Replication of a Plasmid Lacking the Normal Site for Initiation of One Strand

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The origin of replication of the plasmid R1162 contains an initiation site for the synthesis of each DNA strand. When one of these sites (oriL) is deleted, synthesis on the corresponding strand is no longer initiated efficiently in vitro by the R1162-encoded replication proteins, and the plasmid is no longer stably maintained in the cell. However, in vivo the two strands of the plasmid duplex molecule are active at a similar level as templates for DNA synthesis, and newly synthesized copies of each strand are incorporated into daughter molecules at a similar rate. No secondary, strong initiation sites on the ΔoriL strand were detected in the region of the origin. The ΔoriL plasmid induces the SOS response, and this is important for plasmid maintenance even in a recombination-proficient strain. Our results indicate that an SOS-induced host system can maintain an R1162 derivative lacking one of its initiation sites.

For the broad-host-range plasmid R1162 (or the nearly identical RSF1010), replicative synthesis of daughter strands depends on three plasmid-encoded proteins and is initiated at two oppositely facing sites (oriL and oriR) within the origin of replication (19). The two sites are essentially equivalent and interchangeable and can function independently to prime complementary strand synthesis of an M13 derivative (14). Plasmid derivatives that lack either of these sites are unstable, and in cell extracts containing the R1162 replication proteins, there is preferential synthesis of the DNA strand initiated from the remaining site (31).

We have characterized in greater detail the properties of R1162 replication when oriL is deleted. Our observations indicate both DNA strands, the one initiated from the remaining, normal origin and the one without its usual initiation site, are incorporated into daughter molecules at a similar rate. The back-up replication system is likely to be host determined, since it is stimulated by induction of SOS.

MATERIALS AND METHODS

Bacterial strains and plasmids. The Escherichia coli K-12 strains used in this study are given in Table 1. Plasmids are all derivatives of the naturally occurring IncQ plasmid R1162 (5). Sketches indicating significant restriction sites and structural differences are shown in Fig. 1. Plasmid pUT1101 is identical to R1162, IncQ plasmid R1162 (5). Sketches indicating significant restriction sites and study are given in Table 1. Plasmids are all derivatives of the naturally occurring

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CsCl-ethidium bromide centrifugation (26). After visual identification and collection of the DNA bands, the samples were extracted with CsCl-saturated isopropanol, dialyzed against TES, and precipitated with ethanol. Primer extensions were carried out with thermocycling. Reaction mixtures consisted of 30 ng of DNA (\textit{Eco}RI digested), 12 pmol of oligonucleotide primer (20-mer, end-labeled with T4 polynucleotide kinase and \[^{32}\text{P}\]dATP), 375 pmol of each deoxynucleoside triphosphate, and 5 U of \textit{Taq} DNA polymerase (Promega), together in 15 \(\mu\)l of buffer (25 mM Tris HCl [pH 9], 2 mM MgCl\(_2\)). Reactions were carried out at 95\(^\circ\)C for 30 s and 70\(^\circ\)C for 30 s (30 cycles). Prior to addition of the enzyme, samples were heated at 95\(^\circ\)C for 5 min.

Sequencing ladders were prepared similarly, except that the source of the template was supercoiled plasmid DNA isolated by the alkaline method, and the reaction mixtures contained the nucleotide mixtures provided by Promega.

### RESULTS

There are no efficiently utilized, secondary sites for \textit{R1162}-specific initiation on the strand containing \textit{oriL}. Previous observations on the effects of deletions in the \textit{R1162} (and \textit{RSF1010}) \textit{oriV} have been made on substantially modified plasmid derivatives lacking large amounts of \textit{R1162} DNA and including DNA from other sources (27, 31). We first asked whether one of the initiation sites in an essentially intact plasmid can also be deleted, and if this results in the defective replication and instability characteristic of these derivatives. Plasmid \textit{pUT1101} (Fig. 1) is identical to \textit{R1162}, but contains point mutations in the origin of replication (\textit{oriV}). These mutations, which do not affect replication of the plasmid or its stability (19, 31) (Table 2), create a \textit{SmaI} site between \textit{oriL} and \textit{oriR} and an additional \textit{EcoRV} site. An 85-bp fragment containing \textit{oriL} was deleted by digestion with \textit{EcoO109} and \textit{SmaI}; the resulting plasmid, \textit{pUT1110}, could be maintained in the cell but was unstable (Table 2).

![Diagram](https://example.com/diagram.png)

**FIG. 1.** \textit{R1162} and derivatives used in this study. The drawings are not to scale; the region containing the origin of replication has been enlarged to show detail. Arrowheads indicate direction of synthesis from \textit{oriL} and \textit{oriR}. Filled rectangles designate binding sites for Rep\textit{C} protein. Cm\(^r\) indicates DNA encoding resistance to chloramphenicol. Abbreviations for restriction enzyme sites are Bt (BstNI), Ec (\textit{EcoO109}), K (KpnI), N (\textit{BamH}I), P (PstI), R (\textit{EcoRI}), Rv (\textit{EcoRV}), Sm (\textit{SmaI}), and X (\textit{XhoI}). Triangles, site of deletion of \textit{oriL}; diamond, base pair mismatch; open rectangles, \textit{oriL} and \textit{oriR}; DR, direct repeat.

### TABLE 1. Strains used in this study

| Strain | Properties | Source or reference |
|--------|------------|--------------------|
| MV10   | F\(^{-}\) thr-1 leuB6 lacY1 thi-1 rfbD1 supE44 thrA6 deoC1 | 12 |
| CR34   | F\(^{-}\) thr-1 leuB6 lacY1 thi-1 rfbD1 supE44 thrA6 deoC1 | 2 |
| AB1157 | F\(^{-}\) λ\(^{-}\) thr-1 ara-14 leuB6 Δ(gpt-proA) 62 lacY1 tpx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mit-1 argE3 thi-1 | 2 |
| DM49   | AB1157 lacA3 | D. Mount (23) |
| JC9239 | F\(^{-}\) recF143 thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tpx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mit-1 argE3 thi-1 | J. Clark |
| JH137  | λ\(^{-}\) HfrC pheA(Am) lac Z dinD1::MudI (Amp\(^{r}\) lac) | P. Model (11) |
| GR501  | λ\(^{-}\) lig-251 (ts) relA1 spoT1 thi-1 | 7 |
We compared the replication of R1162 and pUT1110 DNA in a cell extract containing the R1162 replication proteins (19, 25). After incubation in the presence of $^{35}$S-dATP, supercoiled DNA was purified by CsCl-ethidium bromide ultracentrifugation. The DNA was then digested with BanII and EcoRI, the resulting small fragment was denatured, and the two, differently sized strands (205 and 213 bases) were separated by polyacrylamide gel electrophoresis. The labeled DNA, visualized by autoradiography, is shown in Fig. 2. The two strands of R1162 DNA (Fig. 2, lane a) have different intensities in the autoradiogram because there are 64 dAMPs in the smaller strand and 47 dAMPs in the larger strand. When pUT1110 DNA was replicated in the extract, there was much less label in the smaller strand, which is normally initiated from oriL (Fig. 2, lane b). Thus, on the strand containing oriL, there are no efficiently utilized, alternative sites for initiation of R1162 replication by the plasmid-specific mechanism. Synthesis on this strand in vivo could be the result of initiation either by the R1162 replication proteins at sites that are poorly recognized or by a second, host-specified system with low activity in the extract.

**TABLE 2. Stability of R1162 and derivatives**

| Host and plasmid | % plasmid-containing cells after growth generation$^a$: |
|------------------|--------------------------------------------------------|
|                  | 0 20 40 60                                             |
| MV10             |                                                        |
| R1162            | 100 100 94 98                                         |
| pUT1110I         | 100 100 100                                          |
| pUT1110          | 82 63 20 12                                          |
| pUT1385          | 100 100 99 98                                         |
| pUT1386          | 94 40 9 1                                            |
| pUT1296          | 100 98 97 97                                         |
| pUT1416          | 95 83 79 72                                         |
| MV12 (MV10 recA456) |                                                    |
| pUT1385          | 100 100 99                                          |
| pUT1386          | 52 0 0 0                                            |
| AB1157           |                                                        |
| pUT1385          | 100 100 100                                          |
| pUT1386          | 94 8 4 4                                            |
| DM49 (AB1157 lexA3) |                                                   |
| pUT1385          | 100 100 92                                          |
| pUT1386          | 44 0 0 0                                            |

$^a$ One hundred colonies tested for each time point.

$^b$ —, not done.

Daughter strands are initiated at a similar frequency in vivo for the ΔoriL plasmid. R1162 derivatives lacking oriR or oriL were previously estimated to be present in the cell at about half the copy number of similar derivatives containing the intact origin (31); the copy number of pUT1110 was also reduced by no more than this amount (data not shown). Nevertheless, synthesis of the two strands of pUT1110 could be initiated in vivo at very different frequencies. Initiation at oriR could occur many times during the period of one cell generation, whereas initiation of complementary strand synthesis could take place much less frequently and at a rate determining the final copy number in the cell. Indeed, for RSFI010 derivatives lacking an initiation site, single strands initiated from the remaining ori have been observed to accumulate in the cell (13). Moreover, replication of the complementary strand could occur either on duplex molecules or on members of the pool of single strands synthesized in excess from the remaining origin.

To estimate the relative initiation rate of new strands appearing in daughter molecules, we labeled R1162 and pUT1110 plasmid DNA for small fractions of a generation in a growing culture and then determined the relative amounts of radioactivity in each strand. Log phase cells of thymine-requiring CR34 strains (2) containing either R1162 or pUT1110 were incubated in minimal-Casamino Acids medium (10) at 25°C in the presence of [3H]thymidine. After 5, 10 and 30 min, cells were sampled and quick-frozen in the presence of sodium azide, and the supercoiled plasmid DNA was extracted (21). The DNA was digested with BanII and EcoRI, and the two, differently sized strands of the resulting small DNA fragment were denatured, separated by polyacrylamide gel electrophoresis, and visualized by autoradiography. The two strands of R1162 DNA (Fig. 3, lanes a to c) have different intensities in the autoradiogram because there are 67 dTMPs in the larger strand and 44 dTMPs in the smaller strand. However, the relative amount of label in each strand was not noticeably changed for pUT1110 DNA (Fig. 3, lanes d to f). Thus, asymmetry in labeling due to an absent initiation site was not observed by this procedure, even at times that were short relative to the generation time under these conditions (approximately 90 min).

The similar initiation potential on each strand of the plasmid molecule was also indicated by a density-shift experiment. CR34 cells containing R1162 or pUT1110 were grown in the presence of [3H]thymidine to uniformly label the DNA, and they were then washed and divided into medium with or without 3 μg of bromodeoxyuridine per ml. After further incubation at 37°C for 40 min, about one generation, the supercoiled plasmid DNA was isolated and fractionated by density on a CsCl gradient. In each case, virtually all the radioactive label
appeared as a discrete peak in the gradient, at the position of hybrid density (Fig. 4A). The plasmid DNA in these peaks was recovered, and the radioactive label in the two strands of the small EcoRI-BamHI fragment was analyzed as before. The amount of label in each strand was similar for both R1162 and pUT1110 (Fig. 4B). If a large number of strands were synthesized on the oriR template and released from the duplex DNA, and these strands were then chosen randomly, at a lower probability per molecule, for synthesis of the complementary strand, then most of the radiolabel in the molecules with hybrid density would be in the strand containing oriR. This is because some of the radioactively labeled, complementary strands would not be selected for replication from the large, single-strand pool.

The labeling experiments might be misleading if pUT1110 DNA was in the form of dimers and higher multimers or was replicated by a rolling circle mechanism. In this case, an unequal potential for initiation could still result in a similar rate of appearance of newly synthesized strands in daughter molecules, since a single (infrequent) initiation on the oriL template could then generate multiple, unit-length copies of one strand. We did not observe a large population of multimers for pUT1110 (data not shown), but it was possible that a small pool of such molecules was replicated preferentially. We therefore also used another assay to measure the relative initiation frequency of each daughter strand. Plasmids pUT1385 and pUT1386 (Fig. 1) contain a 950-bp DNA fragment derived from pBR325 and encoding chloramphenicol resistance (6), but they are otherwise identical to the respective parental plasmids pUT1101 and pUT1110. The additional DNA does not contain a primosome assembly site (pas) or other known sites for initiation of replication (3, 24), and as a consequence it does not increase the stability of the plasmid (Table 2). The apparent copy number of pUT1386 is also less than that of pUT1385 (Fig. 5, lanes a and b) and is similar to that of other ΔoriL plasmids (31).

We inserted at the EcoRI site of pUT1386 a 37-bp, chemically synthesized DNA fragment (see Materials and Methods) that provides unique sites for the restriction enzymes KpnI and XbaI. Plasmid DNA was digested with these enzymes and then ligated with annealed oligomers containing a single base mismatch. The DNA was then used to transform JC9239, a strain containing the recF143 allele and therefore deficient in nonmethyl-directed mismatch repair (9). The oligomers were designed so that replication after transformation would result in two different classes of daughter molecules, one with a Smal site and the other with a BsrNI site in the cloned DNA. If each strand of pUT1386 was used with equal frequency as a template for replication, then both kinds of daughter molecules would be equally frequent in the population of transformed cells. In contrast, if the strand lacking its normal origin was poorly utilized as a template, then the marker restriction site on that strand, the BsrNI site, would appear less frequently among the progeny molecules.

The results following the analysis of 43 independently derived colonies of transformed cells are shown in Table 3. There was an almost equal number of colonies containing plasmids with either the BsrNI site or the Smal site. Moreover, because of the combination of restriction sites used, it is unlikely that such molecules would have arisen from head-to-tail multimers generated during the ligation. Thus, after transformation the probability of initiation on each strand was similar.

R1162 ΔoriL induces the SOS system, which is required for maintenance of the plasmid but also results in plasmid instability. The recA56 mutation, which inactivates all the major activities of RecA (16), causes the more rapid loss of pUT1386 but has no effect on pUT1385 (Table 2). RecA could stabilize pUT1386 by permitting a population of dimers and higher multimers. For such a population, a single initiation on the

| Expt | No. of colonies of transformed cells with cleavage pattern: |
|------|----------------------------------------------------------|
|      | A        | P        | S        | B        | S + B  |
| 1    | 11       | 11       | 10       | 9        | 2      |
| 2    | 6        | 7        | 1        | 0        | 0      |
| 3    | 5        | 5        | 3        | 2        | 2      |
| 4    | 1        | 0        | 1        | 6        | 5      |
| Total| 4        | 11       | 11       | 10       | 9      | 2      |

*Plasmid types are Δ (deletion across cloning site), P (parental molecule), S (Smal site present), B (BsrNI site present), and S + B (both Smal and BsrNI sites present).
defective strand would replicate more than one monomeric equivalent, resulting in a higher apparent unit copy number (31). However, as indicated earlier, a large population of dimers has not been detected. In addition, pUT1386 but not pUT1385 is destabilized by the lexA3 (Ind^-) allele (23), which permits normal RecA-mediated recombination (Table 2). This suggests that SOS is induced in pUT1386-containing cells and is important for maintenance of the plasmid.

We used the reporter strain JH137 (Table 1) to test for induction of SOS. There were 8 U (22) of β-galactosidase in the uninduced strain; in a fully induced strain (1 mg of mitomycin C per ml for 30 min), the enzyme activity increased to 51 U (all values are the averages of two independent assays; 1 standard deviation is <2 U). JH137(pUT1385) produced only 7 U, similar to the plasmid-free strain and significantly lower than the 18 U for JH137(pUT1386). These data indicate that the ΔoriL derivatives cause chronic induction of SOS, which could contribute to maintenance of the plasmid.

The instability of the ΔoriL plasmid pUT1386 (Table 2) is inconsistent with its apparent copy number. This is demonstrated by the behavior of pUT1396 and pUT1416 (Fig. 1), plasmids that contain additional binding sites for RepC, one of the R1162-encoded replication proteins (25). These additional sites reduce the copy number of the plasmids, presumably by titrating the RepC in the cell (18). Plasmid pUT1396, with two additional sites, has a lower copy number than pUT1386 (Fig. 5, lanes b and c) but is stably maintained in the cell (Table 2). Instability is detectable only when there are four cloned binding sites and the copy number is further decreased (Fig. 5, lane d and Table 2). These results indicate that copy number by itself is not responsible for the instability of pUT1110 and pUT1386. It is likely that plasmid instability is a consequence of SOS induction. Because of this induction, division of plasmid-containing cells is inhibited (28), and plasmid-free, uninduced cells are enriched in the population. This would also explain why the copy number of pUT1386 (Fig. 5, lane b) appears lower than would be expected from a similar rate of initiation on each strand. Within a growing culture, cells would appear containing the reference plasmid but not the R1162 ΔoriL derivative.

There are no strong initiation sites on the ΔoriL template in the region of the plasmid origin of replication. Strand synthesis initiated at oriR results in a D-loop extending counter-clockwise (as the plasmids are drawn in Fig. 1). Normally, a second initiation occurs at oriL, within the expanding region of single-stranded DNA (19). We asked whether another initiation site is unmasked in this region when oriL is deleted.

**FIG. 5.** Relative plasmid copy numbers of pUT1385 (a), pUT1386 (b), pUT1396 (c), and pUT1416 (d). The reference plasmid, also present in each strain, is pWSK29 (29), a pSC101 derivative with a copy number of six to eight per cell. Prior to electrophoresis plasmid DNA was digested with EcoRI, which cleaves pWSK29 once, and with the other plasmids twice.

GR501 (lig^-ts) strains (7) containing pUT1385 or pUT1386 were grown at 30 and 42°C, and plasmid DNA was isolated by a neutral lysate procedure (see Materials and Methods). This DNA was then fractionated into open circular and covalently closed forms by ethidium bromide-CsCl ultracentrifugation. The locations of nicks or gaps in the open circular molecules were determined by primer extension analysis with a sequencing ladder generated with the same primer and the supercoiled plasmid template.

When plasmid DNA isolated from cells grown at 30°C was used as template in the primer extension, most of the product was too large to be resolved on the sequencing gel (Fig. 6, lanes a and c). However, DNA from cells grown at 42°C resulted in...
extended primers with low molecular weight, indicating the presence of numerous nicks due to the defective DNA ligase. In agreement with this, pUT1386 shows a similar degree of instability in MV12 (recA) and DM49 (lexA3 but Rec+).

Induction of SOS in the cell by single-stranded forms of replisomes is generally deleterious, and mechanisms have evolved to prevent its occurrence. During conjugal transfer of the F factor, SOS induction by the single-stranded transfer intermediate is inhibited by products of plasmid-encoded genes (4). However, for the broad-host-range plasmid R1162, transient induction of SOS might favor plasmid transmission and establishment. R1162 encodes several of the proteins required for its replication (25), and this allows a greater measure of independence from the host cytoplasm. This also means that when the plasmid enters a cell, these proteins must all be synthesized sufficiently rapidly to allow successful establishment. Recruitment by the plasmid of a supplementary host mechanism for synthesis could increase the chance of establishment during this time, when the concentrations of these proteins are still low. This is particularly true during conjugal transfer, when plasmid transmission generates a single-stranded intermediate in the recipient member of the mating pair (and perhaps in the donor).

DISCUSSION

Although R1162 DNA is normally replicated by synthesis of each DNA strand from its own initiation site within the origin, plasmid derivatives lacking one or the other of these sites have been isolated (31). We have characterized the effect of deleting one of these sites from an otherwise intact plasmid. Initiation of synthesis on the ΔoriL template by the R1162-specific replication proteins is inhibited, since there is no efficiently utilized site for the plasmid-encoded primase. Nevertheless, during replication in the cell newly synthesized copies of both strands appear in daughter molecules at a similar rate (Fig. 3 and Table 3). Furthermore, each strand of the duplex parental molecule appears to be used equally well as template (Fig. 4).

Deletion of oriL induces the SOS system of repair, which contributes to plasmid maintenance. Replicative intermediates of R1162 would consist of two D-loops and therefore contain substantial amounts of single-stranded DNA. If the synthesis of each strand was initiated nearly simultaneously, then the lifetime of these intermediates would be short. However, when one of the initiation sites is deleted, synthesis of the affected strand might sufficiently lag behind the other so that a replicative intermediate is generated with a lifetime long enough to induce the SOS system. In the extreme case, single strands of plasmid DNA, initiated from the remaining origin, would be released into the cell, and these have been detected by others for RSF1101 (13). Induction of SOS in cells containing pUT1386, despite the high potential for initiation on the strand with the deleted origin, indicates that in the intact plasmid, initiation from oriL and oriR is closely balanced.

The single-strand initiation sites in RSF1101 derivatives can be replaced by primosome assembly sites from ϕX174 or pACYC184 (15). We have similarly found that a properly oriented pas from pBR322 restores the stability of pUT1101 (unpublished results). Therefore, synthesis on the ΔoriL template does not involve an efficiently utilized pas. Initiation of synthesis on the ΔoriL template might involve a mechanism similar to SOS-inducible replication of the E. coli chromosome (ISDR) (20), which also does not appear to involve canonical primosome assembly sites (1) and is recA dependent. The recombinase activity of RecA is required for ISDR, probably to generate regions of single-stranded DNA necessary for initiation.

Replication of the ΔoriL R1162 derivatives should not require recombination, since the single-stranded DNA would be formed by initiation from oriR. In agreement with this, pUT1386 shows a similar degree of instability in MV12 (recA) and DM49 (lexA3 but Rec+).

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