to occur.

Several plasmids analyzed to date contain DnaA boxes in their replication origins. Not only can some of these plasmids replicate in conditionally lethal dnaA mutants but they are also able to suppress these mutations by integrating into chromosome (11). It was somewhat surprising, therefore, that F and PI plasmids, which are able to facilitate the integrative suppression of dnaA-temperature sensitive strains (11–13), fail to replicate in the dnaA 'null' strains (14).

Wickner *et al.* (15), studying the involvement of DnaA protein in an *in vitro* replication system, proposed that the roles of DnaA in the replication of oriC and plasmid PI probably differ. In addition to the integrative suppression, two biochemical features appear to support this conclusion. First, the levels of DnaA which can activate PI replication are insufficient to activate replication from oriC *in vitro*. Second, the ADP-bound form of DnaA is active in PI replication, while *in vitro* OriC replication requires the ATP-bound form (7).

Plasmid R6K contains three origins of replication, termed α, β and γ. Replication from each origin requires different cis-acting regions (diagrammed in figure 1), but each origin requires the R6K-encoded protein π for replication (16–19). IHF protein is the only host-encoded factor which is able to bind to the γ origin (20) and is required for replication when normal levels of π protein are made (21). In this study we present evidence that the DnaA protein also binds to the central regulatory segment of plasmid R6K (γ origin) and is required for activity of all three plasmid origins under all conditions examined. After completion of this work we learned that the DnaA protein is also essential for replication of R6K γ origin plasmid *in vitro* (22).

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

Bacterial strains and plasmids are listed in Table 1.

**Transformations**

Competent cells were transformed with supercoiled plasmid DNA using the calcium chloride procedure (23).

**DNase I footprinting**

The plasmid pMF34 (24) was digested with EcoRI or Sall. The EcoRI digests were dephosphorylated with calf intestinal alkaline phosphatase and treated with T4 polynucleotide kinase in the
From the other two, would replicate in the absence of DnaA. Thus to inhibitory levels of R6K initiator protein (20, 21); thus it.

IHF, the 7 origin is more sensitive than the other two origins predominantly in the intact R6K (29, 30), this finding could reflect the requirement for DnaA for activity of these two origins but not the central 7 origin. Also, in the absence of the host protein DnaA, we wished to determine if any single origin, separated from the other two, would replicate in the absence of DnaA. Thus presence of γ32P-ATP. The Sall fragments were treated with the Klenow fragment of DNA polymerase I in the presence of α32P-ATP. The EcoRI-labeled fragments were then digested with Sall; the Sall-labeled fragments, with EcoRI. The conditions for footprinting were as described (6). DnaA purified in the laboratory of Dr. A. Kornberg was obtained from Dr. E. Boye. Sequencing reactions (A and G) were performed as described (25). EcoRI was obtained from New England Biolabs; DNase I, from Boehringer Mannheim; all other enzymes were obtained from International Biotechnologies, Inc.

π Immunoassay and plasmid copy number determination
Immunoblotting analysis of π was carried out as previously described (26). Plasmid copy number was determined in cell lysates obtained by the alkaline lysis procedure (27).

RESULTS
Plasmid R6K and its derivatives cannot be transformed into dnaA-'null' mutant cells.
The intact R6K plasmid is unable to transform the dnaA::Tn10 mutant strain AQ3563 (28). Because the α and β origins are used predominantly in the intact R6K (29, 30), this finding could reflect the requirement for DnaA for activity of these two origins but not the central γ origin. Also, in the absence of the host protein IHF, the γ origin is more sensitive than the other two origins to inhibitory levels of R6K initiator protein π (20, 21); thus it is possible that the origins may also differ in their requirement for DnaA. We wished to determine if any single origin, separated from the other two, would replicate in the absence of DnaA. Thus

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Origin(s)</th>
<th>Markers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC177</td>
<td>p15A</td>
<td>PenR KanR</td>
<td>(44)</td>
</tr>
<tr>
<td>pFW8</td>
<td>R6K γ</td>
<td>PenR</td>
<td>this study; see fig. 2</td>
</tr>
<tr>
<td>pFW10, pFW11</td>
<td>p15A</td>
<td>ConR</td>
<td>this study; the pir gene was inserted in either orientation as a BamHI fragment from pMF39 into pACYC184 (44) digested with BamHI</td>
</tr>
<tr>
<td>pMF26</td>
<td>R6K γ-β</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF26 pir113*</td>
<td>R6K γ-β</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF32</td>
<td>pUC, R6K γ</td>
<td>PenR</td>
<td>this study; the truncated R6K γ origin was inserted as a HindIII-BgIII fragment into pUC9 digested with HindIII and BamHI</td>
</tr>
<tr>
<td>pMF33</td>
<td>pUC, R6K γ</td>
<td>PenR</td>
<td>this study; the R6K γ origin was inserted as an EcoRI-BgIII fragment into pUC8 digested with EcoRI and BamHI</td>
</tr>
<tr>
<td>pMF34</td>
<td>pUC, R6K γ</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF35</td>
<td>R6K γ</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF39</td>
<td>pUC</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF30</td>
<td>R6K β</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF50 pir200</td>
<td>R6K β</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF100</td>
<td>pUC, R6K γ</td>
<td>PenR</td>
<td>(24)</td>
</tr>
<tr>
<td>pMF50</td>
<td>R6K α-γ-β</td>
<td>PenR</td>
<td>(24)</td>
</tr>
</tbody>
</table>

*pir113 is a new designation of the previously described pir1, pir104 (24) and pir50 (32) mutations, all of which were revealed by sequencing to contain a Pro to Ser change at the amino acid 113 (J. Hoffman and M. Rlutowicz, unpublished).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genetic markers</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>AQ634</td>
<td>trpA9605 his-29 argH thyA deo metD88 dnanA*</td>
<td>(53)</td>
</tr>
<tr>
<td>AQ3563</td>
<td>dncA450: Tn10 (Tel+) chr pkn500 (kan*)</td>
<td>(53)</td>
</tr>
<tr>
<td>C2110</td>
<td>F&quot; his-&quot; thy-&quot; rha polA</td>
<td>(54)</td>
</tr>
<tr>
<td>p3478</td>
<td>thyA36 deoC2 IN(rnmD-rmF)1 polA</td>
<td>(55)</td>
</tr>
</tbody>
</table>

Table 2. Transformation efficiency of R6K plasmids into dnaA+ and dnaA::Tn10 competent cells.

<table>
<thead>
<tr>
<th>Transforming plasmid DNA</th>
<th>R6K origin</th>
<th>Penicillin G concentration (μg/ml)</th>
<th>AQ634</th>
<th>AQ3563</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6K pirWT</td>
<td>α-γ-β</td>
<td>250</td>
<td>2.2×10^2</td>
<td>0</td>
</tr>
<tr>
<td>pMF26 pirWT</td>
<td>γ-β</td>
<td>250</td>
<td>5.5×10^2</td>
<td>0</td>
</tr>
<tr>
<td>pMF26 pir113</td>
<td>γ-β</td>
<td>250</td>
<td>2.0×10^3</td>
<td>0</td>
</tr>
<tr>
<td>pMF50 pirWT</td>
<td>β</td>
<td>250</td>
<td>7.2×10^2</td>
<td>0</td>
</tr>
<tr>
<td>pMF50 pir200</td>
<td>β</td>
<td>250</td>
<td>4.3×10^3</td>
<td>0</td>
</tr>
<tr>
<td>pFW8 pirWT</td>
<td>γ</td>
<td>250</td>
<td>1.5×10^3</td>
<td>0</td>
</tr>
<tr>
<td>pACYC177</td>
<td>–</td>
<td>250</td>
<td>5.3×10^4</td>
<td>1.7×10^4</td>
</tr>
</tbody>
</table>

R6K and deletion derivatives containing one or two origins were tested for their ability to transform the isogenic AQ634 dnaA+ and AQ3563 dnaA::Tn10 strains. Although R6K derivatives carrying the β origin (pMF50) or the β and γ origins (pMF26) could be established in the dnaA+ strain, no transformants of the dnaA::Tn10 strain could be obtained, even at low (125 μg/ml) penicillin levels (Table 2). Replication of the isolated γ origin

1.5×10^6 dnaA+ and dnaA::Tn10 competent cells were transformed with R6K or the indicated plasmid deletion derivative (0.25 μg of supercoiled, closed circular DNA). Numbers indicate colonies obtained per μg of DNA per 4×10^7 cells plated on antibiotic-containing LB plates supplemented with 5 μg/ml thymidine.
proposed that IHF restrains the negative control of replication alleles. We have examined if R6K derivatives containing copy-up replication in IHF-deficient strains is dependent on the origin (Table 3). We have shown recently (21) that its deletion derivatives can replicate in the absence of DnaA. Transformation data could indicate that neither R6K nor any of the origin fragments for footprint experiments (see Materials and Methods). In plasmid pMF39, EcoRI sites are indicated: H2, HaeIII (-700, 570 and 1961); E, EcoRI (-106); H, HindIII (0); S, SmaI (243); B, BglII (277); F2, FnuDD (325 and 1595); A, AccI (1653). The EcoRI (-106) site is artificial; the BglII (277) site was destroyed in the process of cloning pMF32, pMF33, pMF34, and pMF35; the SalI site in the adjacent polylinker was used in digesting MF34 to obtain origin fragments for footprint experiments (see Materials and Methods). In plasmid pMF39, EcoRI and BamHI sites in the polylinker adjoin each side of the FnuDD fragment containing the pir gene. For descriptions of the orientation of R6K fragments and construction of plasmid pFW8, see Figure 2.

Table 3. Transformation efficiency of R6K γ origin plasmid pMF35 into competent cells carrying resident r-producing plasmids.

<table>
<thead>
<tr>
<th>Transforming DNA</th>
<th>Penicillin G concentration (µg/ml)</th>
<th>AQ634 dnaA*</th>
<th>AQ634 dnaA*</th>
<th>AQ3563 dnaA::Tn10</th>
<th>AQ3563 dnaA::Tn10</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMF35</td>
<td>250</td>
<td>4.0 × 10^2</td>
<td>6.0 × 10^2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pUC9</td>
<td>250</td>
<td>7.2 × 10^2</td>
<td>5.3 × 10^2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1.5 × 10^6 competent dnaA* or dnaA::Tn10 cells carrying the resident r-producing plasmids pFW10 or pFW11 were transformed with 0.25 µg of closed circular plasmid DNA. Numbers indicate colonies obtained per µg of DNA per 4 × 10^7 cells plated on antibiotic-containing LB plates supplemented with 5 µg/ml thymidine.

**Figure 1.** Replication region of E. coli plasmid R6K (not to scale). Double-headed arrows indicate cis-acting DNA segments required for replication from the α, β and γ origins; the location of each Greek letter marks the approximate site of replication initiation for each origin. Dotted line separates the two cis-acting DNA segments required for a origin replication (18). Sites shown here to bind DnaA (DnaA boxes) are labeled 1 and 2 (consensus sequences at −24 to −16 and 247 to 255, respectively). a-i indicate DnaA consensus sequences that do not bind DnaA; dark hatched boxes contain one mismatch from the 9-bp consensus; lighter hatched boxes contain two mismatches underlined (and nt locations) are: a, TTATAAAAC (32-40); b, TTATATTAA (73-81); c, TTGTTCAAA (87-95); d, TTATCTGAA (1301-1309); e, TTATCTAAA (1457-1465); f, TTATCGACA (673-681); g, TTATTCAAA (829-837); h, TTATCAACT (976-984); i, GTATACAAA (1651-1659). Small squares labeled I and H represent IHF binding sites ihfl (which contains two adjacent fflF consensus sequences, 61-73 and 76-88) and ihf2 (251-263) (20, 21). The seven black triangles indicate the 22-bp repeats that bind Bis proteins, respectively. Thin lines at the bottom of the figure indicate the R6K sequences included in various R6K derivative plasmids. Relevant restriction sites are indicated: H2, HaeIII (−700, 570 and 1961); E, EcoRI (−106); H, HindIII (0); S, SmaI (243); B, BglII (277); F2, FnuDD (325 and 1595); A, AccI (1653). The EcoRI (−106) site is artificial; the BglII (277) site was destroyed in the process of cloning pMF32, pMF33, pMF34, and pMF35; the SalI site in the adjacent polylinker was used in digesting MF34 to obtain γ origin fragments for footprint experiments (see Materials and Methods). In plasmid pMF39, EcoRI and BamHI sites in the polylinker adjoin each side of the FnuDD fragment containing the pir gene. For descriptions of the orientation of R6K fragments and construction of plasmid pFW8, see Figure 2.

was tested using constructs that provide the R6K initiator protein in cis (Table 2) or in trans to the origin (Table 3). The transformation data could indicate that neither R6K nor any of its deletion derivatives can replicate in the absence of DnaA.

Next we determined if DnaA, like MF, was involved in control of R6K replication. We have shown recently (21) that γ origin replication in IHI-deficient strains is dependent on α copy-up mutants. These mutants contain single amino acid changes that enable DnaA-independent replication. A derivative of β-γ origin plasmid pMF26 containing the copy-up mutation pirl13 [formerly pirl1 and pirl04 (24)], and a pMF50 (β origin) derivative containing the pir200 allele were thus transformed into AQ634 and AQ3563. As shown in Table 2, none of the pir mutant proteins permitted replication of these R6K plasmids independently of DnaA protein. Thus the data accumulated so far seem to indicate that a bypass of the requirement for DnaA, if it exists, cannot be achieved under conditions that allow bypass of the requirement for IHF.

DnaA binds to two sites within a minimal R6K replicon

A common mechanism by which DnaA protein affects replication involves binding of DnaA to an origin, with consequent effects on DNA structure and/or the binding of other proteins. There...
enhancements require the presence of the downstream DnaA binding sites in DNase I footprinting experiments. DnaA was (8).

oriC region of the 7 origin as it interacts with the AT-rich region of boxes, or if DnaA is specifically interacting with the AT-rich domain punctuated by periodic enhancements is reminiscent of that seen when multiple DnaA monomers bound cooperatively to oriC (6), to a region extending 40 to 50 bp to either side of a DnaA box in pBR322 (6), and 30-bp region which lies between the two promoters for the dnaA gene and surrounds a DnaA box (35).

A 106-bp segment containing DnaA box 1 is not absolutely essential for R6K replication. An experiment aimed to determine which of the two DnaA boxes is essential for R6K replication was to delete each of the segments containing them, and ask if the resulting truncated origins remain functional. In order to determine if DnaA box 1 is essential for replication, we made use of two isogenic plasmids pMF34 and pMF32 (Fig. 1). The former construct contains the entire EcoRI-BglII 383-bp minimal γ origin, cloned into pUC9; the latter lacks the leftmost EcoRI-HindIII 106-bp segment of this origin and hence lacks DnaA box 1. pMF32 and pMF34 plasmids replicate via the pUC origin if and only if DNA Polymerase I is produced.

Figure 2. Construction of γ origin plasmid pFW8. pMF33 was constructed by inserting the EcoRI-BglII (~106 to 277) γ origin fragment into pUC9 digested with EcoRI and BamHI. pMF35 was derived from plasmid pMF33 by deleting the Has2 fragment containing the pUC origin. FnuDII indicates the junction between FnuDII and HindIII sites in pMF39. To make pFW8, the EcoRI fragment of pMF39 containing the pir gene was inserted into the single EcoRI site of pMF35. pFW8 contains the pir gene in cis to the 383-bp γ origin and therefore contains most, but not all, of the DNA segment required for replication from the β origin (17, 19 and F. Wu, unpublished). The β origin requires the structural integrity of the DNA segment between HindIII (0) and the end of the β origin, which has been alternately mapped to AccI (1652) (19 and F. Wu, unpublished) or HaeII (1961) (17). In an intact β origin, the pir gene lies to the right of the γ origin; however, the pir gene in pFW8 lies to the left of the γ origin. Therefore, we believe that pFW8 is replicating as a γ origin and not a β origin.

Table 4. Transformation efficiency of plasmids carrying the truncated and complete γ origins (pMF32 and pMF34, respectively).

<table>
<thead>
<tr>
<th>Transforming DNA</th>
<th>Δ22-pirWT</th>
<th>Δ14-pirWT</th>
<th>Host</th>
<th>Δ22-pir113</th>
<th>Δ22-pir116</th>
<th>Δ22-pir200</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMF32</td>
<td>0</td>
<td>1.6×10^4</td>
<td>4.8×10^3</td>
<td>1.7×10^4</td>
<td>5.8×10^3</td>
<td></td>
</tr>
<tr>
<td>pMF34</td>
<td>3.0×10^2</td>
<td>7.6×10^3</td>
<td>3.8×10^3</td>
<td>3.8×10^3</td>
<td>4.0×10^3</td>
<td></td>
</tr>
</tbody>
</table>

Plasmids pMF32 and pMF34 (0.25 μg supercoiled, closed circular DNA) were transformed into 1.5×10^8 p3478 competent cells. Host cells harbored resident plasmids producing normal levels of wt (Δ22-pirWT), decreased levels of wt (Δ14-pirWT), or normal levels of β mutations (Δ22-pir113, Δ22-pir116, or Δ22-pir200). Numbers indicate colonies obtained per μg of DNA per 4×10^7 cells plated on LB plates containing 250 μg/ml penicillin G and 5 μg/ml thymidine. As a control, no colonies were obtained when pUC9 was transformed into these cells. Also, similar numbers of colonies were obtained when transformed cells were plated onto LB plates containing 125 μg/ml penicillin G and 5 μg/ml thymidine (data not shown).

were two reasons suggesting that DnaA might directly bind to γ origin DNA. First, sequence analysis revealed that the central R6K γ origin contains 5 putative DnaA consensus binding sites (DnaA boxes) with 1 or 2 mismatches (designated 1, a, b, c, and 2 in Figure 1). Second, the previously described γ111rev1 mutation changes the sequence of DnaA box 2 (nt +247 to +255) from CTATCAACA (two mismatches from the consensus underlined) to CTATCAAT (three mismatches). This mutation impairs replication of a γ origin plasmid (33, 34). Thus we asked whether purified DnaA protein could protect any of these putative binding sites in DNase I footprinting experiments. DnaA was found to protect two areas with poorly defined boundaries but clearly identifiable DnaA boxes within each of protected segments. DnaA box 1 TTGTCCACA (~24 to ~16) has one mismatch from consensus (underlined). The DnaA boxes designated a, b and c in Fig. 1 were not seen to be protected in our assay, however, irregularly spaced enhancements can be clearly seen at the left-most boundary of the AT-rich domain (coordinates 0, +1) and well within it (coordinates +34, +39, +46 and +55) (Fig. 3). It is not yet known if these rightmost enhancements require the presence of the downstream DnaA boxes, or if DnaA is specifically interacting with the AT-rich region of the γ origin as it interacts with the AT-rich region of oriC (8).

DnaA box 2 clearly resides within a second protected area (Fig. 4). The segment immediately upstream of DnaA box 2 shows periodic enhancements (coordinates +213, +221 to +222, +232 to +235, and +240 to +243). This large protected region punctuated by periodic enhancements is reminiscent of that seen when multiple DnaA monomers bound cooperatively to oriC (6), to a region extending 40 to 50 bp to either side of a DnaA box in pBR322 (6), and 30-bp region which lies between the two promoters for the dnaA gene and surrounds a DnaA box (35).
concentration is reduced (26). As shown in Table 4, pMF34, which contains a complete γ origin, can be established in all recipients producing either different concentrations of π or mutated π variants. In contrast the plasmid carrying the truncated γ origin, pMF32, can be established only in cells producing π copy-up mutants or decreased levels of wt π. Consequently, we call the 277-bp HindIII-BgII fragment the core origin. A similar overall pattern of transformation frequencies with plasmids pMF32 and pMF34 was observed (data not shown) in the E. coli C background (C2110 polAI) commonly used in our previous studies (26, 33).

Similar attempts to delete the 34-bp SnaBI-BgIII right-most segment of the γ origin, which contains DnaA box 2, failed to yield a functional origin. We could not rescue an active γ origin replicon when this 34-bp fragment was deleted from pMF100 (Fig. 1) in polAI hosts containing helper plasmids producing normal or decreased levels of wt π (M. Filutowicz, unpublished).

There are three major conclusions from these data. First, DnaA box 1 is not absolutely essential for γ origin activity. Second, the sequence requirements for the minimal R6K replicon (γ origin) can be reduced from 383 bp to 277 bp by altering either quantitatively by reducing concentration of wt protein, or qualitatively by providing copy-up π variants. The latter point sheds important new light on the definition of the boundaries of R6K origins and allows us to reinterpret some puzzling observations published in the past (see Discussion). Finally, the γ origin requires the 34-bp fragment containing the DnaA box 2.

Figure 4. Footprint of the R6K γ origin using DnaA protein and the Sall-labeled EcoRI-Sall fragment of pMF34. Lanes 1, G+A sequencing; lane 2, DNasel control; lane 3, 0.14 μg DnaA; lane 4, 0.07 μg DnaA. DnaA box 2 is bracketed; numbers indicate nt location; (+) indicates an enhancement of cleavage in the presence of DnaA; (−) indicates protection.

DnaA does not alter π production

Several putative DnaA boxes within the pir-bis operon have been identified by computer analysis of the available R6K sequence (Figure 1). Two boxes (d and e) lying within the pir coding segment contain one mismatch from the DnaA consensus; two additional sequences (f and g) each contain two mismatches. The bis gene (17, 19, 36) also contains two putative DnaA boxes (h and i), each having one mismatch from the DnaA consensus sequence. Because DnaA is able to bind to regulatory sites containing DnaA boxes and thereby mediate transcriptional repression of certain genes (35, 37–40), we asked if DnaA protein could also influence the expression of π protein. If it could, this second regulatory circuit, in addition to autoregulation...
Wt Tl —

dnoA
gene expression, the levels of and protein in contains a site, DnaA box 1, to which DnaA protein binds

Instead, we propose that the major role of DnaA is binding to

a origin by two lines of evidence. First, the 106-bp fragment containing

a single site, DnaA box 2, in a 277-bp fragment common to all

production.

deficient strain cannot be explained by an effect of DnaA on

T

the inability of R6K or its derivatives to replicate in the DnaA-

little or no effect on the expression of the pir gene. Therefore,

plasmid copy number (data not shown). Hence, when

gene, was four- to five-fold lower in the absence

of DnaA than in its presence; w production was proportional to

that the copy number of plasmid pMF39 (24), a pUC-derivative

were similar (Fig. 5A). The copy number of each plasmid was

cloning strategy). Immunoblotting assays demonstrated that the

plasmids were measured by immunoblotting. These plasmids

covalently closed circular) DNA bands are indicated.

(41—43), would help in preventing the intracellular concentration from climbing to levels high enough to inhibit replication (26).

To test the possible involvement of DnaA in the regulation of pir gene expression, the levels of protein in dnaA* and

isogenic dnaA ‘null’ cells harboring pFW10 or pFW11 helper plasmids were measured by immunoblotting. These plasmids contain the pir gene, including its regulatory sequences, cloned in either orientation into the pACYC184 vector (See Table 1 for cloning strategy).

Immunoblotting assays demonstrated that the levels of produced from these plasmids in AQ634 and AQ3563 were similar (Fig. 5A). The copy number of each plasmid was also nearly equivalent in both strains (Fig. 5B). We also found that the copy number of plasmid pMF39 (24), a pUC-derivative carrying the pir gene, was four- to five-fold lower in the absence of DnaA than in its presence; production was proportional to plasmid copy number (data not shown). Hence, when synthesis is uncoupled from R6K replication, DnaA protein appears to have little or no effect on the expression of the pir gene. Therefore, the inability of R6K or its derivatives to replicate in the DnaA-deficient strain cannot be explained by an effect of DnaA on production.

DISCUSSION

Plasmid R6K requires DnaA for replication, because neither R6K nor deletion derivatives could replicate in a dnaA:/:7n10 strain (28 and this study). Another, less likely, interpretation of these transformation data is that DnaA may be required not for the replication but for the establishment of these plasmids. DnaA does not alter the level of production of R6K initiator protein . Instead, we propose that the major role of DnaA is binding to a single site, DnaA box 2, in a 277-bp fragment common to all three R6K origins. The adjacent 106-bp origin fragment also contains a site, DnaA box 1, to which DnaA protein binds in vitro. The central role of DnaA binding to box 2 is supported by two lines of evidence. First, the 106-bp fragment containing DnaA box 1 is not required for replication of the R6K origin

(18) or the origin (17, 19). We show here that it is also not necessary for replication of the origin when certain mutants or decreased levels of wt are provided in trans. Implications of these observations will be discussed later. Second, point mutations in the DnaA box 2 that increase or decrease the number of matches to the 9-bp DnaA consensus sequence respectively increase or decrease the copy number of origin plasmids. The previously described 111rev1 mutation changes the sequence of the DnaA box 2 from two mismatches to three mismatches. This mutation impairs R6K replication, reducing the copy number of plasmids replicating from the origin replicating either the wt origin or a origin mutated only in the ihf2 (S. Dellis, T. Schatz and M. Filutowicz, unpublished data).

The role of the 106-bp origin enhancer in origin replication

In this paper we present evidence that the 383-bp origin can be functionally separated into at least two domains: the core origin, which is essential for replication under all conditions so far tested; and the adjacent 106-bp segment, which we call the origin enhancer, which is required for replication of the origin when normal levels (4000—10,000 dimers per cell) (26) of wt are provided in trans. However, the core origin is able to replicate under two conditions: (1) when decreased levels of wt are supplied or (2) when certain mutants are provided in trans. These two conditions allow an increase in origin plasmid copy number over that seen when normal levels of wt are provided, consistent with previous observations indicating that normal levels of wt partially inhibit replication; this inhibition is apparently counteracted by the enhancer.

A role for the enhancer is suggested by an earlier finding that the origin can function when the transposon Tn5 is inserted between the core origin and the enhancer, or within the enhancer itself (45). We propose that the outer end of the transposon may functionally substitute for the enhancer by providing a perfect DnaA box (TTATACACA) (46). Alternatively, or in addition to this, the enhancer may be the origin adopt an unusual structure which permits replication at inhibitory levels of wt. It has been shown (47) that IHF can fold the origin only when the enhancer and the seven repeats are present. Like the enhancer, IHF helps restrain the inhibitory effect of wt (21). Thus an IHF-enhancer nucleoprotein structure may provide a mechanism for alleviating inhibition of replication. Finally, IHF binding may allow long-range interactions between DnaA molecules bound to DnaA box 1 in the enhancer and DnaA box 2 or DnaA and protein. These putative DnaA-IHF-p interactions may be similar to those seen in vitro in the pSC101 origin (48).

The findings reported here also cast new light on previous accounts of origin incompatibility (49). Origin replication requires a cluster of repeats that act as binding sites for , but an extra set of repeats provided in cis may inhibit replication, causing incompatibility depending on location and orientation. molecules can mediate coupling of two repeats clusters on the same plasmid (50), looping out the intervening DNA segment. Like plasmids carrying the core origin, origin plasmids containing an extra set of repeats on the enhancer side of the origin cannot replicate when normal levels of wt are provided.

Figure 5. A, immunoblot assays showing levels of produced by plasmids pFW10 and pFW11 in AQ634 and AQ3563. Two bands crossreacting with IgG's correspond to the 305-amino acid full-size polypeptide (wt ) and 206-amino acid short protein variant (wt*) produced from a second translational start point within the pir gene (D. York, J. Hoffman, J. Gan and M. Filutowicz, ms. in preparation). From the somewhat higher level of produced by pFW11 in AQ634, we suspect that transcription from the TetR gene in the vector may in part override autoregulation of the pir promoter. B, levels of plasmids pFW10 and pFW11 in AQ634 and AQ3563. Chromosomal and plasmid (OC, open circular; CCC, covalently closed circular) DNA bands are indicated.
in trans, but can when decreased levels of wt π, or π copy-up
mutants are provided in trans. Therefore, it seems possible that
cis-association or ‘handcuffing’ of repeats (49) may inhibit
replication not by sterically hindering access of replication
proteins to the origin, as previously proposed (49); rather
handcuffing may prevent replication by looping out, distorting
or constraining the enhancer segment and AT-rich region of the
γ origin.

Finally, the finding that the enhancer is not required for γ origin
replication brings into question the traditional definition of the
β origin. While the γ origin was believed to require the enhancer
(16), the β origin was believed to contain the DNA segment
extending from the core origin rightward (17, 19; see Fig. 1).
Our finding that certain levels of π allow replication of the core
origin suggests that the boundaries of the β origin need to be
reconsidered, using plasmid constructs producing quantified levels
of π.

ACKNOWLEDGEMENTS
We thank E. Boye for DnaA protein and B. Kline for bacterial
strains. Ilya Goldberg was a participant in an honors thesis
program in the Department of Biochemistry, University of
Wisconsin, Madison. This research was supported by grants to
M.F. from NIH (GM40314) and NCS (IN-35-30-24).

REFERENCES
5817–5821.
Acad. Sci. USA 78, 7370–7374.
5. Zyskind, D. W., Cleary, J. M., Brusilow, W. S. A., Harding, N. E. and
161–173.
Sci. USA 81, 6456–6460.
Sci. USA 83, 4423–4427.
Chem. 265, 11622–11627.
17. MacAllister, T. W., Kelcy, W. L., Miron, A., Stenzel, T. T. and Bastia,
Acad. Sci. USA 83, 9645–9649.