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Definition of *oriR*, the minimum DNA segment essential for initiation of R1 plasmid replication *in vitro*

(initiation protein/*repA* protein/replication origin/*cis–trans* actions/BAL-31 deletion analysis)

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ABSTRACT The 3.6-kilobase *Bgl* II–*Eco*RI fragment from R1 plasmid containing *copA*, *repA*, and the replication origin (*ori*) was inserted into the ColE1-type plasmid pUC8. The resulting hybrid plasmid replicates in extracts prepared from both *polA*$^-$ and *polA*$^+$ cells, whereas pUC8 replicates only in a *polA*$^+$ extract. This characteristic provides a method for assaying the *repA* and *ori* functions. Hybrid plasmids that were either *repA*$^-$ or *ori*$^-$ were unable to replicate in a *polA*$^-$ cell extract. Replication of the *repA*$^-$ *ori*$^+$ plasmid was restored by complementation of the *repA* defect by a *repA*$^+$ *ori*$^-$ plasmid *in vitro*. Successful complementation of the *repA* function *in vitro* provides a method for assaying the repA protein. In order to define the minimum DNA segment with origin function (*oriR*), deletions were introduced starting from either side of the insert, and the replication properties of the plasmids carrying these deletions were examined in a *polA*$^-$ cell extract. The right end of *oriR* was located at position 1,611 in the nucleotide coordinates defined previously [Ryder, T., Rosen, J., Armstrong, K., Davidson, D. & Ohtsubo, E. (1981) in *The Initiation of DNA Replication:* ICN-UCLA Symposia on Molecular and Cellular Biology, ed. Ray, D. S. (Academic, New York), Vol. 22, 91–111]. By complementing *repA*$^-$ *ori*$^+$ plasmids with the *repA*$^+$ *ori*$^-$ plasmid, the left end of *oriR* was localized at position 1,424. Therefore, the *oriR* sequence, localized within a region of 188 base pairs, is separate from the *repA* gene. A hybrid plasmid carrying the 206-base-pair segment between positions 1,406 and 1,611 also replicates in a *polA*$^-$ cell extract when the *repA* function is supplied in *trans*. Removal of an additional 66 base pairs (positions 1,406–1,471) inactivates the function of the minimal *oriR* segment.

Drug-resistant plasmids R1, R100, and R6-5 are similiar in genomic organization (1–3). The replication origins of R100 and R6-5 have been mapped to a small region by electron microscopy, and in both cases replication proceeds unidirectionally from this origin (4–6). A subcloned DNA segment containing the origin from R1, R100, or R6-5 is sufficient for autonomous replication, expression of incompatibility, and copy-number control (7–10). Three genes, *repA*, *copA*, and *copB*, are encoded within the replication region of the R1 plasmid. *RepA* encodes a plasmid-specific, *cis*-acting initiation protein that is essential for plasmid replication (9, 11, 12). The M_r of the repA protein of R1 and R100 based on the nucleotide sequence of the gene is 33,000 (12, 13). The gene products of *copA* and *copB*, identified respectively as a small RNA molecule (14, 15) and as a M_r 11,000 polypeptide (16), inhibit the expression of *repA* (17). Although the regulation of *repA* expression has been studied in detail, the mode of action of the repA protein and the precise initiation site in R1 replication are not known. In this report, we studied the mode of action of the repA protein and the replication initiation region of the R1 plasmid by using an *in vitro* replication system (18, 19). As a result, we identified the product of the *repA* gene. We also showed that the replication deficiency due to the loss of a functional *repA* gene is complemented by a helper plasmid that carries the functional *repA* but not the functional origin. By complementation of *repA* function *in vitro*, we located the replication initiation region of the R1 plasmid (*oriR*) to a 206-base-pair (bp) region that is completely separate from *repA*.

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MATERIALS AND METHODS

***Escherichia coli* Strains and Plasmids.** The strains and plasmids used are W3110 and C600 (from R. Fuller), MC1061 (20) and P3478 (*polA1*) (laboratory stock), and C2110 (*his rha polA1*) (from R. Kolter); JM83 Δ(*lac, pro*) (φ80 *lac*ZΔM15) and JM101 Δ(*lac, pro*)/F′ *lac*IqZΔM15 *pro*$^+$ were used as hosts for the pBR322-derived cloning vehicles pUC8 and pUC9 (21). R1 plasmid and its derivatives used are pEO1562 (wild-type mini-R1) (8), pMOB45, and pBEU17 [runaway replication plasmid (22, 23)].

Preparation of Cell-Free Extracts. Fraction I was prepared by the freeze/thaw lysis method of Staudenbauer (24); this fraction usually contains 25–30 mg of protein per ml.

Assay for *in Vitro* DNA Synthesis. The conditions for *in vitro* DNA synthesis, originally developed by Diaz *et al.* (18) and modified by R. Fuller, were used in this work. The standard reaction mixture, 25 μl, contained 40 mM Hepes·KOH (pH 8.0); 40 mM KCl; 11 mM magnesium acetate; 2 mM ATP, 500 μM each of GTP, CTP, and UTP; 100 μM each of dATP, dCTP, dGTP, and dTTP, with [*methyl*-^{3}H]dTTP at 40 cpm/pmol of total deoxyribonucleotide; 2 mM dithiothreitol; 20 mM creatine phosphate; 5% polyethylene glycol 8000; 200 μM each of 20 amino acids; 100 μg of creatine kinase, 27 μg of calcium Leucovorin (folinic acid), 100 μg of *E. coli* tRNA, 27 μg of β-NADP, 27 μg of flavin-adenine dinucleotide, and 100 μg of bovine serum albumin per ml; 500 μM cAMP; 5–10 nmol (as nucleotide) of DNA template; and 150–200 μg of *E. coli* proteins (fraction I). The reaction mixture was incubated for 10 min at 0°C, and the incubation was continued at 30°C for 60 min. DNA synthesis was expressed as the amount of total deoxyribonucleotide incorporated into acid-insoluble material.

Nucleotide Sequence Determination. The end points of the deletions were determined by nucleotide sequence assay according to the modified procedure of Maxam and Gilbert (25, 26).

RESULTS

Cloning of the R1 Plasmid Replication Region into a ColE1-Type Vector. In order to facilitate analysis of the R1 replication region and preparation of plasmid DNA, various R1 plasmid

Abbreviations: kb, kilobase(s); bp, base pair(s).

DNA segments were cloned into the ColE1-type vector pUC8. The structures of four typical plasmids are shown in Fig. 1. pREP803, a hybrid plasmid between R1 and pUC8, is capable of replicating both in *polA*$^+$ and *polA*$^-$ strains. *RepA*$^-$ plasmids pREP811 and pREP821 are derivatives of pREP803, constructed by an insertion and deletion, respectively. As expected, both pREP811 and pREP821 failed to replicate in the *polA*$^-$ strain. The starting plasmid pRP8, which was used to map the origin, is maintained both in *polA*$^+$ and *polA*$^-$ strains because the 1.8-kilobase (kb) insert in pRP8 carries all the necessary information for autonomous replication.

Origin Mapping: 3′ End Mapping. The *in vivo* replication origin of miniplasmids derived from R100 has been mapped at a position around 1,850 (4), whereas the origin of R6-5 plasmid has been located at a position around 1,400 (6). To define the replication origin more precisely, BAL-31 exonuclease deletion analysis was performed. The hybrid plasmid pRP8, which contains the R1 replication region between positions 78 and 1,848, was linearized with *Eco*RI, digested with BAL-31, and recircularized after introduction of *Eco*RI linkers. The reaction mixture was transformed into both C2110 (*polA1*) and MC1061 (*polA*$^+$). The sizes of the deletions were defined by nucleotide sequence determinations. The right end of the shortest plasmid (pRP814) that replicated in a *polA*$^-$ strain was at position 1,611, whereas that of the longest plasmid (pRP848) that was not maintained in the same strain was at position 1,497 (Fig. 2). The replication activities of these hybrid plasmids were examined *in vitro* by using a *polA*$^-$ cell extract (Table 1). Their replication properties *in vitro* were in agreement with *in vivo* replication characteristics. These results indicate that the 3′ end of the minimum R1 replication region is present within the 114-bp segment from position 1,498 to 1,611. Most of the stem-loop structures (13) in the downstream are not essential for autonomous replication of R1 plasmid. A similar observation has been reported recently for R100 *in vivo* (27).

5′ End Mapping. In order to locate the 5′ end of the replication origin, complementation of *repA* function is essential because removal of this region inactivates the *repA* gene. Previous experiments (9) indicate that the repA protein acts only in *cis in vivo*. As will be discussed later, the establishment of complementation of *repA* function *in vitro* provided a method for assaying *repA* and *ori* functions separately. Based on this assay, the 5′ end of the initiation region was located. Because the *repA*$^-$ plasmid pREP821 replicates efficiently in the presence of *repA*$^+$ *ori*$^-$ helper plasmid, the 5′ end of the initiation region should exist downstream of the *Sal* I site. A set of deletions beginning at the *Sal* I site of pREP821 and extending towards the origin was constructed (Fig. 2). Each deletion derivative was analyzed for the extent of the deletion and for replication activity *in vitro*. pREP821-53-13, whose deletion extends to position 1,423, still replicated *in vitro*. pREP821-53-20, whose deletion extends another 300 bp to the right (Table 2), did not replicate *in vitro*. These results indicate that the DNA segment carrying the information for initiation of R1 plasmid replication is present within a region of 188 bp (positions 1,424–1,611) and is completely separate from *repA*. In order to define the minimum DNA segment for initiation of replication, similar deletion derivatives were constructed from pRP814, which contains minimal *ori* DNA to the 3′ side (Fig. 2). Plasmid pRP814-8, which contains only 206 bp of R1 plasmid DNA (positions 1,406–1,611), replicated in the presence of helper plasmid. This indicates that this 206-bp DNA segment is sufficient for the initiation of R1 replication, provided that repA protein is supplied. This segment was designated *oriR* (Fig. 2). The minimum *oriR* sequence may be no shorter than 140 bp because deletion of an additional 66 bp inactivates *oriR* function (pRP814-8-4, Fig. 2; Table 2).

Identification of *in Vitro* Synthesized repA Protein. Proteins synthesized in the *in vitro* replication system were labeled with [^{35}S]methionine and fractionated by NaDodSO$_4$/poly-

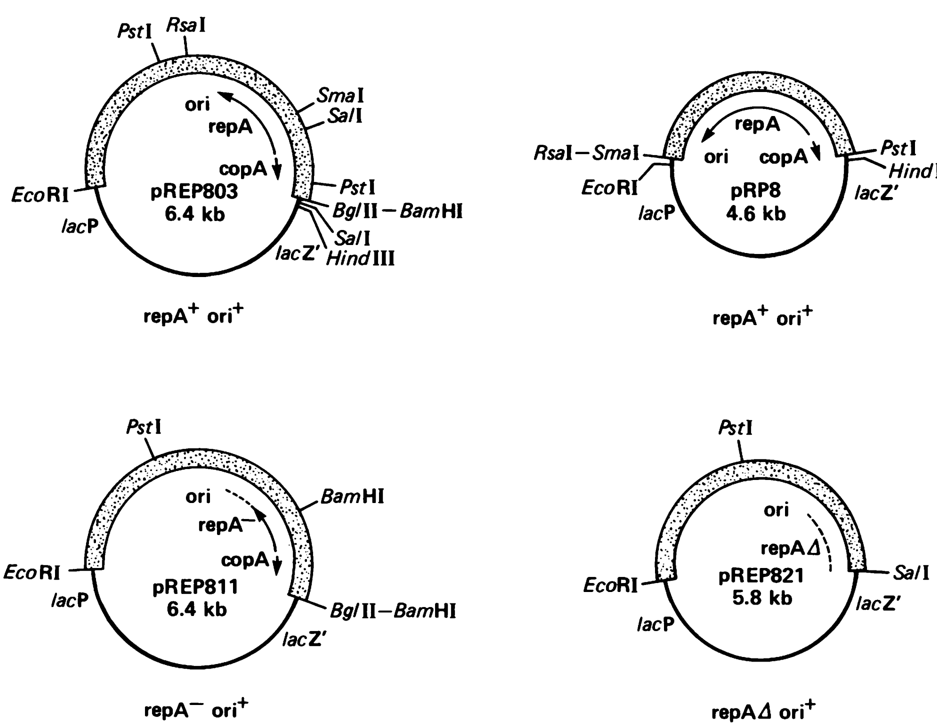

FIG. 1. Structures of R1/pUC8 hybrid plasmids. The boxed portion is derived from mini-R1-plasmid pEO1562. The 3.6-kb *Bgl* II–*Eco*RI fragment containing *copA*, *repA*, and the replication origin was cloned into pUC8 digested with *Bam*HI and *Eco*RI to generate pREP803. pREP811 was constructed by inserting an 8-bp *Bam*HI linker at a unique *Sma* I site in pREP803 located in the *repA* coding region. pREP821 was constructed by deleting a 0.6-kb *Sal* I fragment containing the *repA* promoter, *copA*, and the NH$_2$-terminal region of *repA* gene from pREP803. pRP8 has a 1.8-kb *Pst* I–*Rsa* I fragment inserted at *Pst* I and *Sma* I sites in pUC8.

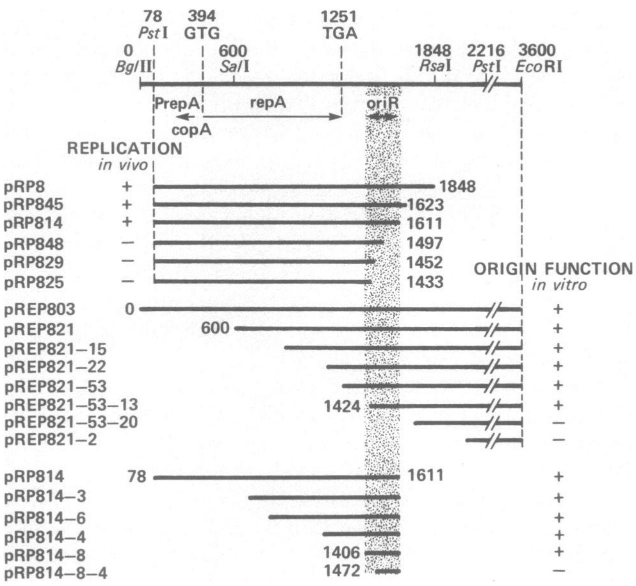

FIG. 2. The structure and replication activity of plasmids carrying deletions of variable sizes with one common end point. Solid lines represent the remaining sequences cloned in pUC8. The shaded area is the *oriR* sequence defined in the present work. The number at the end of some lines is the nucleotide position in the coordinates at the deletion end point. The pREP821 and pRP814 series were constructed by introducing deletions by BAL-31 digestion from the *Sal* I site towards the origin. pREP821 series has the common end point at the *Eco*RI site located 1.4 kb downstream of the *Pst* I site. Replication activity was measured either by maintenance of the plasmid DNAs in C2110 (*polA1*) strain (for pRP8 series) or by replication activity *in vitro* with *polA*$^-$ cell extract in the presence of helper plasmid DNA (for the pREP821 and pRP814 series).

acrylamide gel electrophoresis. Based on the following observations, we have identified protein A (Fig. 3) as the R1 plasmid-encoded repA protein. (*i*) The presence of protein A (lanes 2–4, 7, and 8) correlates with the presence of a functional *repA* gene in the plasmid DNA templates. (*ii*) pREP811, carrying a frameshift mutation in the *repA* coding region, directed the synthesis of protein B with an apparent M_r of 14,000 (lane 5). This is consistent with the nucleotide sequence data (12), which predict that the nonsense mutation within the *repA* gene would yield a smaller polypeptide (123 amino acids). (*iii*) pREP821, which has lost the *repA* promoter and the NH_2-terminal portion of the *repA* gene, did not direct the synthesis of protein A (lane 6). (*iv*) pREP901 and pREP951, carrying the *repA* gene and the *lac* promoter in the same orientation, produced ≈3 times more protein A than did pREP803 in which the *repA* gene and the *lac* promoter have opposite orientations (lanes 7 and 8).

Table 1. Replication properties of plasmids bearing deletions at the right end of *oriR*

Pladmid DNA	Maintenance in *polA1*	DNA synthesis, pmol
pREP803	+	753
pRP8	+	675
pRP845	+	317
pRP814	+	394
pRP848	−	13
pRP829	−	18
pRP825	−	22

Standard reaction mixtures containing 4.5 nmol (as nucleotide) of the plasmid DNA indicated were incubated at 30°C for 60 min with fraction I from P3478.

Table 2. *trans* complementation by *repA*$^+$ *ori*$^-$ DNA and the replication properties of plasmids bearing deletions at the left end of *oriR*

	DNA synthesis, pmol	
repA$^-$ template	Without pPR825	With pPR825
None	—	18
pREP811	31	663
pREP821	23	569
pREP821	ND	19 (+ Cm)
pREP821	ND	18 (+ Rif)
pREP821-15	21	703
pREP821-22	10	556
pREP821-53	21	589
pREP821-53-13	21	671
pREP821-53-15	26	37
pREP821-53-20	17	36
pREP821-2	13	20
pRP814-6	9	367
pRP814-3	16	392
pRP814-4	17	438
pRP814-8	18	319
pRP814-8-4	15	41
pRP814-8-7	15	32
pRP814-8-10	19	18

Standard reaction mixtures containing 2 nmol (as nucleotide) of the *repA*$^-$ template as indicated were incubated at 30°C for 80 min in the absence and the presence of 0.7 μg of *repA*$^+$ *ori*$^-$ plasmid DNA (pRP825) with P3478 fraction I. The concentrations of chloramphenicol (Cm) and rifampicin (Rif) were 150 μg/ml and 30 μg/ml, respectively. ND, not detected.

In Vitro trans Complementation of _repA_$^-$ _ori_$^+$ DNA Replication. Plasmid pREP821 (*repA*$^-$ *ori*$^+$) did not replicate either *in vivo* or *in vitro* in a *polA*$^-$ background (Fig. 4, lane 1, and Table 2). In order to discover whether the defective replication can be complemented *in vitro*, pMOB45 (a derivative of a runaway replication plasmid), carrying both functional *repA* and *ori*, was added to the reaction mixture containing pREP821. A small incorporation into pREP821 DNA was observed (Fig. 4, lane 2), but the extent was low (not more than 10% of that of pMOB45). This suggests that *in vitro* the *repA* protein also acts mainly in *cis*—i.e., on the same DNA template—and that only a limited amount of the repA protein is available to other DNA molecules. This is consistent with the *in vivo* observation that pMOB45 failed to support the replication of pREP821 in *polA*$^-$ cells (data not shown).

A striking difference was found in the extent of replication of *repA*$^-$ *ori*$^+$ plasmid templates when *repA*$^+$ *ori*$^-$ plasmids were used as a source of *repA* function *in vitro*. The pRP848 and pRP825 plasmids (*repA*$^+$ *ori*$^-$) lack part or all of the origin region but contain the *repA* coding region and its promoter intact. Synthesis of the repA protein directed by pRP848 and pRP825 templates was confirmed *in vitro* (data not shown). In the presence of pRP848 or pRP825, pREP821 (the *repA* recipients) replicated *in vitro* as efficiently as did parental plasmid (Fig. 4, lanes 3 and 4). The *repA* donors (pRP848 and pRP825 plasmids) failed to replicate in a *polA*$^-$ extract (Table 1) but supplied the *repA* protein in *trans* to initiate the replication of the *repA*$^-$ plasmid. As expected, replication was totally suppressed by addition of rifampicin or chloramphenicol (Table 2). These results demonstrate that the *repA* function is complemented more efficiently in *trans* when the R1 replication origin in *cis* position is inactivated.

R1 Plasmid Replication Is Efficient When Sufficient Amounts of the repA Protein Are Supplied. In R1 plasmid replication

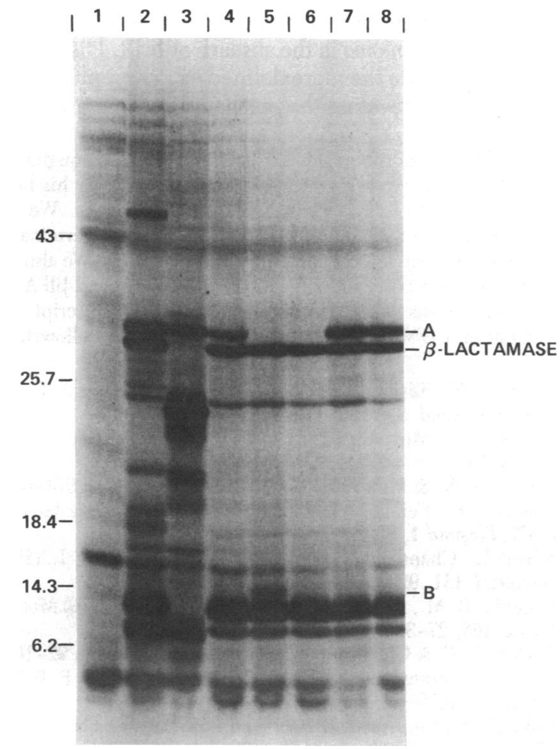

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of proteins synthesized *in vitro*. Reaction mixtures containing 20 μM [^{35}S]methionine (2,000 mCi/mmol) and 100 μM dNTPs were incubated for 30 min under the standard condition for DNA synthesis. The reactions were stopped by adding 5 μl of 1 M NaOH and incubated at 37°C for 20 min. Proteins were precipitated by addition of 1.5 ml of 5% CCl$_3$COOH; the precipitates were washed with 0.5% CCl$_3$COOH and ether, redissolved in 62.5 mM Tris·HCl, pH 6.8/10% glycerol/700 mM 2-mercaptoethanol/2.3% NaDodSO$_4$, and analyzed by 12.5% NaDodSO$_4$/polyacrylamide gel electrophoresis (28). Gels were dried and autoradiographed with intensifying screen at −70°C. Templates in lanes: 1, no template; 2, pBEU17; 3, pMOB45; 4, pREP803; 5, pREP811; 6, pREP821; 7, pREP901; 8, pREP951. M_rs are shown × 10^{-3}.

in vitro, a relatively large amount of DNA was required when a *repA*$^+$ *ori*$^+$ plasmid was used as a template (Fig. 5). With 2 nmol and 6 nmol (as nucleotide) of pBEU17 template, the extent of replication was, respectively, 3% and 10% of input DNA. This low level of incorporation may be due to the inefficient replication at the initiation or elongation stages, or both. Alternatively, a high DNA concentration may be required at the transcription level for the synthesis of the repA protein. Since *trans* complementation *in vitro* provided a method for separating the origin sequence from the *repA* gene, these possibilities were tested directly. DNA synthesis was monitored as a function of the amount of *repA* recipient (*repA*$^-$ *ori*$^+$ plasmid), with the amount of *repA* donor (*repA*$^+$ *ori*$^-$ plasmid) kept constant. The *repA*$^-$ *ori*$^+$ plasmid replicated efficiently at a low DNA concentration in the presence of *repA* donor (Fig. 5): at 1 nmol (as nucleotide) of *repA*$^-$ *ori*$^+$ DNA, as much as 50% of input *repA*$^-$ *ori*$^+$ DNA was replicated. These results indicate (*i*) that the R1 plasmid replicates very efficiently provided that sufficient repA protein is present and (*ii*) that the excess template requirement is simply for the synthesis of repA protein.

DISCUSSION

All the information for autonomous replication, incompatibility, and copy-number control of IncFII plasmids (R1, R100, and R6-5) is clustered within a small region on the genome (7–9). By comparing the nucleotide sequence of replication regions

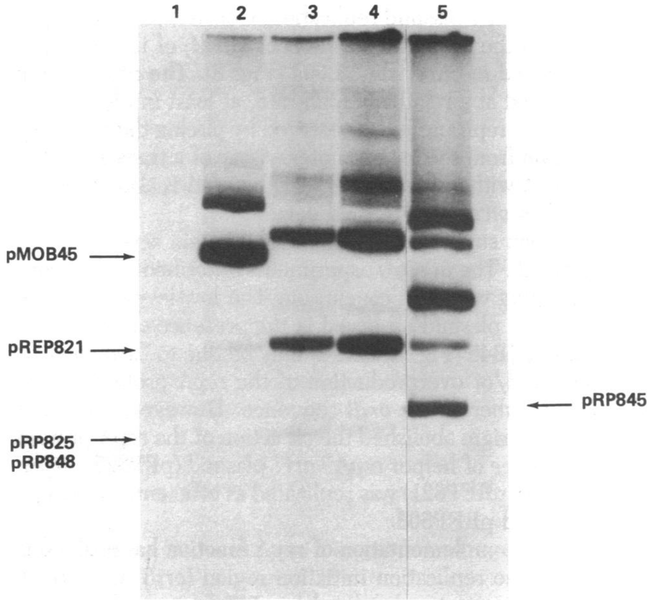

FIG. 4. Complementation of the replication of *repA*$^-$ *ori*$^+$ plasmids with a *repA*$^+$ *ori*$^-$ helper plasmid. Standard reaction mixtures containing 0.5 μg of pREP821 DNA were incubated for 75 min at 30°C in the presence of [α-^{32}P]dCTP (480 cpm/pmol) with fraction I prepared from P3478 cells. In addition to pREP821, pMOB45 (lane 2), pRP848 (lane 3), pRP825 (lane 4), or pRP845 (lane 5) was included at 30 μg/ml. The reactions were terminated by adding 0.2 ml of 10 mM EDTA. The samples were extracted with phenol, precipitated with 0.5 ml of 95% ethanol, washed once with 70% ethanol, dried, and redissolved in 20 μl containing 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, and RNase A at 100 μg/ml. The samples were analyzed by 1.0% agarose gel electrophoresis and autoradiographed.

from R1 and R100, Ryder *et al.* (12) have identified the coding frame which they named *RepA1* (*repA* in this paper). The observation that the inactivation of *repA* gene by insertion or deletion results in the loss of replication activity *in vivo* and *in vitro* (Fig. 2 and Table 2) supports the idea that repA protein

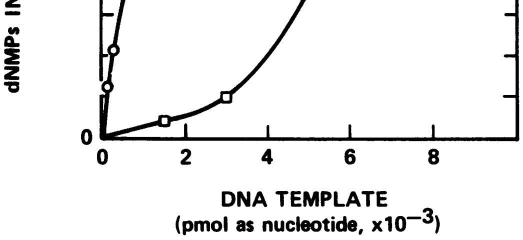

FIG. 5. Effect of plasmid DNA concentration on DNA synthesis. Standard reaction mixtures were incubated at 30°C for 80 min. (○) Reaction mixtures containing 0.6 μg of pRP925 (*repA*$^+$ *ori*$^-$), various amounts of pRP821-53-13 (*repA*$^-$ *ori*$^+$) DNA, and fraction I from P3478; □, reaction mixtures included various amounts of pBEU17 DNA with fraction I from W3110 harboring pBEU17. pRP925 was constructed by inserting the *Eco*RI–*Hin*dIII fragment of pRP825 carrying the *repA* promoter and the coding region into *Eco*RI/*Hin*dIII-digested pUC9.

is essential for R1 plasmid replication. Consistent with the prediction from the nucleotide sequence, the M_r of the repA protein identified *in vitro* was 33,000 (Fig. 3). The repA protein is synthesized at a relatively high rate, at least *in vitro*. Overproduction of repA protein is observed by placing the *repA* gene downstream from the *lac* promoter. Assay of a translational fusion of *repA* with *lacZ* suggests that the *repA* is also efficiently expressed *in vivo* (unpublished data).

It has been suggested that the repA protein acts only in *cis in vivo* (9, 12). The *in vitro* experiments described in this paper are in keeping with this conclusion. The low-level replication of $repA^-$ ori^+ plasmid observed in the presence of $repA^+$ ori^+ plasmid pMOB45 (Fig. 4, lane 2) may be due to "leakiness" of *cis* action and/or overproduction of the repA protein beyond the copy number of the *oriR* sequence. However, the inactivation of *cis* origin abolished the *cis* action of the repA protein. In the presence of helper $repA^+$ ori^- plasmid (pRP825), $repA^-$ ori^+ plasmid (pREP821) was replicated as efficiently as was parental plasmid pREP803.

The *trans* complementation of *repA* function has enabled us to separate the replication initiation region (*oriR*) from *repA*. The *oriR* sequence was localized to a 206-bp segment starting 158 bp downstream from the termination codon of *repA*. This segment is sufficient for the efficient replication of R1 plasmid in the presence of repA protein *in vitro*. These results suggest that there are few possibilities that repA mRNA acts as a primer for the initiation of R1 replication and that plasmid incompatibility is mediated by the interaction of the copA RNA with primer RNA as was suggested for ColE1 replication (29). The 206-bp segment is largely identical in both R1 and R100, suggesting that this region is important. Consistent with our *in vitro* results is the observation that deletions of part of the region between *repA* and *oriR* sequence (positions 1,328–1,358) do not impair replication activity *in vivo* (H. Ohtsubo and E. Ohtsubo, personal communication).

Based on these observations, the following features of repA protein action have emerged. Synthesis of the repA protein and the initiation of replication are normally tightly coupled on the R1 template. The *cis*-acting repA protein recognizes the origin sequence on the same template. In this way, the origin titrates the active repA protein. The inactivation of the origin uncouples the linkage, and the accumulated active repA protein can interact in *trans* with the origin sequence on a different template. Our results demonstrate unequivocally that, at least *in vitro*, the repA protein synthesized on one template interacts with the *oriR* sequence on the other plasmid to initiate replication. This result suggests that the repA protein synthesized from $repA^+$ ori^- plasmid will interact with the *oriR* sequence in *trans in vivo* as well, although we have not yet succeeded in this experiment. However, the maintenance of the plasmid carrying the *oriR* sequence upstream of *repA* in $polA^-$ cells suggests that *oriR* is also functional *in vivo* (our unpublished results).

The initial stage of R1 replication probably involves specific recognition of the *oriR* sequence by the repA protein, but the nature of the interaction and the mechanism of the *cis* action are unknown. The complementation of *repA* by *in vitro* synthesized repA protein provides the basis for an assay for repA protein synthesized *in vivo* in the absence of *oriR*. Purified repA protein could provide the more defined *in vitro* replication system essential to unraveling the mechanism of initiation.

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