

Origin and direction of DNA replication of plasmid RSF1030

(*in vitro* replication/dideoxy NTPs/electron microscopy/restriction enzyme mapping/gel electrophoresis)

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ABSTRACT An *in vitro* replication system has been used to study the origin and direction of replication of the covalently closed, circular DNA of plasmid RSF1030, a nonconjugative R factor. We have enriched for replicative intermediates in these studies either by isolating them on the basis of their unique structure or by limiting the extent of synthesis in the *in vitro* system. Circular molecules that have replicated to various extents migrate to characteristic positions in agarose gels, thus providing a rapid and efficient method for isolating partially replicated forms. Alternatively, replicative intermediates can be isolated directly from reaction mixtures that contain dideoxyTTP (ddTTP), a compound that limits the average extent of synthesis *in vitro*. Electron microscopic analysis of such intermediates linearized with either *Hpa* I or *Bam*HI indicates that RSF1030 replicates *in vitro* from a unique origin located 70% from one end of *Hpa* I-cleaved molecules and 47% from the *Bam*HI site. The unidirectional mode of replication has been confirmed by the order in which the six *Hinc*II fragments of RSF1030 DNA are labeled *in vitro* when synthesis is limited to various extents with ddTTP. Finally, a physical map of RSF1030 has been constructed using the restriction endonucleases *Bam*HI, *Hpa* I, and *Hinc*II, and the origin and direction of replication have been defined relative to the map.

The various known species of bacterial plasmids vary widely in their modes of replication. The plasmids ColE1 (1-3) and pSC101 (4), for example, replicate unidirectionally from a unique origin. The conjugative plasmids R6K and RSF1040, however, possess two initiation sites and one termination site. Replication of these plasmids usually proceeds exclusively from one or the other of the two initiation sites, although both are occasionally used simultaneously (5, 6). These plasmids are unusual in that replication is asymmetrically bidirectional; that is, it begins at an origin and proceeds in one direction to the terminus before proceeding in the opposite direction from the same origin to the same termination point. Yet another plasmid, RSF1010, replicates either unidirectionally or bidirectionally with equal probability from a unique origin (7).

We have recently developed an *in vitro* system capable of replicating the plasmid RSF1030 (unpublished data). RSF1030 is a nonconjugative plasmid of molecular weight 5.5×10^6 [8.3 kilobase pairs (kb)], carrying the gene for ampicillin resistance on the transposable element Tn2 (8). *In vivo* replication of RSF1030 resembles that of ColE1 in several respects. Both replicate as a multicopy pool and continue to replicate in the presence of chloramphenicol so that up to 3000 copies accumulate per cell. Replication is inhibited by rifampicin and requires DNA polymerase I. In spite of these similarities, RSF1030 has no detectable sequence homology with ColE1 (9), so the plasmids appear to have evolved independently.

Synthesis in the *in vitro* system mimics *in vivo* replication faithfully in all respects that have been tested. Most striking are the similar protein requirements for *in vivo* and *in vitro* rep-

lication, including requirements for both RNA polymerase and DNA polymerase I. In addition, the proteins encoded by *dnaB*, *dnaC*, *dnaG*, *dnaP*, *polC* (DNA polymerase III), *dnaZ*, and *dnaI* and DNA gyrase, all of which are required for *Escherichia coli* DNA synthesis, are also required for plasmid replication *in vitro*. In this report we show that RSF1030 replication *in vitro* begins at a unique origin and proceeds by an overall unidirectional mechanism. The origin of replication has been positioned on a restriction map of RSF1030 DNA and the direction of replication has been established.

MATERIALS AND METHODS

Bacterial Strains, Media, and Cell Growth. Extracts for the *in vitro* replication system were prepared from *E. coli* strain W3110 (RSF1030) grown in L broth. Covalently closed, circular RSF1030 DNA was prepared from strain HMS174 *recA1* (RSF1030) grown in Vogel-Bonner medium.

Materials. Reagents and sources were as follows: Seakem Agarose (ME) from Marine Colloids, Inc. (Rockland, ME); deoxynucleoside triphosphates and ribonucleoside triphosphates from Sigma; 2',3'-dideoxyTTP (ddTTP) from P-L Biochemicals; [α - 32 P]dTTP (300 Ci/mmol) from New England Nuclear; nitrocellulose paper (0.45 μ m) from Millipore; chloramphenicol from Parke, Davis (Detroit, MI); *E. coli* DNA polymerase I from C. C. Richardson; pancreatic DNase from Sigma; and restriction endonucleases from New England BioLabs.

Preparation of *In Vitro* Replication Extracts. Extracts for replication of DNA *in vitro* were prepared as described in the legend to Fig. 2.

Gel Electrophoresis and Restriction Enzyme Mapping. Agarose gels, 0.7 or 1.4%, were run in E buffer (40 mM Tris/5 mM Na acetate/1 mM EDTA at pH 7.4) in slab gel apparatuses equipped with fans. Gels were stained with 0.1 μ g of ethidium bromide (EtdBr) per ml in H₂O before photography. DNA was removed from agarose gels by freezing a slice of the gel and then squeeze-thawing in parafilm and collecting the extruded buffer in a 50- μ l pipet. Gels that were to be autoradiographed were dried on a Peter Hofer gel dryer. Films were preflashed and exposed at -70°C with intensifying screens. Autoradiograms were scanned on a Joyce-Loebl densitometer.

Restriction enzyme digestions were carried out in the buffers suggested by New England BioLabs. Double blots were done by the method of Sato *et al.* (10).

Electron Microscopy. Samples were spread for electron microscopy by the formamide-Kleinschmidt technique (11). A Philips 300 electron microscope was used to take photomicrographs on 35-mm film from which molecule lengths were measured by overhead projection by using a Hewlett Packard 9280A calculator and a Hewlett Packard digitizer.

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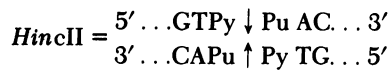
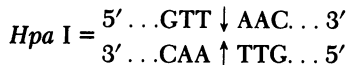
Abbreviations: kb, kilobase pair; ddTTP, dideoxyTTP; EtdBr, ethidium bromide.

RESULTS

Restriction Enzyme Mapping of RSF1030 DNA. A restriction enzyme map of the *Bam*HI, *Hpa* I, and *Hinc*II sites on RSF1030 DNA has been constructed. Both *Bam*HI and *Hpa* I cleave RSF1030 DNA only once, producing linear molecules. *Hinc*II cleaves the molecule into six fragments, the sizes of which are shown in Fig. 1. Molecular weights were determined by using *Hpa* I fragments of phage T7 DNA as size standards (12).

The map positions of the *Hinc*II fragments have been determined by the double-blotting technique (10). A partial *Hinc*II digest of RSF1030 (10 µg) was run on an agarose gel and transferred to nitrocellulose filter paper. A second gel, containing 1 µg of a complete *Hinc*II digest that had been labeled with [α -³²P]dTTP in a nick translation reaction using pancreatic DNase and DNA polymerase I, was run. The DNA was transferred under hybridizing conditions from the second gel to the filter paper containing the partial digest, at a 90° angle to the first gel. By determining the combinations of ³²P-labeled fragments that hybridize to each partial fragment, one can construct a restriction map. The sets of fragments DF, CDF, CDE, AF, and BE existed in the set of partial digests, indicating a map order of A,B,E,C,D,F.

In order to position the *Bam*HI and *Hpa* I cleavage sites relative to the *Hinc*II map, we analyzed *Bam*HI/*Hpa* I and *Bam*HI/*Hinc*II double digests by gel electrophoresis. The *Bam*HI site is located within the *Hinc*II E fragment, and the *Hpa* I site is the same as the *Hinc*II site between *Hinc*II fragments C and D. *Hpa* I cleaves at a subset of *Hinc*II sites, due to the overlapping sequence recognitions of the two enzymes (13):



The restriction map derived from these results is shown in Fig. 1.

Isolation of Replicative Intermediates. In order to char-

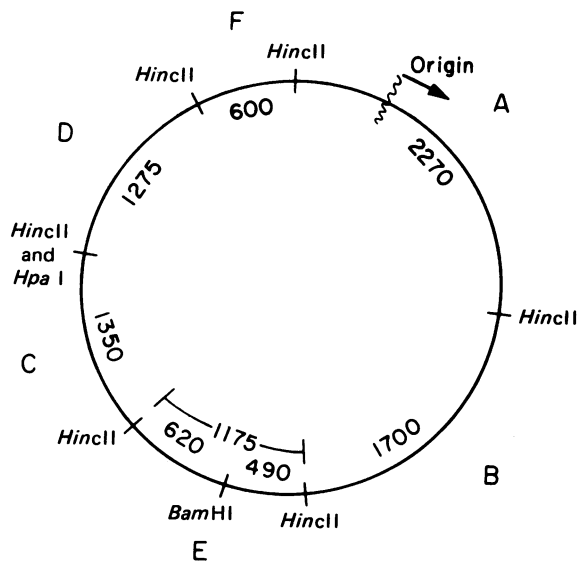


FIG. 1. Restriction map of RSF1030 DNA. Sizes of restriction fragments were determined by using an *Hpa* I digest of T7 DNA for size standards. Molecular weight determinations were carried out on a 30-cm, 1.5% agarose gel run at 60 V for 6 hr. Details of the mapping procedures are given in the text.

acterize the mode of replication of RSF1030, we studied replicative intermediates by electron microscopy. Replicating molecules were prepared in an *in vitro* plasmid replication system. Synthesis was carried out with [α -³²P]dTTP as described in the legend to Fig. 2, and the products were run on a 0.7% agarose gel (Fig. 2). The EtdBr-stained gel shown in lane b represents both template and product DNAs, while the autoradiogram shown in lane c represents only product DNA. Labeled product is seen at the positions of supercoiled (form I) and nicked circular duplex (form II) DNAs and in two additional bands labeled RI α and RI β . There is also a smear of radioactivity from the position of form I to the position of the RI β band (Fig. 2).

DNA was extracted from the RI bands and from intermediate positions between the form I and RI β bands and mounted for electron microscopy. Replicative intermediates appeared as relaxed circular molecules with replicated loops of various lengths. The extent of replication increased with decreasing distance of migration in the gel. Supercoiled intermediates were not seen, probably because the gel had been stained and photographed under conditions that nick DNA. The fact that DNA extracted from the supercoiled form I band also appeared as relaxed circles in the electron microscope supports this contention. On the other hand, under the conditions of the formamide-Kleinschmidt method used, negative superhelical form I DNA may be partially unwound, and this could account for

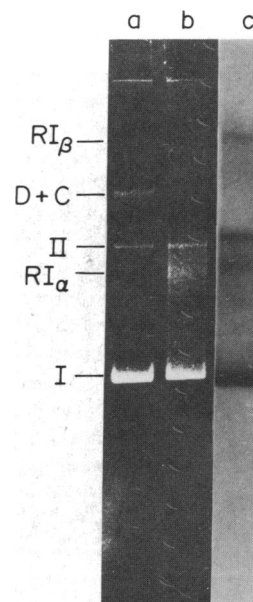


FIG. 2. Agarose gel electrophoresis of RSF1030 DNA synthesized *in vitro*. Extracts for *in vitro* DNA synthesis reactions were prepared from chloramphenicol-treated cultures of W3110(RSF1030). Cells were lysed by lysozyme treatment followed by freezing and thawing and centrifugation to remove chromosomal DNA. FxII was prepared by precipitation of endogenous plasmid DNA with streptomycin sulfate followed by ammonium sulfate precipitation of the streptomycin supernatant. RSF1030 DNA was synthesized in a 0.05-ml reaction mixture containing 0.02 ml of W3110/RSF1030 FxII, 40 mM Hepes (pH 8.0), 100 mM KCl, 12 mM magnesium acetate, 50 µM each of dCTP, dGTP, dATP, and [α -³²P]dTTP (200 cpm/pmol), 2 mM ATP, 0.5 mM each rCTP, rGTP, and rUTP, and 0.5 µg of RSF1030 DNA. The reaction was carried out for 60 min at 30°C. Samples were extracted with phenol, precipitated with ethanol, and run on a 0.7% agarose gel at 40 V for 12 hr. Lane a, RSF1030 marker; lane b, EtdBr-stained gel; lane c, autoradiograph of lane b. The bands in the autoradiograph do not line up exactly with those in the stained gel because the pictures are at slightly different magnifications. RI α and RI β , replicative intermediate DNAs; I, form I DNA; II, form II DNA; D + C, dimers plus catenanes.

the open appearance of the closed molecules. This result, in addition to our previous findings, establishes that RSF1030 molecules are not replicating by a rolling circle mechanism.

Electron Microscope Studies on Origin and Direction of RSF1030 Replication. Replicative intermediates were made linear with either *Hpa* I or *Bam*HI for electron microscope studies of the origin and direction of replication. Replicating molecules were either extracted from agarose gels as described above or obtained by carrying out the *in vitro* DNA replication reaction in the presence of various amounts of ddTTP. Because ddTTP halts DNA chain growth when it is incorporated, this is an efficient way to limit the extent of DNA synthesis to varying degrees. When ddTTP was used, reaction mixtures were extracted with phenol and the products were digested with the appropriate restriction enzyme before being mounted for electron microscopy.

Molecules isolated from agarose gels, cleaved with *Bam*HI, and observed by electron microscopy are shown in Fig. 3 *a-e* in order of increasing extents of replication. Replication begins at a point 47% from one end of the linear molecules. Molecules that are greater than about 50% replicated appear as double forks, or H forms, as seen in Fig. 3 *d* and *e*. From an analysis of 89 molecules, replication appeared to be unidirectional. It was difficult to orient the molecules, however, because the origin is located near the center of *Bam*HI-cleaved DNA. For this reason, replicating molecules were cleaved with *Hpa* I and examined. Replicative intermediates were generated with

ddTTP, cleaved with *Hpa* I, and observed by electron microscopy. Molecules containing internal loops were scored. The lengths of both unreplicated arms and the average length of the replicated loop were measured. In molecules that were <5% replicated, the unreplicated arms were approximately 70 and 30% of the total length. The long arm was designated L_1 , and the short arm L_2 . Fig. 4 is a plot of the lengths of L_1 and L_2 measured as a percentage of the total length of the molecule against the extent of replication. The length of L_1 decreases as replication proceeds, while the length of L_2 stays constant until the molecule is 70% replicated. Thus, replication is unidirectional for at least 70% of the molecule.

These data indicate that in this *in vitro* system replication of RSF1030 DNA begins at a unique origin located 70% from one end of *Hpa* I-cleaved molecules and 47% from one end of *Bam*HI-cleaved molecules and proceeds by a unidirectional mechanism for at least 70% of the molecule. We have observed only four molecules that are greater than 70% replicated. These include one *Hpa* I-cut molecule with the termination point 9% of the molecular length away from the origin and three *Bam*HI-generated double-forked molecules like the one shown in Fig. 3*e*. The structures of these molecules suggest that the termination point is, in fact, at or near the origin of replication.

The origin of replication has been positioned on the restriction map shown in Fig. 1. A unique position can be determined because the *Hpa* I and *Bam*HI sites were physically mapped

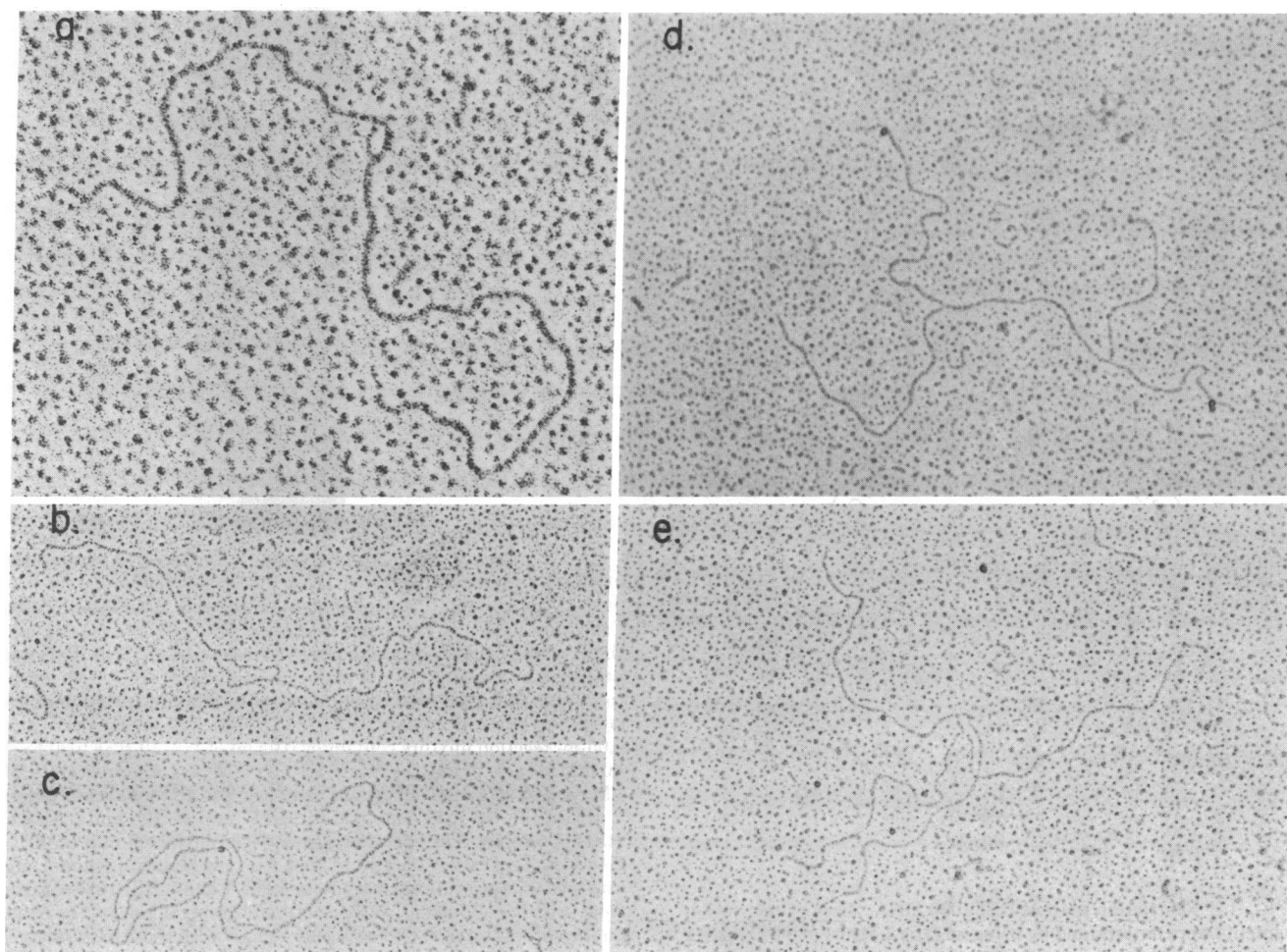


FIG. 3. Replicative intermediates cleaved with *Bam*HI. Replicating molecules were prepared as described in the text. Molecules are arranged (*a-e*) in order of increasing extents of replication. Magnification: 1 cm = 0.16 μ m (*a*) or 1 cm = 0.311 μ m (*b-e*).

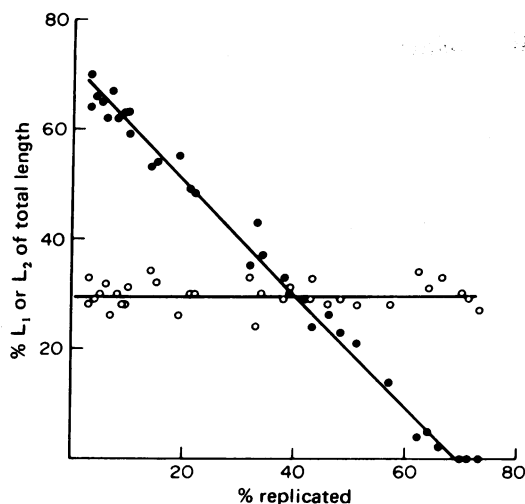


FIG. 4. Analysis of the mode of replication of RSF1030. An *in vitro* DNA replication reaction was carried out with FxII in the presence of 25 μ M ddTTP. *Hpa* I-cleaved replicating molecules with internal loops were photographed and measured. L_1 and L_2 are the unreplicated arms, and R_1 and R_2 the two halves of the replicated loop. The total length of a molecule with an internal loop is then: $T = L_1 + L_2 + (R_1 + R_2)/2$. The extent of replication is $(R_1 + R_2)/2T$. ●, L_1 ; ○, L_2 .

relative to the *Hinc*II cleavage sites. The *Bam*HI site is approximately 50% and the *Hpa* I site approximately 70% of the molecular length of RSF1030 away from the origin in the direction of replication.

Gel Electrophoresis Mapping of Origin and Direction of Replication. We have also studied the origin and direction of RSF1030 replication by determining the order in which the *Hinc*II fragments are labeled *in vitro*. DNA synthesis reactions were carried out *in vitro* in the presence of different amounts of ddTTP in order to allow replication to proceed to 16, 19, 29,

53, and 76% of the maximum obtained in the absence of ddTTP. Products were digested with *Hinc*II, run on agarose gels, stained with EtdBr, photographed, and autoradiographed. The results are shown in Fig. 5. The EtdBr-stained gel (Fig. 5a) shows the normal *Hinc*II pattern of six bands regardless of the extent of replication. In addition, at greater extents of replication a new, high molecular weight band designated A' appears (lanes k and l). The autoradiogram in Fig. 5b shows a very different pattern. The first material to be labeled (lane h) appears in a diffuse band above the position of the A fragment. This material probably represents fragments containing replication bubbles. Next (lane i), the amount of diffuse label increases and extends up to fragment A', and fragment B becomes labeled. A' and E both appear as heavily labeled bands in lane j. Densitometer tracing of the autoradiogram (lane j) indicates that the C fragment has a higher specific activity than D at this extent of replication. Thus, the order in which the first three fragments are labeled is BEC and is consistent with the unidirectional mode of replication proposed from electron microscope studies.

According to the electron microscope mapping, however, the origin of replication lies within the A fragment, so the A fragment should be the first to be labeled. In fact, significant label does not appear in the A fragment until lane k, where 53% of maximal synthesis has occurred. The appearance of the new band A', however, accounts for this apparent discrepancy. If the origin is within the A fragment and if replication is completely unidirectional, then the A fragment would not be completely replicated until the entire molecule was replicated. The fact that significant label does not appear in the A fragment until fragment F, the last one predicted to be replicated by a unidirectional mechanism, is also labeled (lane k) suggests that the origin of replication does lie within the A fragment and that replication is completely unidirectional. We suspected that the fragment A' was a branched molecule that had been replicated from the origin to the AB junction, but not in the direction of the AF junction. A *Hinc*II digest of newly replicated DNA was

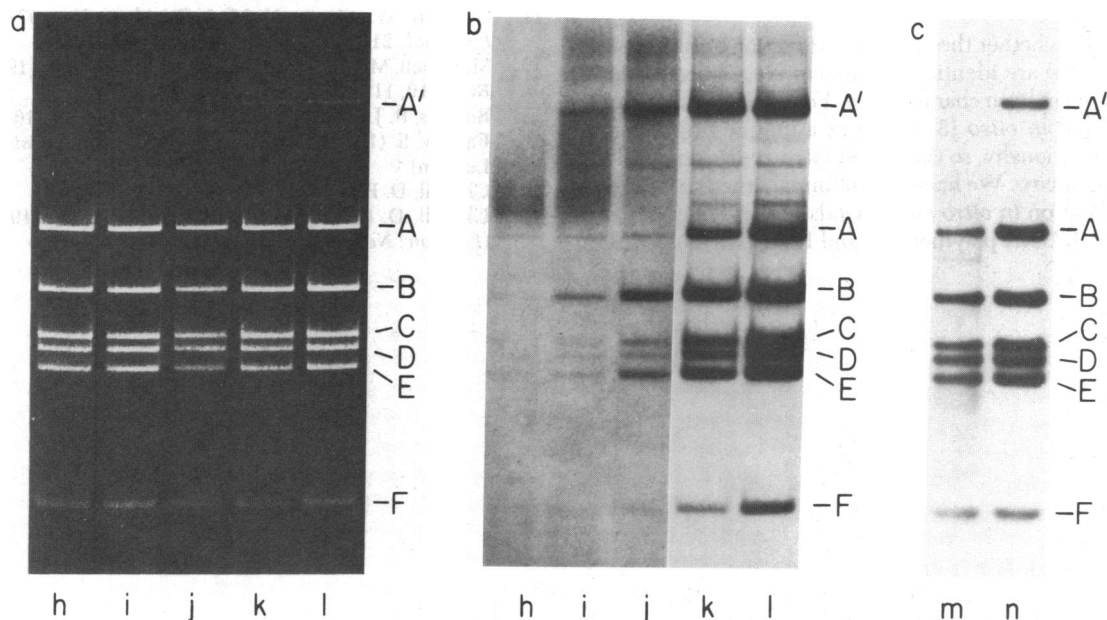


FIG. 5. Analysis of replicative intermediates by gel electrophoresis. DNA synthesis was carried out *in vitro* in the presence of various amounts of ddTTP, as indicated, and of [α - 32 P]dTTP (300 cpm/pmol). Products were digested with restriction endonuclease *Hinc*II and run on a 1.4% agarose gel at 90 V for 5 hr. The gel was stained (a) and autoradiographed (b). Amounts of ddTTP and percentages of maximal synthesis were: lane h, 250 μ M, 16%; lane i, 100 μ M, 19%; lane j, 50 μ M, 29%; lane k, 25 μ M, 53%; and lane l, 10 μ M, 76%. (c) *Hinc*II digest of the products of DNA synthesis *in vitro* in the absence of ddTTP with (lane m) and without (lane n) prior denaturation. DNA in lane m was heated to 100°C for 3 min in gel buffer and reannealed for 10 min at 65°C before being loaded on the gel.

therefore carried out and the products run on an agarose gel with (lane m) and without (lane n) prior denaturation and subsequent renaturation (Fig. 5c). If A' is in fact a branched molecule, then it should disappear upon denaturation. This does occur, and a new band appears between the A and B fragments. We conclude that this is the fragment covering the region between the origin and the AB junction. Several other faint bands that have not been characterized appear between the E and F fragments. These data are consistent with the interpretation that replication begins within the A fragment and moves clockwise around the map. If there is any counterclockwise synthesis, it is limited to the region between the origin of replication and the AF junction.

DISCUSSION

The origin and direction of plasmid RSF1030 replication *in vitro* have been assigned by both traditional electron microscopic procedures and by novel applications of agarose gel electrophoresis, restriction enzymology, and the DNA synthesis inhibitor, ddTTP. *In vitro* replication begins at a unique site on the DNA. The origin of replication used *in vitro* has been located on a restriction map, and replication has been shown to proceed by an overall unidirectional mechanism. The results do not, however, rule out the possibility of some synthesis in the opposite direction. In fact, among 89 *Bam*HI-cleaved molecules examined, two were seen with a replication loop spanning the putative origin of replication. These could have been generated by partially bidirectional synthesis, by incorrect initiation, or by random breakage of the DNA. In addition, six molecules were seen with a replication loop at a site other than the usual origin of replication. These also could be the products of incorrect initiation or of breakage of the DNA at sites other than the *Bam*HI site during extraction from the gel. Since the *Bam*HI digestion was not complete, molecules broken at random sites could still be full length and would therefore be scored during electron microscope mapping. The majority of the evidence, however, including the structures of very late replicating intermediates, indicates that replication is unidirectional.

It is not known whether the origin and direction of RSF1030 replication *in vitro* are identical to those *in vivo*, since the *in vivo* events have not been characterized. For ColE1, replication *in vivo* (1, 2) and *in vitro* (3) begins at a unique origin and proceeds unidirectionally, so that the *in vitro* events do mimic those occurring *in vivo*. We have shown (unpublished data) that RSF1030 replication *in vitro* corresponds to that seen *in vivo* in requiring both DNA polymerase I and RNA polymerase (9,

14). In addition, at least eight other proteins known to be required for host chromosome replication are also required for *in vitro* RSF1030 replication (unpublished data). We therefore anticipate that *in vivo* replication of this plasmid will begin at the same origin used *in vitro* and proceed primarily by a unidirectional mechanism.

RSF1030 resembles ColE1 in many of its replicative properties. Both are present as multicopy pools in logarithmic phase cells (9, 15) and continue to replicate in the presence of chloramphenicol so that up to 3000 copies accumulate per cell. The two plasmids are also similar in their requirements for DNA polymerase I (14) and RNA polymerase (9, 16). We can now add a unidirectional mode of replication to this list of similarities. Although these two plasmids appear unrelated on the basis of sequence homology, only future experiments will determine if they share some limited common sequences or structural features or both near the origin of replication.

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