DNA synthesis is initiated at two positions within the origin of replication of plasmid R1162

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ABSTRACT

DNA synthesis of broad host-range plasmid R1162 is initiated from two positions, flanking a large (40 bp stem, 40 bp loop) inverted repeat. Each start-point is located within a highly conserved, but oppositely oriented, 10 base-pair sequence. Synthesis from the two positions converges within the intervening inverted repeat. An analysis of deletions suggests that both start positions must be present for synthesis. A model describing possible early events in replication of plasmid R1162 is presented.

INTRODUCTION

In common with replicons generally, broad host-range plasmid R1162 (1) contains a region required in *cis* for replication of its DNA (2). This region maps with the origin of DNA replication that was determined by electron microscopy (3) for the plasmid RSF1010, which is very similar or identical to R1162. Dissection of the origin region by molecular cloning has revealed that the DNA required in *cis* is located within two adjacent HpaII restriction fragments of 210 and 370 bp (Fig. 1) (2). Although these fragments are both required, they may be inverted or separated from each other. Thus, each fragment contains a separate essential domain.

The domain within the 370 bp HpaII fragment contains 3 1/2, 20 bp direct repeats that are required both for replication and for expression of plasmid incompatibility (4). Similar direct repeats are found in other, unrelated replicons, and serve as targets for binding of plasmid-specific proteins for replication (5-8). It is likely that they function similarly in R1162. The base sequence within the 210 bp HpaII fragment includes a large inverted repeat with a 40 bp stem and 40 bp loop (2). We show here that DNA synthesis is initiated at each of the outer ends of this inverted repeat, at a conserved (9/10 bp) sequence. Our results indicate that replication of R1162 DNA requires initiation of DNA synthesis from both these positions.

MATERIALS AND METHODS**Plasmids, bacterial strains and bacteriophage**

A map of R1162, and sketches of the derivatives used in this study, are given in Fig. 1. The derivatives have a common BamHI - EcoRI segment,

containing genes encoding proteins required for maintenance of R1162 in the cell (RepI and RepII), and also a gene encoding kanamycin-resistance (Km). The fragment consists of R1162 DNA extending rightward on the R1162 map from a HaeII site at 0.7 kb to an MboI site at 5.7 kb. The MboI end reforms a BamHI site when joined to BamHI-cleaved DNA. At the HaeII end, the DNA is linked to a HaeII-generated portion of an EcoRI fragment derived from R6-5 (9) and containing Tn903, a transposon encoding kanamycin-resistance (10). The junction lies within the transposon, so that it is no longer active, but the Km gene is intact. The plasmids also contain the HpaII fragments required in cis for replication. The 210 bp HpaII fragment was bracketed with EcoRI and HindIII restriction sites by first cloning this DNA into the ClaI site of pBR322 (11), and then excising the small EcoRI - HindIII fragment from the resulting plasmid. The 370 bp HpaII fragment was cloned by replacement of the small ClaI - BamHI fragment of pBR322 with a HpaII - MboI fragment (an MboI site is located at 6.51 kb); the BamHI site is reconstructed in this cloning. The EcoRI polylinker from M13mp7 (12) was then cloned into the resulting plasmid at the EcoRI site, and the 370 bp fragment excised by BamHI digestion. The BamHI fragment containing the 210 + 370 bp fragment was prepared similarly, except that a HpaII + MboI fragment made up of both the 210 and 370 bp fragments was used in the initial cloning into pBR322. In most of the plasmids, the 210 and 370 bp HpaII fragments are separated by 130 bp DNA. This DNA is derived from portions of pBR322 and the EcoRI fragment from R6-5; it does not contain DNA involved in the replication of either plasmid.

All plasmids were constructed by standard cloning procedures. Plasmid DNA was prepared from strains of *E. coli* MV10 (thr leu thi lacY supE44 fhuA ΔtrpE5) (13) by the method of Holmes and Quigley (14) or Marko *et al.* (15), treated with restriction endonucleases and T4 polynucleotide ligase obtained from commercial sources, and used to transform MV10 by the method of Cohen *et al.* (16). Bacteria were grown in 1% tryptone, 0.5% yeast extract, 0.5% NaCl. Derivatives of M13 bacteriophage were routinely propagated in JM103 (Δ[lac pro] strA thi supE endA sbcB hsdR, F' traD36 proAB lacI^q lacZ ΔM15) (12). RZ1032 (dut ung) (obtained from T. Baldwin) was used for the preparation of bacteriophage with uracil-containing DNA.

Replication of plasmid DNA in cell-free extracts

Preparation of cell-free extracts and assays of DNA synthesis were basically carried out according to methods described by Diaz and Staudenbauer (17) and Scherzinger *et al.* (18). Strain MV10 containing plasmid pMS94, which encodes all the proteins required for replication of R1162 (4), was the source of the extract. Bacteria were lysed by treatment with lysozyme and freeze-thawing. Particulate matter was then removed by centrifugation at 130,000 x g for 30 min, and nucleic acids precipitated from the supernatant with 2.7% streptomycin sulfate. Soluble protein was finally concentrated by precipitation with (NH₄)₂SO₄ (final concentration 73%) and dialyzed against 25 mM HEPES, pH 8, 50 mM KCl, 0.1 mM Na₂EDTA, 1 mM dithiothreitol. For assay of DNA synthesis, reaction mixtures (25 μl) contained: dATP, dGTP, dCTP,

dTTP (each 50 μ M), GTP, CTP, TTP (each 400 μ M), ATP (2 mM), dithiothreitol (800 μ M), NAD (50 μ M), cyclic AMP (50 μ M), creatine PO₄ (15 mM), magnesium acetate (11 mM), KCl (80 mM), HEPES - KOH, pH 8 (40 mM), creatine kinase (100 μ g/ml), polyethylene glycol (2.5 %), 9.5 μ l extract, and supercoiled plasmid DNA, purified by two successive centrifugations in CsCl-ethidium bromide gradients. Reactions were carried out at 30 C for 1 hr.

Localization of nascent DNA fragments synthesized in the presence of dideoxycytidine triphosphate (ddCTP)

Reaction mixtures (25 μ l) for DNA synthesis contained between 0.1 - 0.3 μ g supercoiled plasmid DNA, 10 μ Ci α -³²P-dATP (800 Ci/mmol), and 150 pmoles ddCTP. The amount of plasmid DNA used in each case was that giving maximal incorporation, determined from experiment. The ddCTP reduced overall DNA synthesis about 85%. Reactions were stopped by the addition of 3 μ l 0.25 M Na₂EDTA, 2.5 % SDS solution. Proteinase K (3 μ l, 5 mg/ml) was added, and the mixtures incubated at 37 C for 1 hr. The solutions were then extracted with an equal volume of phenol (neutralized with 0.5 M Tris-HCl, pH 7.8), and the nucleic acid precipitated and washed with ethanol. The DNA was resuspended in 100 μ l 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, and divided into four 25 μ l portions. Three of these were digested with 20 units EcoRI, BamHI, or HindIII at 37 C for one hour. The fourth sample was incubated under identical conditions, but no restriction enzyme was added. After incubation the DNA was reprecipitated by ethanol, resuspended in Tris buffer (3 μ l), and mixed with 5 μ l of a solution containing 0.3 % xylene cyanol, 0.3 % bromophenol blue, 0.37 % Na₂EDTA in formamide. The samples were finally immersed in boiling water for 3 min to denature the DNA, and applied to a 6% polyacrylamide - urea gel (19) for electrophoresis. Radioactively labelled DNA fragments were visualized by autoradiography of the dried gel.

Hybridization of nascent DNA fragments to M13 recombinant bacteriophage DNA

DNA synthesis and subsequent digestion with restriction endonucleases were carried out as described above. Following digestion with enzyme, each sample was resuspended in 12 μ l 10 mM Tris-HCl (pH 7.4), 100 mM NaCl and divided into three equal portions. One sample was saved for application to a polyacrylamide gel as described, the others were mixed separately with 3 μ g M13 mp8 or mp9 bacteriophage DNA (20) containing one of the two strands of DNA making up the 210 bp HpaII fragment. The mixtures were heated in boiling water for 3 min, then allowed to cool to room temperature for 30 min prior to loading onto the gel for electrophoresis.

Oligonucleotide-directed mutagenesis

A 580 bp R1162 DNA fragment, made up of the 210 + 370 bp HpaII fragments and bracketed by BamHI cleavage sites (see pUT741, Fig. 1), was first cloned into the DNA of bacteriophage M13 mp9. Uracil-containing phage DNA was then prepared from this mp9 derivative after propagation in *E. coli* strain RZ1032 (dut⁻, ung⁻). A 20-base oligonucleotide containing a SmaI site, and a 19-base oligonucleotide with an EcoRV site, hybridizing to different positions within the 210 bp HpaII fragment, were prepared by an oligonucleotide-synthesizing facility at the University of Texas. Prior to use, 10 ng of each

oligonucleotide were phosphorylated by incubation with 2 units T4 polynucleotide kinase (New England Biolabs) in 20 μ l 0.05 M Tris HCl, pH 7.5, 15 mM MgCl₂, 5 mM dithiothreitol and 1 mM ATP at 37 C for 1 hr. The phosphorylated DNA was mixed directly with 1 μ g of the single-stranded phage DNA, containing the cloned R1162 DNA, held at 100 C for 3 min, and then allowed to cool to room temperature for 30 min. Half the annealed mixture was diluted to 100 μ l in 20 mM HEPES buffer, pH 8, containing 12 mM MgCl₂, 2 mM dithiothreitol, 1 mM ATP, 500 μ M each deoxyribonucleoside triphosphate, and 1.25 % polyethylene glycol 8000. Five units DNA polymerase I (large fragment) and 120 units T4 DNA ligase were then added, and the mixture incubated at 37 C for 2 hrs. The DNA sample was then used directly to transfect JM103 made competent by the method of Cohen *et al.* (16). The DNA from purified plaques was screened for the presence of the mutations by dideoxy DNA sequencing (21).

RESULTS

Nascent DNA synthesis is initiated from two positions within DNA required in cis

The plasmid pUT716 (Fig. 1) contains the two HpaII fragments required in cis for maintenance of R1162, but these are at separate locations and bracketed with useful restriction endonuclease cleavage sites. Supercoiled pUT716 DNA was replicated in vitro in the presence of α -³²P-dATP and ddCTP (MATERIALS AND METHODS), then cleaved with various restriction enzymes, denatured, and subjected to polyacrylamide-urea gel electrophoresis. The radioactively-labelled DNA fragments were visualized by autoradiography. The results are shown in Fig. 2, and are schematically represented in Fig. 1. The sizes of the labelled nascent fragments that are produced following cleavage with different restriction enzymes indicate that there are two end positions within the 210 bp HpaII fragment. The locations of these can be determined to within a few base-pairs, indicated by asterisks in Fig. 3, from the sizes of the restricted DNA fragments. The base sequence at each endpoint is conserved (9/10 bp, boxed in Fig. 3), but in inverted orientation.

The endpoints of the nascent DNA fragments are located either at start sites for DNA synthesis, or at sites for specific termination of strand elongation. If we assume that synthesis is in the 5' to 3' direction, then we can distinguish these possibilities since the polarities of the two DNA strands of the 210 bp HpaII fragment are known from DNA sequencing (Fig. 3). Newly synthesized fragments of pUT716 DNA were prepared as before, but prior to electrophoresis a third of the sample was hybridized separately to excess M13 mp8 or mp9 single-stranded bacteriophage DNA containing one of the two DNA strands from the 210 bp fragment (MATERIALS AND METHODS). The results are shown in Fig. 4. The upper DNA strand in Fig. 3 hybridizes specifically to the 199 base fragment generated by EcoRI digestion, causing a reduction in the amount of this fragment at its usual position in the gel; the lower strand hybridizes to each of the remaining small DNA fragments. This pattern of hybridization indicates that the 5' ends of the nascent DNA fragments, and

FIG. 1. Structures of key plasmids used in this study. A map of R1162 (top) shows the locations of the origin region (oriV), regions encoding proteins required for maintenance of R1162 (RepI, RepII), and genes for resistance to sulfonamides (Su) and streptomycin (Sm). The origin is expanded to show the positions of the 210 and 370 bp HpaII fragments and the locations of the inverted repeat (IR) and direct repeats (DR). Distances are in kilobase-pairs of DNA (kb) from the EcoRI cleavage site. The sketches of the remaining plasmids show the locations of the 210 and 370 bp HpaII fragments, which are indicated by double lines. These fragments are in their normal relative orientations with the exception of pUT717, in which the 210 + 370 bp fragment has been inverted. The small arrows represent the IR in the 210 bp fragment. The measured size and deduced locations of the nascent DNA fragments observed in Figs. 2 and 3 are indicated for each plasmid. Additional abbreviations: Km, kanamycin-resistance; B, H, Hp, R, S, V, restriction endonuclease cleavage sites for BamHI, HindIII, HpaII, EcoRI, SmaI and EcoRV.

FIG. 2. Polyacrylamide gel electrophoresis of plasmid DNA fragments synthesized in cell extracts in the presence of ddCTP. Prior to loading onto the gel, the samples were either cleaved with EcoRI (R), BamHI (B), or HindIII (H), or left undigested (N), and then denatured. Fragment sizes (in bases) are given at the left. In this gel, the fragments arising from EcoRI digestion of pUT815 are migrating overall slightly faster than corresponding EcoRI fragments for other plasmids, and there is a second band below the 305 base fragment. These are artifacts and not seen in other gels. Additional bands in the gel that are not marked are full-length restriction fragments (see Fig. 1), or fragments of newly replicated DNA too large for accurate measurement. Dideoxy sequencing reaction products of the 210 bp HpaII DNA fragment were used as size markers.

therefore the start-points of synthesis, lie within the 210 bp fragment.

The locations of the start-points for the nascent DNA synthesis are determined by the base sequence within the 210 bp HpaII fragment, and not by their distance from other DNA, such as the DNA essential in cis within the 370 bp HpaII fragment. If the intervening spacer DNA is deleted, so that the 210 bp and 370 bp fragments are in their normal, adjacent location, the population of the nascent DNA fragments changes to reflect this (pUT741, Figs. 1,2). If the plasmid contains two copies of the 210 bp DNA (pUT718), then DNA is initiated from within each (Figs. 5 and 1). Inversion of the 210+370 bp fragment in pUT718, so that the two copies of the 210 bp DNA are

FIG. 3. Base sequence of 210 bp *Hpa*II fragment showing start-points for DNA synthesis. The sequence has been previously published elsewhere (2). Base-pairs at the possible start positions are indicated by asterisks; the large arrows above these show the direction of synthesis. Base changes introduced by oligonucleotide-directed mutagenesis are also shown in the figure. The deletions in pUT831, pUT832 and pUT833 extend from the indicated base-pairs to the introduced *Eco*RV site. The inverted repeat in the fragment is shown by the arrows below the sequence.

in opposite orientation, does not affect initiation of synthesis from within each copy (pUT717, Figs. 2 and 1). In every case the deduced locations for the origins of synthesis are consistent, and in agreement with those determined for pUT716.

The nascent DNA contains little or no RNA. Plasmid pUT718 DNA was replicated and cleaved with restriction endonucleases. Half of each sample was then incubated in 30 μ l 0.15 N NaOH, 1 mM EDTA (pH 11) for 20 hrs at 37 C. Following neutralization of the sample by the addition of 170 μ l 0.5 M Tris-HCl (pH 7.8), the DNA was precipitated, resuspended in buffer, heated, and applied to a polyacrylamide-urea gel as before. The migration rate of the alkali-treated DNA was not significantly different from that of the control samples (data not shown). Thus, the newly synthesized DNA is not covalently bound to RNA, although the presence of a small number of ribonucleotides is not excluded.

Nascent DNA synthesis reflects plasmid DNA replication

The specific locations of the ddCTP-terminated DNA fragments make it likely that these fragments arise during early events in plasmid DNA replication. Alternatively, however, the fragments could be an artifact of the cell extract system, resulting, for example, from adventitious initiation

FIG. 4. Polyacrylamide gel electrophoresis of DNA fragments synthesized in cell extracts in the presence of ddCTP, and hybridized to M13 bacteriophage DNA containing each DNA strand from the 210 bp HpaII fragment. The upper and lower strands are those in Fig. 3. The figure is labelled as described in Fig. 2.

at positions not utilized for replication *in vivo*. Additional observations support the interpretation that the nascent DNA arises from replicative DNA synthesis.

The ddCTP-terminated fragments initiated from within the 210 bp fragment are not obtained if the 370 bp HpaII fragment is deleted. We constructed pUT828, identical to pUT716 except that the small BamHI fragment, containing the essential DNA within the 370 bp HpaII fragment, has been replaced by pBR322 (Fig. 1). This plasmid is now replicated by means of the pBR322 replicon, although the 210 bp DNA is unchanged. No ddCTP-terminated DNA synthesis of this plasmid is initiated from within the 210 bp fragment (Fig. 6).

FIG. 5. Polyacrylamide gel electrophoresis of DNA fragments synthesized in cell extracts in the presence of ddCTP. Additional description is given in Fig. 2.

We introduced *Sma*I and *Eco*RV cleavage sites into the 210 bp portion of a 210+370 bp fragment by means of oligonucleotide-directed mutagenesis. A plasmid containing the altered fragment, pUT814, shows the same pattern of labelled DNA fragments as pUT718, identical in structure but lacking the mutations (Figs. 5 and 1). Thus, synthesis is initiated both from the unaltered, isolated 210 bp fragment, and from the second, modified 210 bp DNA adjacent to the 370 bp region. In addition, the altered DNA is fully functional in vivo. We transformed *E. coli* with pUT814 DNA cleaved with *Eco*RI, and obtained kanamycin-resistant cells containing plasmids consisting of the large *Eco*RI fragment of pUT814.

We next inverted the *Sma*I-*Eco*RV fragment in pUT814, to generate plasmid pUT815 (Fig. 1). Digestion of this plasmid with *Eco*RI and transformation of *E. coli* resulted in only a few kanamycin-resistant transformants, and these all had the structure of the parental plasmid. No transformed cells

FIG. 6. Polyacrylamide gel electrophoresis of DNA fragments synthesized in cell extracts in the presence of ddCTP. Additional description is given in Fig. 2.

containing plasmids consisting of the single large EcoRI fragment of pUT815 were obtained. The modified 210 bp fragment was therefore no longer active in vivo, although no essential DNA had been interrupted or deleted by the inversion. Further, when pUT815 DNA was incubated in a cell extract, only labelled DNA mapping with the unaltered 210 bp fragment was observed (Figs. 2 and 1). There is thus a correlation between maintenance of a DNA fragment as a plasmid in vivo, and the pattern of nascent DNA synthesis observed from within that fragment in a cell extract.

The properties of Bal31-generated deletion derivatives of pUT814 also support the idea that the DNA fragments generated in vitro are utilized in vivo for plasmid DNA replication. These derivatives, pUT831, pUT832, and pUT833, have deletions extending a variable distance from the EcoRV site to the positions, shown in Fig. 3, within the 210 bp fragment. Plasmid DNAs digested with EcoRI were again used to transform MV10 to kanamycin-resistance. Transformants were obtained at high frequency (approximately 1000 per μ g DNA) for pUT831 and pUT832, and these contained plasmids composed of the large EcoRI fragment. In contrast, cells transformed with EcoRI-

FIG. 7. Possible sequence of early events in replication of R1162 DNA. The locations and orientation of the conserved 10 bp sequence at the start positions are indicated by the boxes containing the arrows. The broken circles indicate hypothetical binding sites for replication proteins.

cleaved pUT833 DNA were obtained about on hundred-fold less frequently, and these never contained plasmids consisting solely of the large *EcoRI* fragment. This suggests that the remaining 210 bp DNA in the large *EcoRI* fragment of pUT833 is no longer functional in vivo. In addition, nascent DNAs are initiated from within both of the 210 bp domains of pUT831 and pUT832, but only from within the intact 210 bp fragment in pUT833 (Fig. 6). Again, therefore, there is a correlation between maintenance of plasmid DNA in vivo and the pattern of nascent DNA synthesis. Importantly, pUT831 and pUT833 differ only in that the latter lacks one of the start-points for DNA synthesis (see Fig. 3).

DISCUSSION

Our results indicate that DNA synthesis is initiated from two positions within the 210 bp HpaII fragment, at a conserved sequence that is located at each end of a large inverted repeat. Synthesis from each initiation point is convergent and directed into the inverted repeat. Initiation of DNA synthesis is dependent on a plasmid-specific mechanism, because (a) no nascent DNA is generated in extracts lacking plasmid-encoded proteins for replication (22), and (b) additional DNA within the 370 bp HpaII fragment is required (pUT828, Fig. 6). We have been able to delete a large region of the intervening inverted repeat (bp 78-146 in Fig. 3), or to insert additional DNA (81 bp at the introduced SmaI site), without interfering with the ability of the remaining DNA to be replicated (unpublished results). Thus, the normal distance between the initiation points need not be maintained for replication. However, if one of these is deleted (pUT833), or the DNA containing it inverted (pUT815), then the origin DNA is disabled, suggesting that both must be present and in their proper orientation. In these cases, no DNA synthesis is initiated from within the altered 210 bp fragment (Figs. 2, 6). DNA synthesis is therefore not initiated independently from each position.

If the two initiation points for replication are dependent, then how do they interact? Each initiation point is located within a conserved 10 bp sequence; the two sequences together form an inverted repeat. One possibility is that initiation is triggered from a cruciform structure, shown in Fig. 7, that would be stabilized by the intervening inverted repeat. The protein replication complex might recognize such a structure (and promote its formation), and simultaneously initiate DNA synthesis from both strands. Alternatively, the two initiation points could be brought together by the looping of intervening DNA, brought about by the interaction of protein molecules simultaneously bound at both points.

In the cell, RSF1010 DNA is replicated either bidirectionally or unidirectionally (3). R1162 is identical, or at least very similar, to the plasmid RSF1010: the known DNA base sequences of the two plasmids within the origin region, or within the genes encoding the replication proteins, are the same (2, 23-25). It is reasonable to assume that R1162 and RSF1010 have the same mode of replication. Unidirectional replication of R1162 would then occur from left to right in Fig. 3. An initiation complex such as that proposed in Fig. 5 is essentially symmetric, and it is difficult to understand how unidirectional replication in a unique direction would be determined from within the complex. There are approximately equimolar amounts of newly synthesized DNA extending across the restriction enzyme cleavage sites at each side of the 210 bp HpaII fragment (pUT716, Fig. 2). If a substantial proportion of the molecules of pUT716 is replicated unidirectionally, then perhaps commitment to this mode occurs after the first events of initiation (Fig. 7). The 210 and 370 bp HpaII fragments at the origin each contain an essential domain. Unidirectional replication might be affected by the relative orientation of these domains, so that newly

synthesized DNA closer to the domain in the 370 bp fragment is more likely to be extended. If this is true, then inverting the 210 bp fragment should not affect the direction, a prediction now being tested.

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