JOURNAL OF BACTERIOLOGY, June 1982, p. 1077–1084
0021-9193/82/061077-08$02.00/0

Vol. 150, No. 3

Origin and Direction of Mini-R1 Plasmid DNA Replication in Cell Extracts of *Escherichia coli*

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Received 2 October 1981/Accepted 11 January 1982

Replication of the mini-R1 plasmids pKN177 and pKN182 can be carried out efficiently in cell extracts of *Escherichia coli* and depends on both transcription and translation. Heteroduplex and restriction analyses indicate that both plasmids are derived from the R1 copy mutant pKN104 by IS*1*-mediated recombination events without involving structural alterations in the replication region. To ascertain whether the in vitro replication of these miniplasmids corresponds to R1 replication in vivo, the origin and direction of replication were analyzed by electron microscopy of replicative intermediates. It was found that replication starts at a unique origin located within the RepA region at R1 coordinate at 82.4 kilobases and proceeds unidirectionally toward the IS*1*b sequence. The specification of the origin and the direction of in vitro replication are therefore in full agreement with the pattern observed previously for the in vivo replication of the closely related plasmids R100 and R6-5. This agreement provides additional evidence that R1 DNA synthesis in vitro employs the same replication mechanism as it does in vivo.

Plasmid R1 is a conjugative plasmid of 63.5 megadaltons (95.8 kilobases [kb]) specifying resistance to several antibiotics (kanamycin, ampicillin, sulfonylamide, streptomycin, chloramphenicol) and belonging to the incompatibility group FII. The IncFII plasmids (R1, R100, R6-5) are composed of a cluster of drug resistance genes (r-determinant) flanked by two directly repeated IS*1* elements and of a region (designated resistance transfer factor) which carries the genes responsible for autonomous replication and conjugal transfer (1). The replication region of the IncFII plasmids consists of a DNA segment of about 5 kb, which is situated adjacent to the IS*1*b sequence and includes the replication origin and genes involved in replication and copy number control. In case of R1 this region has been subdivided into a 2.5-kb basic replicon (RepA), which is defined by the *Pst*I fragments F2, F1, and E (7, 10) and a secondary replication region (RepD) contained within the *Pst*I D fragment (3). The replication region of R1 and the closely related plasmids R100 and R6-5 is highly conserved as indicated by heteroduplex and restriction analyses (26). Evidence has been presented that the replication origin is located within the *Pst*I E fragment (8, 14). Replication of R100 and R6-5 starts in the same region and proceeds unidirectionally toward IS*1*b and into the r-determinant region (15, 20, 25).

The replication of IncFII plasmids has been extensively studied at the physiological and genetic level (26). A characteristic property of IncFII plasmid replication is its dependence on de novo protein synthesis. This has been attributed to the requirement for a nonreusable, plasmid-encoded replication protein (5, 28). An essential replication gene designated *repA* has been identified in R100 by Yoshikawa (29), and the structure of the gene product has been deduced from the nucleotide sequence of the replication region (18). Furthermore, plasmid mutants have been isolated that exhibit a severalfold increase in plasmid copy number (27). The sequence of the replication control region of the R1 copy mutant pKN104 has been determined and shown to differ by a single base substitution from the corresponding wild-type sequence (23). In contrast to the parent plasmids, these copy mutants are somewhat unstable and frequently give rise to the formation of miniplasmids (9, 12, 16). Due to their small size and increased copy number, such miniplasmids offer experimental advantages for biochemical studies of plasmid DNA synthesis.

We have recently reported that cell extracts of *Escherichia coli* can carry out the replication of the mini-R1 plasmids pKN177 and pKN182, both of which derive from pKN104 (4). The in vitro replication of these plasmids resembles R1 replication in vivo by being strictly dependent on protein synthesis directed by plasmid DNA. It was nevertheless important to ascertain that in vitro DNA synthesis employs the basic replicon

of R1 and is not due to the activation of secondary replication origin(s), which are of little or no relevance for in vivo replication. In this communication we present a structural characterization of pKN177 and pKN182 and show that the origin and direction of in vitro replication are the same as those determined previously for IncFII plasmids in vivo.

MATERIALS AND METHODS

Bacterial strains and medium. The *E. coli* strains used were C600(pKN177) and 1100(pKN182) (11). Cells were grown in L broth supplemented with 0.2% glucose.

Isolation of DNA. Plasmid DNA was isolated by CsCl-ethidium bromide equilibrium centrifugation from cleared lysates (2) after precipitation with polyethylene glycol (6). Ethidium bromide was removed by extraction with isopropanol and subsequent ion-exchange chromatography through a Dowex-50 column (4 ml) equilibrated with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 8.0)–1 mM EDTA.

Preparation of cell extracts. Cultures (2 liters) of *E. coli* C600(pKN177) were grown in 5-liter flasks with shaking at 37°C until an optical density at 600 nm of 1.0 was reached. Cells were then poured onto 600 g of crushed ice, sedimented, washed once with 30 ml of buffer A (25 mM HEPES-KOH [pH 8.0], 100 mM KCl, 1 mM dithiothreitol, 1 mM *p*-aminobenzamidine), and suspended in buffer A (1 ml/g of wet cells). All operations were performed at 0 to 2°C as quickly as possible. Lysis was carried out by the freeze-thaw method (21) in the presence of 1 mM EDTA. Extracts were divided into small aliquots and stored in liquid nitrogen.

Assay of DNA synthesis. Standard reaction mixtures contained final concentrations of 50 mM HEPES-KOH (pH 8.0), 100 mM KCl, 11 mM magnesium acetate, 15 mM creatine phosphate, 0.1 mg of creatine kinase per ml, 2 mM ATP, 0.4 mM each CTP, GTP, and UTP, 0.05 mM NAD, 0.05 mM cyclic AMP, 0.025 mM each dATP, dCTP, dGTP, and [^{3}H]dTTP (500 cpm/pmol), 0.5 mM each of the 20 amino acids, and 2.5% polyethylene glycol 6000. The optimal amounts of extract and exogenous plasmid DNA had to be determined for each extract preparation.

Alkaline CsCl gradient centrifugation. Standard reaction mixtures were layered directly on preformed 4.2-ml alkaline CsCl gradients (density, 1.2 to 1.4 g/ml in 0.2 M NaOH) and centrifuged for 35 min in a Beckman SW50.1 rotor at 5°C. Fractions were collected from the bottom of the tubes and assayed for acid-insoluble radioactivity.

Enzymatic digestion of DNA. Replicative intermediates were cleaved with *Eco*RI or *Sal*I restriction endonuclease for 1 h at 37°C in reaction mixtures (25 μl) containing 50 mM Tris-hydrochloride (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$ or 50 mM Tris-hydrochloride (pH 7.5), 150 mM NaCl, and 10 mM $MgCl_2$, respectively. The reactions were stopped by adding EDTA to a final concentration of 25 mM.

Electron microscopy. Internal duplex formation with plasmid pKN177 was performed in a volume of 10 μl containing 0.1 μg of DNA, 0.1 M sodium phosphate (pH 7.0), and 50% formamide. The mixture was boiled for 2 min and then incubated at 40°C for 30 min in the presence of 0.2 M $NaClO_4$. Heteroduplex molecules of pKN177 and pKN182 were prepared in a similar way by using equimolar amounts of these DNAs.

Spreading of DNA was carried out in the presence of formamide as described by Morris et al. (13). The protein nucleic acid film was picked up on freshly prepared Parlodion-coated copper grids, rotary shadowed with platinum-iridium, and examined in a Philips EM-301 electron microscope at a magnification of 11,000. DNA contours were measured from enlarged photographs with an electronic planimeter (Numonics Graphics Calculator). φX174 DNA (5.375 kb) (19) and

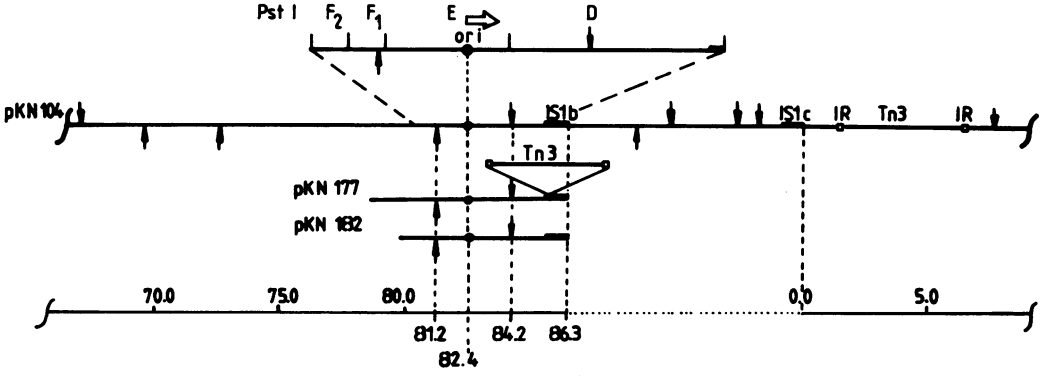

FIG. 1. Physical maps of pKN177 and pKN182 and partial physical map of pKN104 (10–12, 15, 17; this work). Circular plasmid DNA is depicted in a linear representation. The replication region of pKN104 as defined by *Pst*I fragments is shown in an expended scale. Indicated are the direct repeats of the IS*1* element (IS*1*b, IS*1*c), the Tn*3* transposon flanked by inverted repeats (IR), the targets for the restriction enzymes *Eco*RI (↓), *Sal*I (↑), and *Pst*I (|, only in the expanded scale), and the replication origin used in vitro (ori). The direction of replication is denoted by an open arrow. The numbers indicate the R1 coordinates in kb as defined by Ohtsubo et al. (16). Note that this coordinate system is based on a variant of R1 which had lost the 9.5-kb kanamycin resistance segment between the right ends of IS*1*b and IS*1*c (indicated by a dotted line).

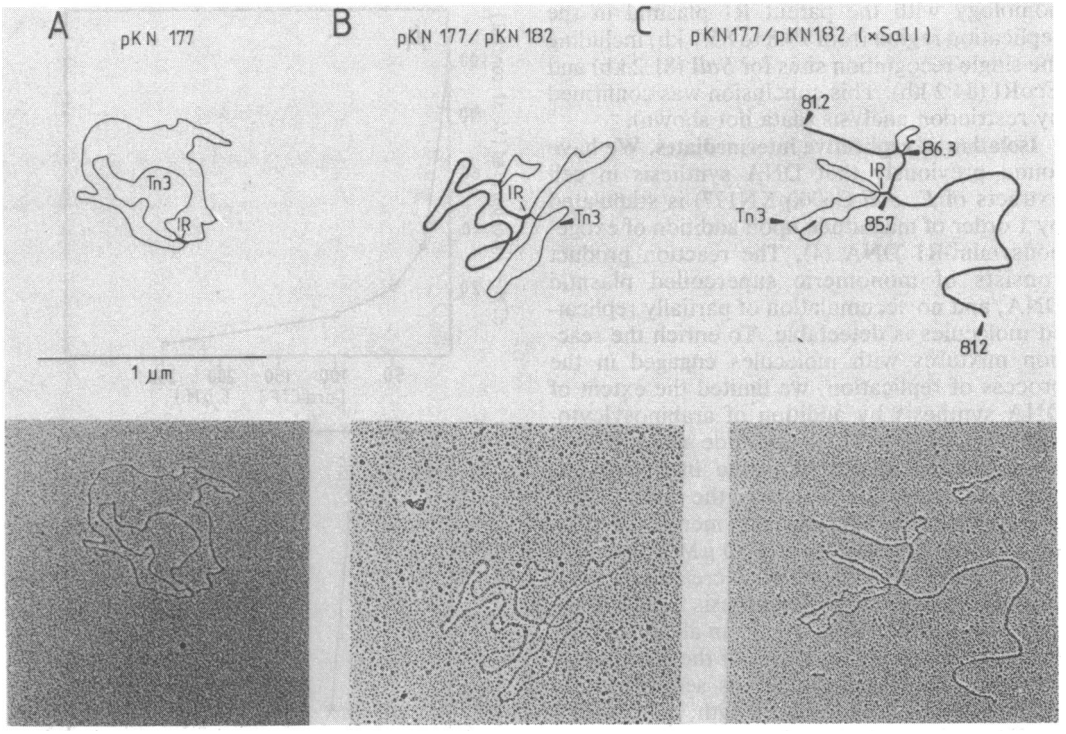

FIG. 2. Heteroduplex analysis of pKN177 and pKN182. (A) Internal duplex of pKN177, (B) pKN177/pKN182 heteroduplex (circular form), (C) pKN177-pKN182 heteroduplex cut with *Sal*I. Numbers refer to R1 coordinates (kb). The tracings indicate double-stranded regions (heavy lines) and inverted repeat (IR) sequences.

pBR322 DNA (4.362 kb) (24) were used as internal length standards for single-stranded and double-stranded DNA, respectively.

Chemicals and enzymes. Arabinosylcytosine triphosphate and *p*-aminobenzamidine were purchased from Sigma Chemical Co. (St. Louis, Mo.). The restriction enzymes *Eco*RI and *Sal*I were obtained from Boehringer (Mannheim, Federal Republic of Germany).

RESULTS

Structure of the mini-R1 plasmids pKN177 and pKN182. The plasmids pKN177 and pKN182 belong to a large group of miniplasmids that were formed spontaneously from pKN104 by in vivo recombination events (12). In the case of pKN182, the recombination event generated a 4.3-megadalton (6.5-kb) miniplasmid (11) that contains a continuous stretch of the parental plasmid including both the RepA and RepD regions and the IS*1*b element (85.6 to 86.3 kb). For better comparison with previous work all map positions are given in the R1 coordinate system of Ohtsubo et al. (16), in which the edge of IS*1*b adjacent to the r-determinant is defined as 86.3/0.0 kb (Fig. 1).

For pKN177, which contains the ampicillin resistance gene of R1, a molecular mass of 8.5 magadaltons (12.8 kb) was determined by both electron microscopy and gel electrophoresis (data not shown). Intrastrand renaturation of pKN177 single strands resulted in the formation of a 5.0-kb loop flanked by two inverted repeats indicating the presence of the Tn*3* transposon (Fig. 2A). Inspection of heteroduplex molecules of pKN177 and pKN182 revealed that all of the DNA of pKN182 is homologous to pKN177 and that the extra DNA of pKN177 is accommodated in the Tn*3* loop and in a 1.3-kb deletion loop (Fig. 2B). The coordinates of these loops were determined with respect to the unique *Eco*RI and *Sal*I site (Fig. 2C) as 85.7 kb (Tn*3* loop) and 86.3 kb (deletion loop).

A plausible interpretation of these data is given in Fig. 1. Both plasmids are depicted as having common endpoints at 86.3 kb (right terminus of IS*1*b) and extending into the resistance transfer factor region to coordinates 79.8 kb (pKN182) or 78.5 kb (pKN177). The deletion loop seen in pKN177-pKN182 heteroduplexes is therefore due to the DNA segment at the left end of pKN177, which is lacking in pKN182. Additionally, the Tn*3* transposon has been inserted into pKN177 within the IS*1*b element at 85.7 kb. Note that both miniplasmids share complete

homology with the parent R1 plasmid in the replication region from 79.8 to 85.7 kb, including the single recognition sites for *SalI* (81.2 kb) and *EcoRI* (84.2 kb). This conclusion was confirmed by restriction analysis (data not shown).

Isolation of replicative intermediates. We have found previously that DNA synthesis in cell extracts of *E. coli* C600(pKN177) is stimulated by 1 order of magnitude upon addition of exogenous mini-R1 DNA (4). The reaction product consists of monomeric supercoiled plasmid DNA, and no accumulation of partially replicated molecules is detectable. To enrich the reaction mixtures with molecules engaged in the process of replication, we limited the extent of DNA synthesis by addition of arabinosylcytosine triphosphate. This nucleotide analog causes an accumulation of replicative intermediates, probably as a result of slowing the rate of DNA chain elongation by DNA polymerase III (22). We chose a concentration of 50 μM arabinosylcytosine triphosphate, which decreased the total amount of pKN177 DNA synthesis to 22% of the untreated control (Fig. 3A). In an alkaline gradient (Fig. 3B), more than 85% of the label incorporated under these conditions sedimented as fragments of less than unit length, as expected for DNA chains derived from replicative intermediates.

For further characterization of the mode of R1 replication in vitro, we analyzed the replicative intermediates by electron microscopy. To distinguish endogenous from exogenous plasmid DNA by size, the DNA was isolated from reaction mixtures containing C600(pKN177) extract supplemented with pKN182 DNA. Plasmid DNA synthesized in the presence of arabinosylcytosine triphosphate was fractionated by CsCl-ethidium bromide equilibrium centrifugation and material banding at a density between closed-circular and open-circular DNA examined in the electron microscope. The preparations were found to contain 3 to 5% of the total DNA molecules as Θ-shaped replicative intermediates representing all stages of the replication cycle (data not shown). The relative amount of pKN182 intermediates was less than that of pKN177 as expected on the basis of the different molecular masses of the two plasmids (4.3 and 8.5 megadaltons, respectively).

Determination of origin and direction of replication. The single *EcoRI* and *SalI* restriction sites present in both pKN177 and pKN182 were used to linearize the DNA molecules and to introduce convenient reference points. Figure 4 shows electromicrographs of replicating pKN177 DNA molecules digested with either *EcoRI* or *SalI*. After cleavage with *EcoRI* (Fig. 4A through D), most replicative intermediates appear as double-Y-shaped structures with two

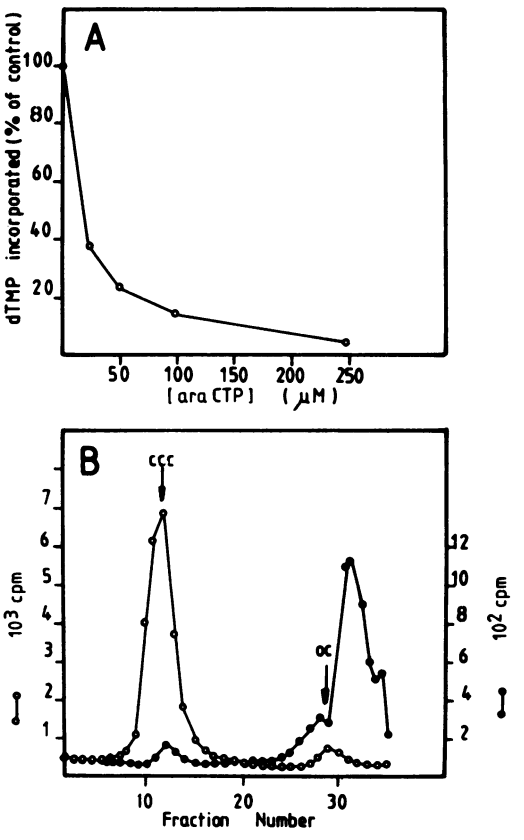

FIG. 3. Effect of arabinosylcytosine triphosphate (araCTP) on pKN177 replication. (A) Inhibition of dTMP incorporation by arabinosylcytosine triphosphate; 100% corresponds to 82 pmol of dTMP incorporated in 60 min in a standard reaction mixture (25 μl) supplemented with pKN177 DNA. (B) Alkaline CsCl velocity gradients. Reaction mixtures were incubated in the absence (○) or presence (●) of 50 μM arabinosylcytosine triphosphate and analyzed by alkaline CsCl gradient centrifugation. For better comparison the sedimentation profiles are superimposed. Sedimentation is from right to left. The arrows indicate the positions of closed-circular (CCC) and open-circular (OC) pKN177 DNA.

replication forks connected by an unreplicated DNA segment. A minor portion of the intermediates contained a small internal replication bubble and two unreplicated arms. In the double-Y-shaped molecules, one fork was of approximately constant length (1.7 kb), whereas the length of the other fork was variable. Similarly in the molecules with a bubble the distance between the enzyme cutting site and the distal branch point of the bubble remained constant. This distance corresponded to the constant length of one replication fork in the double-Y-

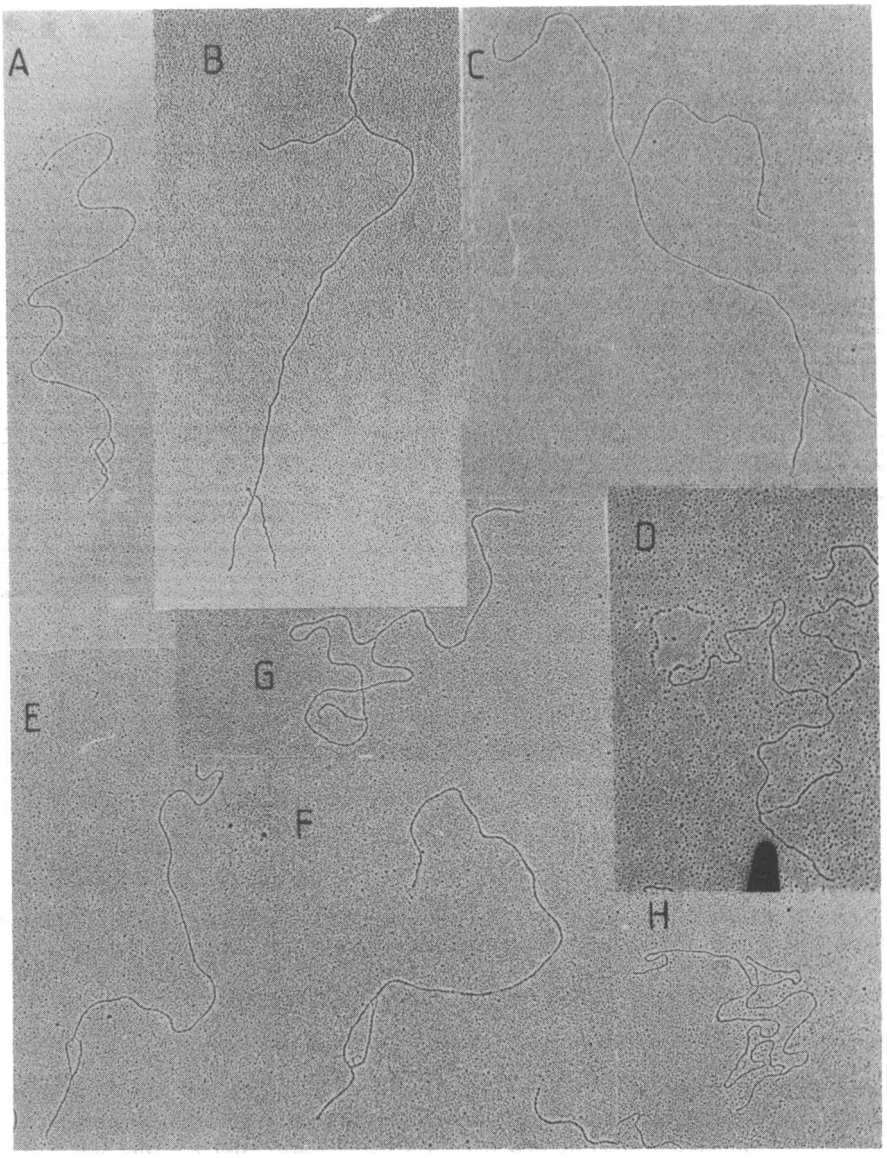

FIG. 4. Electron micrographs of replicating pKN177 DNA molecules treated with *Eco*RI (A through D) or *Sal*I (E through H). In measuring the replicated portions of these molecules a correction was made for displaced single-stranded regions at some of the replication forks.

shaped molecules and was equal in length for both pKN177 and pKN182 replicative intermediates. The replicating molecules could thus be arranged in a sequence compatible with a unique origin located at a distance of 1.7 ± 0.2 kb from the *Eco*RI restriciton site with replication progressing unidirectionally toward this site (Fig. 5A).

Digestion of the replicative intermediates with *Sal*I gave exclusively molecules with bubbles of various sizes (Fig. 4E through H). The distance between the *Sal*I site and one branch point of the bubble was approximately constant (1.2 kb) in both pKN177 and pKN182 intermediates. The replicating molecules could therefore be oriented with respect to the unreplicated arm of constant length and ordered in a sequence consistent with a single origin located at a distance of 1.2 ± 0.1 kb from the *Sal*I restriction site with replication proceeding unidirectionally away from the *Sal*I site (Fig. 5B). By combining the results obtained with the two restriction en-

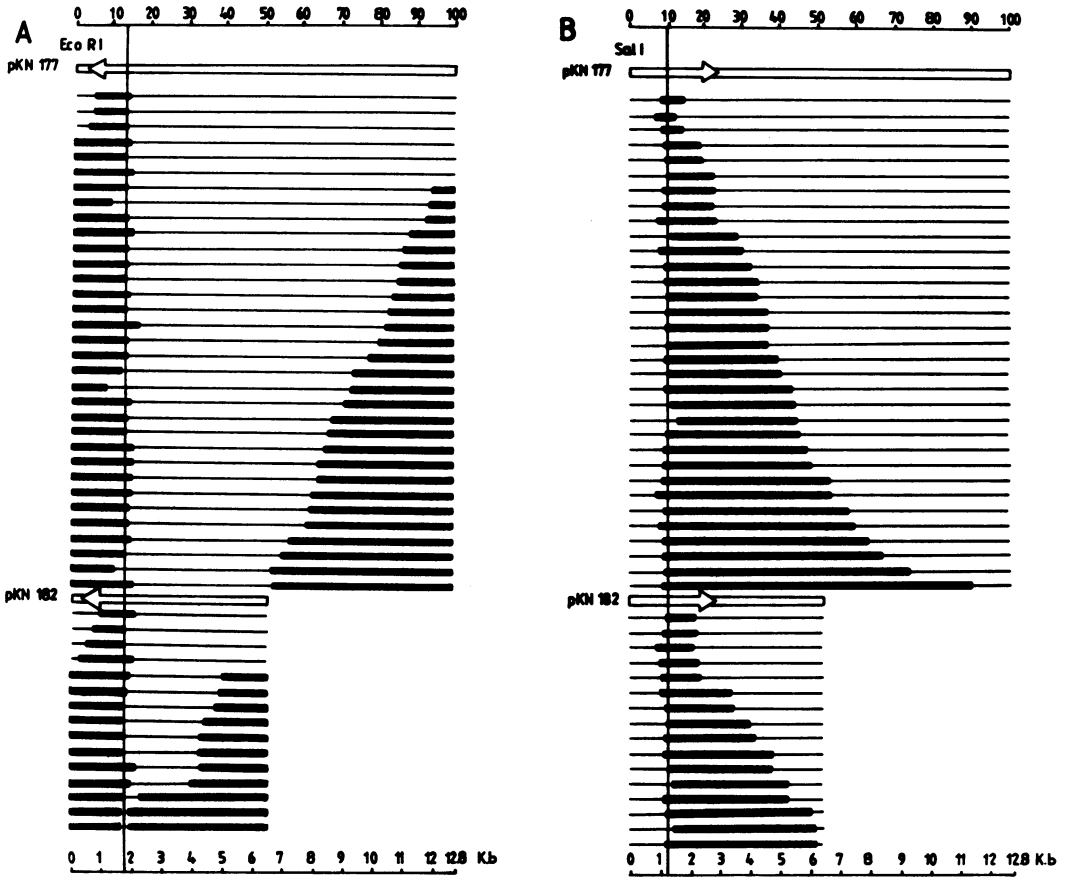

FIG. 5. Line diagrams of replicating pKN177 and pKN182 DNA molecules cleaved with *Eco*RI (A) or *Sal*I (B). The replicated portions of the molecules are indicated by heavy lines. Molecules were aligned as described in the text. The average position of the replication origin is denoted by a vertical bar, and the direction of replication is shown by an open arrow. The length of pKN177 DNA represents 100%.

zymes and correlating them with the physical map (Fig. 1), it can be concluded that the replication origin is located at 82.4 kb and that replication proceeds toward the right as depicted in Fig. 1.

DISCUSSION

The heteroduplex data presented in this paper indicate that the miniplasmids pKN177 and pKN182 were generated from the R1 copy mutant pKN104 by site specific recombination involving the IS*1*b insertion sequence. In case of pKN177 a transposition of the Tn*3* transposon into the IS*1*b element has also taken place. Site-specific recombination events involving IS*1* and Tn*3* have previously been implicated in the formation of mimiplasmids from copy mutants of other IncFII plasmids (9, 16). Thus copy mutants are prone to undergo size reductions by deletions and transpositions. For the purpose of replication studies it is important to note, however, that both pKN177 and pKN182 show no structural alterations within the replication region.

The origin of replication of the mini-R1 plasmids was determined in a cell-free in vitro replication system (4) by electron microscopy of replicative intermediates linearized with the restriction endonuclease *Eco*RI or *Sal*I. It was found that in vitro replication initiates at a unique origin located inside the RepA region at 82.4 kb employing the R1 coordinate system defined by Ohtsubo et al. (16). Replication proceeds from this origin in a unidirectional mode toward the resistance transfer factor-proximal edge of IS*1*b, i.e., clockwise on the genetic map of R1 (1). No differences in the mode of replication were noticed between endogenous and exogenous plasmid DNAs.

The origin determined in vitro appears to be

identical with the in vivo origin of other R1-derived miniplasmids, which has been defined by cloning and deletion analysis as a site within the RepA region required in *cis* for replication (8). The in vitro origin also coincides at the resolution level of electron microscopy with the location of the in vivo origin of R100 (20) and of copy mutant miniplasmids derived from R100 (15) and R6-5 (25). Furthermore the directionality of mini-R1 replication observed in vitro is the same as that reported for IncFII plasmid replication in vivo (15, 20, 25). This agreement provides further evidence that the replication pattern of pKN177 and pKN182 has not been altered by the in vitro conditions.

Although the RepA system constitutes the principal replication system of IncFII plasmids, it is not the only one. Danbara et al. (3) have recently identified by cloning of the *Pst*I D fragment a secondary replication region, designated RepD, which is located between RepA and IS*1*b. However, the role of RepD in plasmid replication is unclear. No evidence for the initiation of DNA synthesis within the RepD region was obtained in the in vitro system. In case of pKN182, which contains an intact *Pst*I D fragment, this could be due to less frequent initiation from this region not detected in our analysis. As far as pKN177 is concerned, it is conceiveable that the transposition of Tn*3* into IS*1*b could have adverse effects on the expression of RepD functions. Alternatively, activation of this secondary origin might only occur after inactivation or deletion of the primary replication system.

The data presented in this paper and previously published results (4) demonstrate that the in vitro replication of mini-R1 plasmids in cell extracts of *E. coli* employs the primary R1 origin located in the RepA region and depends strictly on the synthesis of an unstable plasmid-specific protein (presumably the *repA* gene product). Therefore, the in vitro replication system exhibits the same characteristics as in vivo R1 replication and appears to be well suited for a biochemical analysis of R1 replication. Studies are currently in progress to exploit this system for the determination of the exact starting point of DNA synthesis within the known sequence of the replication region.

ACKNOWLEDGMENTS

We thank Heinz Schuster for support and encouragement. We are grateful to Günther Hillenbrand and Rudolf Lurz for advice with the electron microscopy.

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