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Transcription Events in the Origin of Replication of Plasmid pSC101

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Insertion mutations were isolated in the origin fragment of the plasmid pSC101 after random cleavage with DNase I. The replication properties of the resulting plasmids confirmed previous findings and extended the characterization of the essential regions. Using these plasmids, we analyzed by various methods the transcription events in the pSC101 origin. In addition to the mRNA of *repA*, a gene coding for the self-regulated RepA protein which is essential for replication of the plasmid, we characterized a transcript, which we called RNA Y, that runs in the opposite direction and that starts in the middle of the second repeated sequence in the origin region. Like the self-regulated *repA* mRNA, RNA Y is weakly expressed. It does not code for a complete protein within the origin fragment but may do so in the wild-type plasmid. We also found indications for one or, possibly, two small RNA species, called RNA X, which run in the same direction as RepA and which are partially complementary to RNA Y. We postulate that RNA Y and, possibly, RNA X are implicated in the initiation of replication of pSC101.

Plasmid pSC101 is a naturally occurring plasmid which was isolated from *Salmonella panama* (9, 10). It is 9.2 kilobases in length and confers tetracycline resistance on its host. Its replication is unidirectional and proceeds in the absence of DNA polymerase I (6). It has an absolute requirement for two host proteins, DnaA and IHF (integration host factor) (12, 16, 19, 41), as well as for the plasmid-encoded RepA protein. The DnaA protein is involved in the initiation of replication of the bacterial chromosome (5, 14, 21). IHF is needed for integration of bacteriophage lambda DNA into the chromosome (35) and intervenes in a number of cellular functions, although it is not essential for growth (see reference 13 for a review).

All of the genes that are essential for replication of pSC101 are located on a 2.2-kilobase *Hin*cII-*Rsa*I restriction fragment (Fig. 1). Mutations caused by the insertion of Tn*1000* into this fragment have been used to map its major components (27). This study, as well as other studies (1, 34, 43, 47, 48), has delimited three main regions. They are *par*, a locus involved in plasmid partitioning; *ori*, the locus where replication starts (6) and which contains sequences required for replication; and *repA*, which encodes the RepA protein. Within *ori* is a shorter segment which, when cloned onto a high-copy-number vector, inhibits replication of a pSC101 plasmid in *trans* and thus defines an incompatibility locus, *inc*.

The origin region shows two striking features (Fig. 1): an AT-rich region composed of a long stretch of T residues followed by a stretch of A residues; these two segments are separated by an IHF-binding site and are preceded by a DnaA-binding site and followed by three 20-base-pair direct repeats (8, 43). Bramhill and Kornberg (5) pointed out the presence of two tandem repeat sequences, located 7 base pairs from the DnaA-binding site, which fit the consensus sequence that is proposed to be an entry site for the DnaB helicase in the *Escherichia coli* chromosomal origin, *oriC*. The three 20-base-pair repeats are the major components of the incompatibility locus (8, 27).

The RepA protein is *trans*-acting, and its synthesis is autoregulated at the level of transcription (28, 45, 46). Vocke and Bastia (44) have observed that a RepA–β-galactosidase fusion protein binds specifically to the three direct repeats within the minimal origin, as well as to related sequences which overlap the *repA* promoter. Binding of RepA to the promoter displaces RNA polymerase (45).

The regulation of plasmid replication is a complex problem that is still largely unexplored. In a number of cases, small RNA molecules have been found to be involved in plasmid replication: as a primer initiating replication, as a regulator of the expression of a replication gene, or as a regulator of the priming event itself (see references 38 and 17 for reviews). We have sought to identify transcription events in the pSC101 origin of replication by several approaches. We have cloned DNA fragments into the promoter probe plasmid pKO-4 (30) and monitored expression of the galactokinase activity caused by transcription initiating within the cloned fragment, we have performed Northern blot analysis and RNase protection experiments (32) to identify transcripts produced from intact pSC101 plasmids, and we have used primer extension experiments (22) to identify the 5′ ends of these transcripts.

The results of this study show that there are three classes of transcripts: the *repA* gene transcript; a transcript, which we call RNA Y, that runs across the origin in the direction opposite that of the *repA* transcript; and, possibly, two small transcripts running in the same direction as *repA* and complementary to regions of RNA Y.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media. Cultures were grown at 37°C in LB medium (26). The following antibiotics were used at the indicated concentrations: tetracycline, 25 μg/ml; ampicillin, 25 μg/ml; spectinomycin, 100 μg/ml. To test for the presence of the *polA* mutation, LA plates containing 0.05% methyl methanesulfonate were used. M9 medium supplemented with 0.4% (wt/vol) glucose and 0.2% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.) was used for galactokinase assays.

DNA methodology. Enzymes were purchased from commercial suppliers, and reactions were carried out under the recommended conditions. Cloning procedures, transforma-

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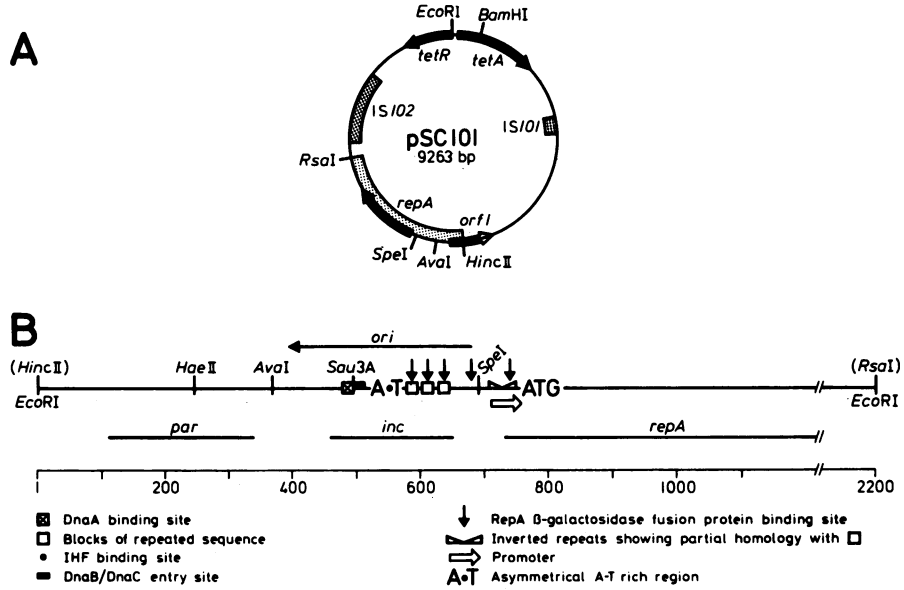

FIG. 1. (A) Physical map of plasmid pSC101 and location of relevant restriction sites. (B) Relevant features of the pSC101 origin fragment (*Hin*cII-*Rsa*I). bp, Base pairs.

tion in *E. coli*, and preparation of plasmid DNA were performed as described previously (27).

Isolation of omega insertions. DNA of plasmid pLC712 (see Fig. 2) was digested with DNase I in the presence of Mn^{2+}, as described by Schenk et al. (39). After phenol extraction and ethanol precipitation, 1 μg of this DNA (20 to 30% linear molecules) was treated with the large fragment of DNA polymerase I in a 30-μl reaction containing 10 mM Tris hydrochloride (pH 7.4), 50 mM NaCl, 10 mM $MgSO_4$, 6 mM mercaptoethanol, 16 μM of each of the deoxynucleotide triphosphates, and 0.5 U of enzyme. After incubation at 15°C for 15 min, the reaction was terminated by phenol extraction and ethanol precipitation. We found this polymerase treatment to be critical for a good yield of plasmids carrying insertions. DNase I-treated DNA contains many single-stranded nicks, and incubation for longer periods of time or in the presence of higher amount of enzyme results in significant strand displacement with a subsequent reduction in the transforming efficiency. The purified omega fragment (37) was ligated with the linear pLC712 molecules (see Fig. 2). Plasmids containing insertions of the omega fragment were recovered by transformation of strain DH1 and selection on plates containing ampicillin, tetracycline, and spectinomycin.

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Genotype or phenotype	Source or reference
Bacteria		
C600	F⁻ *thiA thrA leuB6 lacY1 tonA21 supE44*	R. Appleyard
DB841	F⁻ *thy polA1 endA*	D. Berg
DH1	F⁻ *recA1 endA1 gyrA96 thiA thrA hsdSR17* (r_K^- m_K^+) *supE44 relA1*	18
BW360	*polA1 zig-219*::Tn*10*	40
N100	*galK recA pro rpsL* (Sm^r)	30
Plasmid		
pKO-4	Ap *galK*$^+$	30
pKG1800	Ap *galK*$^+$	30
pSC101	Tc	10
pLC712	Ap Tc	P. Linder, unpublished data
pGB2	Sp/Sm	7
pHP45-Ω	Ap Sp/Sm	37
pLC716	Cm	T. Goebel, unpublished data
pSP64, pSP64	Ap	32

Replication tests. The capacities of the different insertion mutants to replicate were tested by measuring their efficiency of transformation of the *polA* strains DB841 and BW360. In these strains the pBR322 origin of replication cannot function, and transformation depends on the activity of the pSC101 replication origin carried by the plasmid. To test whether a replication defect could be complemented in *trans*, BW360 containing a Cm^r derivative of pSC101, pLC716, was transformed and selection was made for both the incoming and the resident plasmid.

Plasmid constructions. Natural restriction sites and restrictions sites provided by random omega insertions were used to subclone pSC101 origin into the promoter probe plasmid pKO-4 (30) (see Fig. 3 and 4) or into plasmids pSP64 and pSP65 (32).

Galactokinase assays. The plasmids described above were introduced into strain N100, in order to assay for galactokinase activity, as described by McKenney et al. (30).

RNA extraction. An overnight culture was diluted 1 to 100 into LB medium and grown to an optical density at 600 nm of 0.8. A mixture of 3 ml of the culture sample and 300 μl of 10× lysis buffer (0.5 M Tris hydrochloride [pH 6.8], 20 mM EDTA, 10% sodium dodecyl sulfate) was boiled for 2.5 min and chilled at room temperature for 5 min. Then, 200 μl of 3 M sodium acetate (pH 5.2) was added. The preparation was extracted by vortexing it for 2.5 min with 1.2 ml of water-saturated phenol and then with 1.2 ml of chloroform. After it was spun at 6,000 × *g* for 5 min, 3 ml of water-saturated phenol-chloroform was added to the supernatant and was vortexed for 2.5 min. After ethanol precipitation, the RNA

was suspended in 500 μl of water, digested with RQ1 DNase (20 μg/ml; Promega-Biotec, Madison, Wis.) for 20 min at 37°C, extracted with phenol, and precipitated with ethanol. The final pellet was suspended in 10 mM Tris hydrochloride (pH 8)–1 mM EDTA at a concentration of 2 μg/μl.

Northern blot analysis. Northern blot analysis was performed by the method of Khandjian (24).

Preparation of labeled probe. Linear DNA templates (2 μg) were transcribed in 20 μl of a solution containing 40 mM Tris hydrochloride (pH 7.5); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM dithiothreitol; 20 U of Rnasin; 0.5 mM each of ATP, UTP, and CTP; 0.1 mM GTP; 10 μM ^{32}P-GTP; and 10 U of SP6 polymerase for 60 min at 40°C. GTP instead of UTP was used as a label because the AT-rich region of the DNA template causes a premature stop of transcription if the UTP concentration is too low (unpublished data). The DNA template was removed by incubation with RQ1 DNase (20 μg/ml) for 20 min at 37°C. The reaction was stopped by adding 20 μl of stop mixture (1% sodium dodecyl sulfate, 10 mM EDTA, 2 mg of phenol-extracted tRNA per ml) and heating for 5 min at 70°C. Purification of the probe was achieved by running the sample through a column (Bio-Gel P60; Bio-Rad Laboratories, Richmond, Calif.). When it was used for protection experiments, the probe was ethanol precipitated and suspended in 50 μl of hybridization mixture, which contained 80% formamide, 40 mM PIPES, [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.7], 0.4 M NaCl, and 1 mM EDTA.

Protection experiments. The cell RNA extracted as described above (1 μg) was dried and suspended in the hybridization mixture (see above), and then 1 μg of a ^{32}P-labeled RNA probe prepared as described above was added. The sample was heated for 5 min at 80°C for denaturation and was immediately incubated overnight at 30°C. Digestion with 0.1 mg of RNase A at 25°C for 1 h in 300 μl of buffer containing 0.3 M NaCl, 0.01 M Tris hydrochloride (pH 7.5), and 8 mM EDTA was followed by incubation for 20 min at 35°C with 4 μl of proteinase K (10 mg/ml) and 20 μl of 10% sodium dodecyl sulfate. Proteins were then extracted with 300 μl of water-saturated phenol-chloroform. Carrier tRNA (5 μg) was added before ethanol precipitation. Reaction products were suspended in 12 μl of loading dye (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol), denatured for 5 min at 80°C, and chilled on ice before loading. The running gel was either a 6% acrylamide–7 M urea gel (thickness, 0.8 mm) or a sequencing gel of 6% acrylamide–8.3 M urea.

Synthesis and labeling of the 5′ ends of oligonucleotides. Synthetic oligonucleotides were made by using a DNA synthesizer (model 381A; Applied Biosystems). When needed, they were labeled by phosphorylation of the 5′ ends with T4 polynucleotide kinase. The reaction was performed in a 10-μl solution containing 0.5 μM oligonucleotides, 3 μl of [γ-^{32}P]ATP (3,000 Ci/mmol, 10 mCi/ml), 1 μl of 10× PNK buffer (0.5 M Tris hydrochloride [pH 7.5], 0.1 M $MgCl_2$, 1 mM spermidine, 1 mM EDTA), 5 mM dithiothreitol, and 1 μl of T4 polynucleotide kinase (5 μg/μl). After incubation for 30 min at 37°C, 90 μl of stop solution (0.1 M sodium acetate, 0.01 M EDTA) was added. Reaction products were run through a 1-ml column of Sephadex G25-fine in TE buffer (pH 7.5; spin column [29]). An equal volume of ethanol was added, and labeled oligonucleotides were stored at −20°C.

Primer extension. The primer extension method was adapted from that of Inoue and Cech (22). The annealing reaction was performed in 20 μl containing 10 μg of RNA, 2 μl of 5× hybridization buffer (0.5 M KCl, 0.25 M Tris hydrochloride [pH 8.3]), and 2 μl of labeled primer. The solution was incubated for 1 min at 90°C, for 2 min at 60°C, and for 15 min on ice. The extension of the primer was then performed by adding 4 μl of 5× RT buffer (0.3 M Tris hydrochloride [pH 8.3], 0.375 M NaCl, 37.5 mM $MgCl_2$, 2.5 mM dithiothreitol), 1 μl of avian myeloblastosis virus reverse transcriptase (24 μg/μl), and 1 μl of deoxynucleoside triphosphates (10 mM each) and incubating for 15 min at 50°C. After extraction with water-saturated phenol, 2 μg of tRNA carrier was added before ethanol precipitation. The pellet was suspended in 8 μl of denaturing buffer (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol).

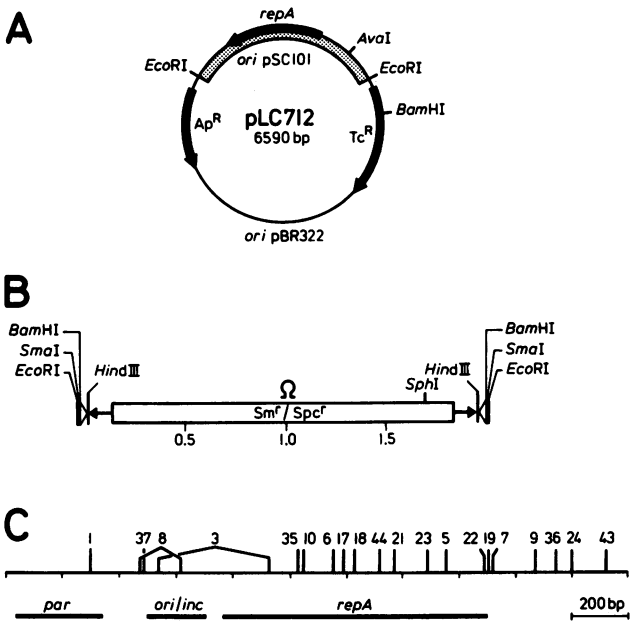

FIG. 2. (A) Physical map of plasmid pLC712. The *Hin*cII-*Rsa*I fragment of pSC101 was cloned into the *Eco*RI site of pBR322. (B) Structure of the omega element (37). (C) Sites of omega element insertions into the replication origin of pSC101. Symbols are as described at the bottom of Fig. 1. bp, Base pairs.

Computer analysis. Computer analysis of the pSC101 sequence was carried out by using the PCGENE software package of Amos Bairoch (Genofit S. A.) on a microcomputer (Vectra RS/20; Hewlett-Packard Co., Palo Alto, Calif.) and the IDEAS software package of Minoru Kanehisa on a computer (VAX 8700; Digital Equipment Corp.).

RESULTS

Isolation of omega insertions into pLC712. The pLC712 plasmid consists of pBR322 and a 2.2-kilobase *Hin*cII-*Rsa*I fragment of pSC101 cloned into the *Eco*RI site of pBR322 (Fig. 2). The pSC101 segment carries the *par* locus and all of the region essential for plasmid replication. DNA from pLC712 was made linear at random sites by digesting it with DNase I in the presence of Mn^{2+} (39); this linear DNA was ligated to a purified DNA fragment specifying resistance to spectinomycin (Fig. 2B, fragment omega [37]). Insertions were mapped to the pSC101 segment of pLC712 by digestion with *Eco*RI and *Bam*HI (the omega fragment carries a *Bam*HI site at each extremity). The map of the insertions is shown in Fig. 2.

The distribution of insertions was not random. It appeared

TABLE 2. Location of Ω insertions in the origin fragment

Plasmids	Insertion position[a]	Replication in the DB841 s	Complementation
1[b]	303–313 (R)	±	−
8[b]	477–619 (D)	−	−
37[b]	502–547 (R)	−	−
3[b]	537–926 (D)	−	−
35[b]	1028	−	+
10	1050	−	+
6[b]	1155	−	+
17[b]	1188	−	+
18	1225	−	+
44	1325	−	+
21	1370	−	+
23[b]	1489	−	+
53[b]	1602–1635 (D)	−	+
22[b]	1692	−	+
19[b]	1705	+	
7[b]	1747	+	
9	1875	+	
36	1947	+	
24	2000	+	
43[b]	2054–2121 (R)	+	

[a] Abbreviations: R, the sequence between the indicated coordinates was repeated at either side of the insertion; D, the insertion was accompanied by deletion of the sequence btween the indicated coordinates.

[b] Insertion position was determined by sequencing.

that DNase I cuts took place preferentially in regions where the local concentration (averaged over a 25-base-pair window) of A and T bases was over 60%. This might be the reason why there were few insertions in the first 1,000 base pairs, where such regions were rare, while insertions seemed to be randomly distributed in the segment of coordinates 1,000 to 2,200, where the average AT concentration was almost uniformly above 60%.

We determined the precise site of insertion of the omega fragment in several of the plasmids by DNA sequencing. In those cases in which we sequenced both sides of the insertion, we found that the insertion was accompanied by deletions or short duplications of 10 to 40 base pairs (Table 2). These presumably arose because of the combined action of DNase I and DNA polymerase; they have been observed in similar experiments in which oligonucleotides were inserted into plasmid DNA (20).

Replication of pLC712::omega plasmids. All of the omega insertion plasmids recovered were tested for replication by transformation of a *polA* strain. The pBR322 origin of replication is DNA polymerase I dependent, and thus, transformation of such a strain requires that the pSC101 replication origin carried by pLC712 be active. The results in Table 2 indicate that all insertions of the omega fragment into the regions previously defined as being essential abolished replication activity. In particular, insertions downstream of the *repA* gene had no effect on replication.

We also tested whether any of the insertion plasmids could be rescued by complementation with a wild-type plasmid. Only those insertions into the open reading frame (ORF) encoding the *repA* gene showed complementation in *trans*. Insertions 3, 8, and 37 into the *ori* region could not be complemented. Insertion 3 carried a deletion from coordinates 537 to 926 which removed the *inc* locus and the *repA* promoter. Insertion 8 carried a deletion from coordinates 477 to 619 which removed the consensus *dnaA* protein-binding site and the AT-rich segment within the *ori* region.

Insertion 37 carries a duplication of the sequence between coordinates 502 and 547 covering the putative entry site for DnaB and the first half of the AT-rich region, but excluding the DnaA box. When the transcription termination signals and the Sp^r gene of the omega fragment were excised from this plasmid, replication activity was restored to the wild-type level. Excision of the omega fragment by digestion with *Bam*HI and religation left an insertion of 10 base pairs, implying either that the structure of this region can be perturbed without affecting replication or that transcription of DNA to the right of the site of insertion from a promoter located leftward is required for replication.

Excision of the omega fragment from the other plasmids did not change their replication properties, except in the cases of insertions 35 and 23 within the *repA* gene. We did not sequence the DNA on both sides of these insertions, but the most likely explanation for the restoration of replication activity is that a deletion or duplication, coupled with the 10-base-pair insertion, restored the correct reading frame.

Cloning fragments of the origin into the promoter probe plasmid pKO-4. We have cloned fragments of the pSC101 origin into the promoter probe plasmid pKO-4 (30). If there is a promoter in the cloned fragment in the proper orientation, it should express the galactokinase gene on pKO-4. The introduction of such plasmids in strain N100 ($galE^+T^+K$) revealed whether the galactokinase gene was expressed. This subcloning was done in both orientations, in order to detect the promoters on each strand. We used natural restriction sites as well as restriction sites provided by omega fragment insertions (see Materials and Methods; Fig. 3 and 4). Two series of plasmids, X and Y, were produced. Transcription in the same direction as *repA* (rightward) in the X series or in the opposite direction (leftward) in the Y series would produce galactokinase. In our galactokinase assays the reference value was that of the original galactokinase gene promoter expressed in pKG1800. In each case we subtracted the background level obtained with pKO-4 alone and expressed the result as a percentage of the value obtained with pKG1800 (Fig. 3 and 4).

In the rightward direction (Fig. 3), the major promoter was the *repA* promoter, in plasmids 35X and GX. Because the autoregulated RepA protein was not made in plasmid 35X, its promoter was not repressed (28). The unregulated *repA* promoter was then so strong that the cells (N100) eventually died from an excess of galactokinase; the values given in Fig. 3 are therefore underestimates.

We detected, also in the rightward direction, a weak promoter between the *Ava*I site (base pair 370) and base pair 477, as shown by a low level of galactokinase activity with plasmids HX and P500X. No galactokinase activity was detected with plasmid 3X, which indicates the possible presence of a transcription stop before base pair 537. Yet, as shown by plasmid 37X, an RNA seemed to exist at base pair 547. This RNA could be driven by a second promoter before base pair 547, or the stop detected between base pairs 502 and 537 could have been due to an artifact of construction. One possibility is that the DnaA-binding site at base pair 483 functions as a transcription terminator (33) in plasmid 3X but not in plasmid 37X.

In the leftward direction no transcription was observed on the noncoding strand of the *repA* gene (Fig. 4, plasmid 3Y). A weak promoter was detected between base pair 682 (the *Spe*I site) and base pair 619 (plasmids 8Y and LY). When the fragment was extended to base pair 502, the level of transcription increased threefold (plasmid 37Y). The transcription level did not change when RepA was inactivated by an omega fragment insertion or by a deletion (plasmids 375-Ω-Y and 37-12Y). If the fragment was extended 4 base pairs to

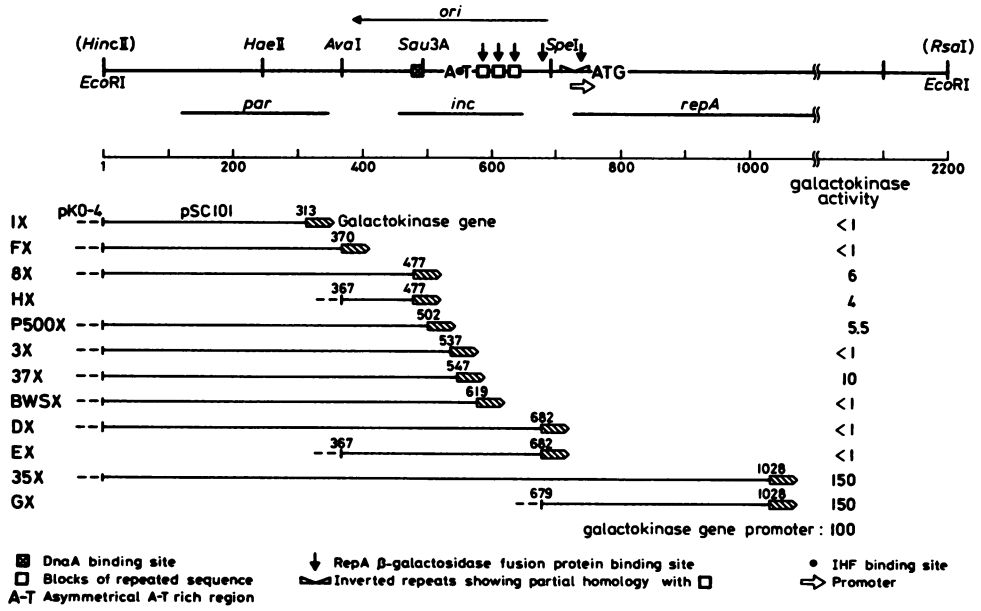

FIG. 3. Cloning of pSC101 origin fragments in plasmid pKO-4 in order to detect the presence of promoters for rightward transcription. The cloned segments are drawn to scale relative to the map of the origin region shown at the top. The solid lines represent pSC101 DNA, the dotted lines represent pKO-4 DNA, and the hatched boxes represent the galactokinase gene. The level of galactokinase synthesized by each plasmid was calculated as described in the text and is indicated here.

coordinate 498, as in the *repA* plasmid P1-12Y, the level of transcription decreased threefold. Restoration of *repA* gene activity, as in P1-37Y, did not change the transcription level. It would thus appear that the few bases near the DnaA-binding site affect the level of transcription in the pKO-4 construction plasmids. Transcription seemed to stop completely between the *Sau*3A site (base pair 498) and the *Ava*I site (base pair 367).

Northern blot analysis. In vivo analysis with the promoter probe pKO-4 involves cloning of a fragment of the origin out of its normal context. This might disturb regulatory events that occur in the wild type. By Northern blot analysis and

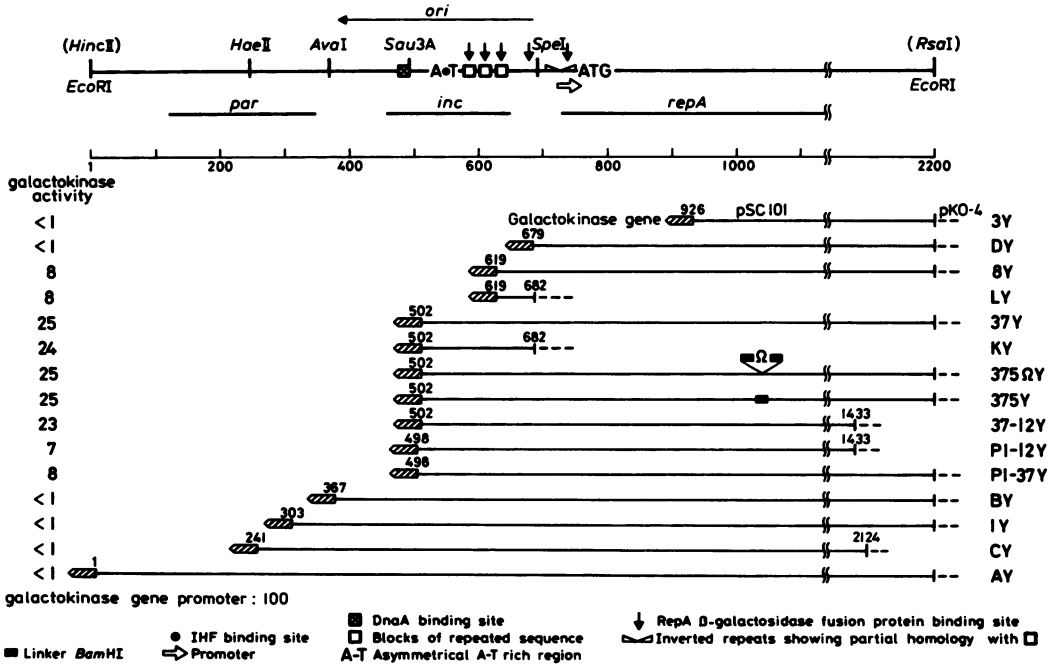

FIG. 4. Cloning of pSC101 origin fragments in plasmid pKO-4 in order to detect the presence of promoters for leftward transcription. Symbols are as described in the legend to Fig. 3. The omega element was inserted in the *repA* gene of plasmid 375-Ω-Y. Excision of the omega fragment by digestion with *Hin*dIII left a linker in the *repA* gene of plasmid 375Y.

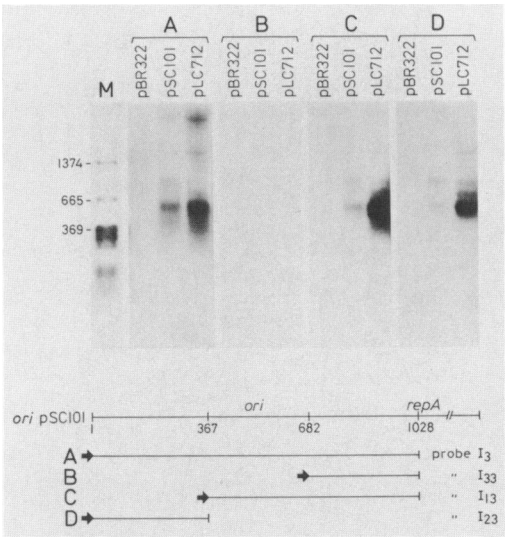

FIG. 5. Northern blot analysis for detection of leftward transcription in the pSC101 origin fragment. The maps at the bottom show the fragments that were cloned, in order to produce various probes, under the control of the SP6 promoter in plasmid pSP65 (32). Thick arrows represent SP6 promoters. From these constructions we obtained probes of single-stranded RNAs that were synthesized in vitro. RNAs extracted from cells carrying pBR322, pSC101, or pLC712 were hybridized with I_3 (A), I_{33} (B), I_{13} (C), or I_{23} (D) probes. These probes should detect the leftward transcription that occurred between coordinates 1028 and 1. M indicates the marker lane, with numbers given in base pairs.

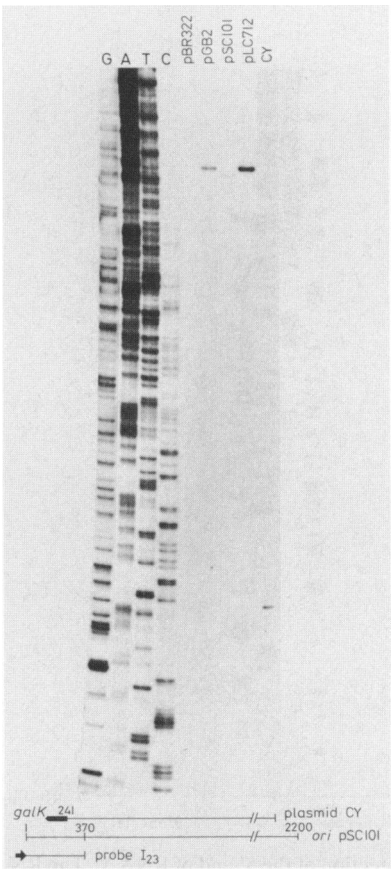

FIG. 6. Protection of RNA Y in the *par* locus by an antisense RNA probe. The products of the reactions were run on a sequencing gel. The first four lanes (G, A, T, and C) show results for a known sequence which was used as a size marker. Plasmid CY is the fusion of a fragment of the pSC101 origin that was cut with the *galK* gene at base pair 241 (see diagram at the bottom; see also Fig. 4). Lanes pBR322, pGB2, pSC101, pLC712, and CY, Protection by the single-stranded RNA probe I_{23} (see Fig. 5) of the RNA extracted from cells carrying the indicated plasmids.

hybridization with different probes of RNA extracted from cells carrying pSC101, we tried to obtain a better evaluation of the situation in the wild type. Northern blot studies were therefore performed by using total RNA from cells carrying pBR322, pSC101, and pLC712, the hybrid plasmid. The probes were antisense RNAs made in vitro under the control of an SP6 promoter, as described in the Materials and Methods. The DNA templates and the RNA probes used are shown in Fig. 5. They yielded transcripts that were able to hybridize with the leftward transcripts in the origin. Hybridization of total RNA extracted from cells carrying pSC101 and performed with the I_3 probes revealed two transcripts of approximately 600 and 900 nucleotides. When the hybridization was done with the I_{33} probe, no transcript was detected. When it was done with I_{23} or I_{13}, we saw the same two bands. This result localizes the transcription start between the *Spe*I site (base pair 682) and the *Ava*I site (base pair 367). We detected three bands with RNA extracted from cells carrying pLC712. The lower band was at approximately the same position as that found with pSC101. The upper one was slightly lower than its pSC101 counterpart. A band of intermediate position was also seen. The length of the detected transcripts suggested that, in vivo, transcription extends beyond the *par* locus.

In similar experiments we were unable to detect any transcript corresponding to the galactokinase expression observed with the X series of pKO-4 plasmids: 8X, HX, P500X, and 37X.

RNase protection and primer extension experiments. Protection experiments were performed with probes and RNAs analogous to those used in the Northern blot experiments. A ^{32}P-labeled RNA probe complementary to the test RNA was synthesized in vitro with SP6 RNA polymerase and hybridized in solution with RNA, in order to form a ^{32}P-labeled RNA-RNA hybrid. Following digestion of the single-stranded RNA extremities with RNase A, the labeled RNA that was present in the resistant hybrid was detected by gel electrophoresis. We hybridized RNA extracted from cells carrying pBR322, pSC101, pGB2, and pLC712 with the RNA probe I_{23} (Fig. 6). Assuming that RNA Y extended over the *par* region, a pSC101 fragment of 367 nucleotides should have been protected by the RNA probe I_{23}. The homology between the I_{23} probe and plasmids pGB2 and pLC712 was extended by 8 base pairs compared with the homology between I_{23} and pSC101, because of additional nucleotides that were introduced by the construction of the plasmids. This is why the protected RNA for these plasmids was slightly longer than that for pSC101.

The size of the protected RNA observed with plasmids pSC101, pGB2, and pLC712 suggested that leftward transcription continues through the *par* locus, in agreement with the sizes of the transcripts that were observed in the Northern blot experiments. In contrast, the measurements of galactokinase activity in the Y series of plasmids seemed to show the presence of a transcription stop between base pairs 498 and 366. In order to clarify this point, we studied the

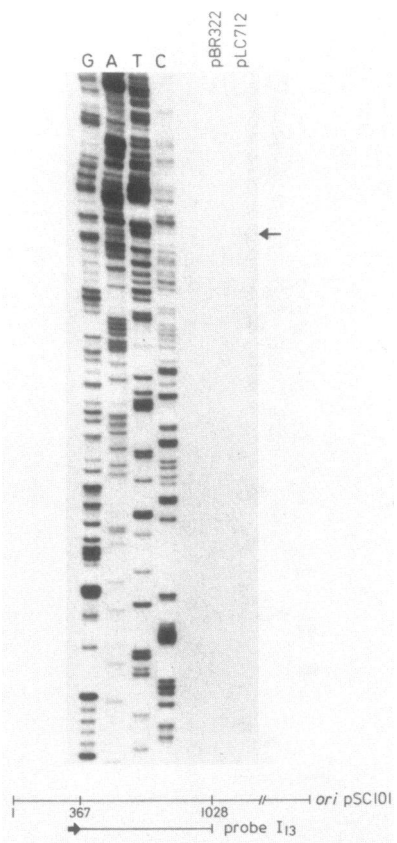

FIG. 7. Protection of the 5′ end of RNA Y. The RNA probe used was I_{13} (see Fig. 5). Lanes G, A, T, and C are the same sequences as those used as size markers in Fig. 6. Lanes pBR322 and pLC712, Protection by the single-stranded RNA probe I_{13} of the RNA extracted from cells carrying the indicated plasmids. The arrow points to the band of protected probe RNA.

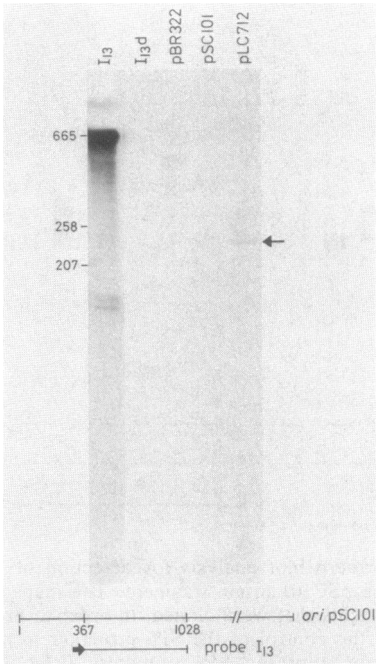

FIG. 8. Protection of the 5′ end of RNA Y. The RNA probe used was I_{13} (see Fig. 5). The conditions were similar to those described in the legend to Fig. 7. The presence of RNA Y in the wild-type pSC101 plasmid is demonstrated here. Lane I_{13}, Probe RNA alone incubated in the hybridization buffer; lane I_{13}d, probe RNA alone incubated in the hybridization buffer and digested with RNase A; lanes pBR322, pSC101, and pLC712, protection by the single-stranded RNA probe I_{13} of the RNA extracted from cells carrying the indicated plasmids. Numbers to the left of the gels are in base pairs. The arrow points to the band of protected probe RNA.

protection by the I_{23} probe of the RNA synthesized from the CY plasmid (Fig. 4). This is a fusion plasmid in which the pSC101 fragment is joined to the *galK* gene at coordinate 241. Protection of the RNA extracted from cells carrying CY by the I_{23} probe is shown on Fig. 6. A 137-base band was detected. Taking into account the specific cleavage by RNase A at bases U and C and the fact that the homology between I_{23} and CY was 130 base pairs, we expected to observe a protected fragment of 137 bases if transcription continued through the *par* locus. A fragment of this size was observed (Fig. 6). The reasons for a premature termination of the RNA in the pKO-4 plasmids are not obvious.

In order to localize the 5′ end of RNA Y, we hybridized the RNA extracted from cells carrying pBR322 or pLC712 with the RNA probe I_{13}. A precise evaluation of the size of the protected fragment was obtained by running the products of the reaction together with a known sequence (Fig. 7). The band observed in pLC712 was 249 nucleotides. Because of the nature of the sequence in this region and because of the specificity of cutting of RNase A, the 5′ end could not be located exactly but was estimated to lie within the second 20-base-pair repeated sequence found in the origin of replication. The same band could be observed with pSC101 RNA (Fig. 8), indicating the existence of this transcript in the wild-type plasmid.

To determine precisely the 5′ end of the RNA Y, we performed a primer extension experiment. We hybridized a synthetic oligonucleotide corresponding to coordinates 498 to 508 of the sequence of RNA extracted from cells carrying pBR322 and pLC712 and we extended it with reverse transcriptase. Dideoxy sequence reactions done on the same pLC712 plasmid with the same oligonucleotide were run together with the denatured product of the reaction (49). Figure 9 shows that the transcript begins at coordinate 606, within the second 20-base-pair repeated sequence. A potential promoter structure corresponding to this start site is present upstream and is shown in Fig. 10.

As with the Northern blot experiments, we were unable to identify, using either RNase protection or primer extention, any rightward transcript corresponding to the galactokinase expression observed with the X series plasmids 8X, HX, p500X, and 37X. Either the amount of transcript was too low or its size was too small for the methods that we used.

Demonstration of promoter activity within the repeated sequences RSII and RSIII. A 51-base-pair DNA fragment comprising base pairs 603 to 654 of the origin sequence, i.e., the entire repeated sequences RSI and RSII along with their intervening sequence (Fig. 10), flanked by a *Bam*HI site on the left and by a *Hin*dIII site on the right, was synthesized. The first four bases of this fragment therefore corresponded to the 5′ end of RNA Y. This fragment was cloned into the pKO-4 promoter probe vector in the proper orientation. Cultures of strain N100 containing the resulting plasmid gave red colonies on MacConkey galactose agar. GalK assays were performed as described above (see Materials and Methods) on three independently isolated clones and showed the presence of galactokinase activity at a level of

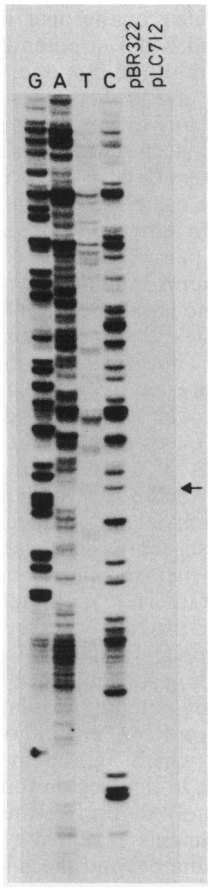

FIG. 9. Primer extension experiment. Lanes G, A, T, and C, Sequence of plasmid pLC712 done with the primer 5'-CACAGGGC TGGCATCT (base pairs 488 to 503); lanes pBR322 and pLC712, extension of the same primer hybridized with RNA extracted from cells carrying the indicated plasmids. The arrow points to the extended primer DNA.

approximately 10% of that given by the *galK* promoter. We concluded that this 51-base-pair fragment contains an active promoter.

DISCUSSION

Omega fragment insertions. We introduced insertions of the omega fragment into the *Hin*cII-*Rsa*I fragment of pSC101 by cleaving the DNA with pancreatic DNase I in the presence of Mn^{2+} ions. This procedure results in cleavage of the double-stranded DNA (31). Sequence analysis of the junctions in the resulting insertion mutations revealed two drawbacks to the use of this technique as a general method for producing insertion mutations.

First, the double-strand breaks do not occur at random. Since the technique of DNase I footprinting was first introduced (15), it has been apparent that there is considerable heterogeneity in the rates of cleavage of individual phosphodiester bonds by pancreatic DNase. The distribution of the insertion mutations that we isolated suggests, in fact, that certain regions of a long template molecule are preferentially cleaved by DNase I. We recovered a single insertion mutation in the first 480 base pairs of the pSC101 segment which is 50% AT, on average, but we recovered 16 insertions between coordinates 920 and 2000 which is 60 to 70% AT, i.e., 1 insertion every 67 base pairs. Most of the insertions occurred at sites with a higher than average AT content; thus, it seems that DNase I has a marked preference for AT-rich regions.

The second drawback was that all the insertions were accompanied by short deletions or duplications (20). We do not know the precise mechanism that generated these alterations, but during incubation with DNase I there was extensive single-strand cutting. By examining molecules in an electron microscope, we observed that strand displacement occurred in the DNA that was treated with DNase I during incubation with the Klenow fragment of DNA polymerase I, particularly at high polymerase concentrations.

Deletion analysis and subcloning experiments have shown that the sequence that is absolutely required for pSC101 replication extends form coordinates 462 to 628 (27, 47; unpublished data). These results were confirmed by the analysis of the omega fragment insertions described here. Deletion mutations which removed part of this segment (deletions 8 and 3) were defective in replication and could not be complemented in *trans* by plasmids which supplied the RepA protein.

We found that the entire sequence of the *repA* gene is required for replication activity. Insertion 22, which was located immediately before the penultimate codon of the *repA* gene, abolished replication. This result was somewhat surprising, since replacement of the final codon does not affect replication (43), but is consistent with the observation that deletion of the final 26 codons inactivates replication (43). The recent observation that a mutation in the third codon of the gene alleviates the requirement for the IHF protein for replication (4) suggests that the entire coding sequence of the protein, rather than a smaller DNA-binding domain, is required for overall activity.

Transcription events in the origin. To detect the presence of promoters in the origin region, our first approach was to clone origin fragments in the pKO-4 promoter probe plas-

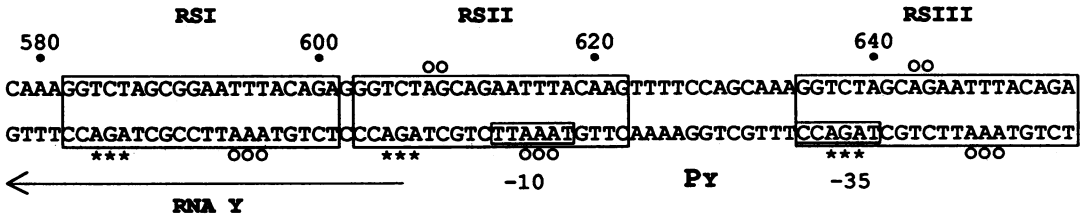

FIG. 10. Location of the 5' end of RNA Y and of its putative promoter. The boxes indicate the positions of the repeated sequences RSI, RSII, and RSIII. A synthetic DNA fragment containing the sequence from base pair 603, the first base of RSII, to base pair 654, the last base of RSIII, was found to have promoter activity. Nucleotides with which RepA interfered strongly (asterisks) and weakly (circles) with DNA methylation are indicated (data were from Vocke and Bastia [44]). Py, RNA Y promoter.

mid. If the cloned fragment contains a promoter, it expresses the *galK* gene; the assumption was that the enzyme level is related to the strength of the promoter. In fact, several types of artifacts were inherent to the method. In our case, the presence or absence of important regulation sites, such as the DnaA-, the RepA-, and the IHF-binding sites or the perturbation of secondary structures in the DNA or RNA could reveal or mask the expression of potential promoters or could introduce spurious termination signals. The observed strength of a promoter in this system could differ significantly from that in the wild-type plasmid. Moreover, the length and the nature of the DNA sequence between the promoter and the *galK* gene could influence the transcription rate, while those of the mRNA could influence the translation rate. Nevertheless, this approach remains useful in providing a convenient first assay for detecting the presence of promoters in the region of concern.

Leftward transcription. The region of pSC101 extending between the *Ava*I site (base pair 367) and the *Spe*I site (base pair 680) contains structures that are found in several other replication origins, although they are rarely found simultaneously: three repeated sequences which are binding sites for the RepA replication protein, an AT-rich region, a DnaA-binding site, an IHF-binding site, and sequences homologous to regions of the *E. coli* chromosome replication origin. The cloning of pSC101 origin fragments in the pKO-4 plasmid revealed the existence of a weak promoter that was localized in the repeated sequences region and that transcribed in the leftward direction, i.e., the direction of replication (Fig. 4). The level of GalK expression varied with the length of the fragment that was cloned. This modulation of expression levels was not caused by copy number variations in the plasmids; control experiments (data not shown) revealed no such variations. It could have been an artifact or an effect of the sequences that were inserted between the promoter and the *galK* gene on transcription or translation. It could also reflect the multiple protein-DNA interactions which take place in that region, with the variations being caused by the presence or absence of protein-binding sites on the fragment that was cloned. In this respect, the differences in the levels of GalK expression between the plasmids (Fig. 4) ending at base 502 (25%) and those ending at base 498 (7 to 9%) may be highly significant. The four bases by which they differed, G, A, T, and C, constitute the sole *dam* methylase site in the origin region.

Northern blot experiments localized roughly the 5′ end of RNA Y between the *Ava*I and the *Spe*I sites. Protection of RNA Y against RNase digestion by various antisense RNA probes localized the major point of initiation within the second repeated sequence. Because of the cutting specificity of the enzyme, this method left some uncertainty as to the exact length of the protected region. The 5′ end of RNA Y was precisely determined by extension of a DNA primer that was hybridized with RNA extracted from cells carrying the pLC712 plasmid. This method was the most reliable of all those we used and demonstrated that the 5′ end of RNA Y is at nucleotide 606.

The cloning of a synthetic DNA fragment containing repeated sequences RSII and RSIII in a pKO-4 plasmid revealed the presence of an active promoter within the 47-base-pair sequence upstream of the RNA Y starting point. Figure 10 shows the sequences of that segment and indicates the most likely promoter structure that was present in that region. The expression of GalK activity in the pKO-4 construction plasmid LY (Fig. 4) indicated, however, that a second promoter might be located a short distance upstream of the first one. Because of the near identity between the sequences of RSII and RSIII, a potential −10 region exists 32 base pairs upstream of the first one. Yet, neither protection experiments nor the primer extension experiment reported above showed any evidence for a species of RNA Y other than the one that started at base pair 606. Whether both potential promoters or only one is used in the wild-type plasmid remains to be established. There is no evidence for either the presence of a leftward RNA species or of promoter activity beyond base pair 682.

The presence of RepA-binding sites in the repeated sequences has been demonstrated by Vocke and Bastia (44). In Fig. 10, the nucleotides where RepA interferes with DNA methylation in RSII and RSIII are indicated. The RepA attachment sites are present over the bases coding for the 5′ end of RNA Y and over the −10 and −35 regions of its putative promoter. Yet, as pointed out above, we detected no difference in RNA Y transcription in the presence or absence of RepA. Plasmid 37Y, which produced RepA, and plasmid 375-Ω-Y, which did not produce RepA, had identical levels of galactokinase expression (Fig. 4). It may be that RepA and RNA polymerase bind to opposite sides of the DNA helix and do not interfere with each other, or that these binding sites are not usually occupied by RepA in vivo. Since, however, the pKO-4 constructions used for this test lacked the DnaA-binding site and since DnaA may have had a decisive effect on any potential regulation system, it might also be that an effect on RNA Y could only be observed in a wild-type system in which all the multiple protein-DNA interactions occurring in that region took place.

We did not accurately determine the 3′ end of RNA Y. Northern blot experiments revealed two major classes of RNA Y, both extending beyond the *par* locus (Fig. 5); this result was confirmed by protection experiments (Fig. 6). The significance of this transcription that extends beyond the replication fragment is not clear (see below).

Rightward transcription. Two considerations led us to expect a transcription running rightwards, in addition to the RepA message. One was the existence of a promoter consensus sequence around coordinate 450 revealed by computer analysis. The other was the demonstration by Linder (P. Linder, Ph.D. thesis, University of Geneva, Switzerland, 1984) of an in vitro transcript, approximately 170 nucleotides long, in the *inc* region. Using different methods, we sought to detect the in vivo presence of such a transcript. By cloning different fragments of pSC101 in the pKO-4 plasmid, we detected a weak transcription between base pairs 370 and 502, a stop between base pairs 502 and 537, and then again, a low level of transcription between base pairs 547 and 619. In spite of these indications, however, none of the other techniques used, i.e., Northern blots, protection experiments, or primer extension, was able to detect any promoter activity in that region. Either the expression of that promoter(s) is drastically repressed in vivo in the wild-type situation or the RNAs that are produced are so short that they remain undetectable. The existence of an antisense RNA in the origin of pSC101 must, therefore, remain an open question.

Conclusions. What might the role of RNA Y be? Its presence in a region that has been shown to be vital for replication would indicate a function related to that process. Its integrity is not, however, required for replication since mutations in the *par* region did not abolish replication (insertion 1), since a Tn*1000* insertion that includes a transcription stop signal at coordinate 450 (insertion 14 of Linder et al. [27]) did not alter significantly the replication properties and since an inversion of the *Ava*I-*Spe*I segment at base

pairs 370 to 682 had no marked effect (T. Goebel, unpublished data).

Computer analysis of the complete pSC101 sequence showed that the *Hin*cII site at the left end of the replication segment lies within a 180-codon ORF, ORF1, in the correct orientation so that it is transcribed by RNA Y, starting with an ATG codon at base pair 144 and preceded by a ribosome-binding site. ORF1 is followed by ORF2, a 371-codon ORF (3), with a ribosome-binding site and in the same orientation as ORF1. ORF2 presents a strong homology to the RSF1010 *mobA* sequence (unpublished data) which is involved in mobilization in that plasmid (11). A nick-relaxation site, *nic*, that is presumably involved in conjugal transfer replication during mobilization of pSC101 lies between the two reading frames (3, 36). The Northern blots in Fig. 5 show that ORF1 is transcribed partially or perhaps completely by RNA Y. Both RNA Y and the putative protein may play a role in conjugal transfer and replication. Evidence for a coupling between vegetative and conjugative replication comes from the observation that the frequency of conjugal mobilization is reduced by approximately 50-fold by conditions which inhibit vegetative replication (36). If ORF1 is indeed translated in pSC101, a fact that has not yet been established, its product is clearly not essential for replication in *E. coli* under the conditions used so far. Moreover, the structure of the message, with a 454-base leader between the 5′ end and the beginning of the coding sequence, would be highly unusual.

Another possible role for RNA Y might be that it acts as a primer, like RNA II of ColE1 (42). If that were the case, in view of the considerations presented above, the primer end would be found before the *Ava*I site and the RNA Y species observed would be abortive, unprocessed molecules. In that case, RNA X, if its existence is confirmed, could probably have a negative regulatory role by interacting with its RNA Y complementary strand. Another possible role for RNA Y might be to help open DNA in the origin and facilitate the installation of an initiation complex. Such a role for transcription events has been postulated for initiation in the origins of replication of the *E. coli* chromosome, OriC (2, 5, 25), and of the bacteriophage lambda (see reference 23 for a review). The analogies between pSC101 and OriC are striking: the presence of a DnaA-binding site, a potential DnaB-DnaC entry site, a binding site for gyrase, and an AT-rich region. Those with lambda are also strong: an AT-rich region and repeated sequences. These resemblances suggest that RNA Y could also be an "activator" of pSC101 replication.

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