The replication initiator protein of plasmid pSC101 is a transcriptional repressor of its own cistron

(DNA replication/recombinant DNA/DNA-protein interaction/transcription *in vitro*)

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ABSTRACT The plasmid-encoded replication initiator protein of pSC101 specifically repressed initiation of transcription of its own cistron from its natural promoter. Addition of the purified initiator had little or no visible effect on transcription initiated from a heterologous promoter. DNase protection experiments revealed that the RNA polymerase recognition sequence was overlapped by the initiator protein recognition sequences, which are vicinal to the replication origin. Using the labeled promoter sequence, we have performed competitive DNase protection experiments in two ways: by adding RNA polymerase and initiator protein simultaneously or by sequentially adding first RNA polymerase and then initiator protein to the DNase reaction mixture. The RNA polymerase protection pattern was recessive to that of the initiator regardless of whether the two proteins were added simultaneously or sequentially. This observation suggests that the mechanism of autoregulation is due to competition of the two proteins for the sequences in and around the promoter region. Furthermore, the sequential addition experiments raise the possibility of displacement of RNA polymerase from the promoter by the initiator protein.

The low-copy-number plasmid pSC101, discovered by Cohen *et al.* (1, 2), has become an interesting system for the study of DNA replication. The plasmid replicates unidirectionally from an unique origin (3), which is located in an 1800-base-pair (bp) stretch of DNA (4). The initiation of pSC101 replication is dependent on both the host-encoded *dnaA* protein (5) of *Escherichia coli* and a plasmid-encoded 37.5-kDa initiator protein (6, 7). The plasmid-encoded initiator has been isolated by tagging it with β -galactosidase and purifying the tagged protein by affinity chromatography through a *para*-aminophenyl β -D-thiogalactoside column (6, 8). The protein binds to five contiguous sites that are vicinal to the plasmid replication origin (8). At the lowest protein concentration, the plasmid-encoded initiator binds to a sequence that has dyad symmetry and was thought to contain the promoter of the initiator protein cistron (called $P_{pSC101-I}$ hereafter; see ref. 8).

The potential overlap of the strongest initiator protein binding site(s) and $P_{pSC101-I}$ suggested to us that the initiator protein could sterically hinder the RNA polymerase of *E. coli* from binding to $P_{pSC101-I}$, thereby autoregulating initiator synthesis at the transcriptional level. We have investigated this hypothesis by performing *in vitro* transcription experiments in the presence of various concentrations of purified pSC101-encoded initiator protein. In this paper we show that the plasmid-encoded initiator specifically repressed transcription initiated from $P_{pSC101-I}$.

On the basis of competitive DNase protection experiments, we have shown that exogenously added purified initiator protein inhibits RNA polymerase from binding to

$P_{pSC101-I}$. Using a deletion mutant and *in vitro* transcription experiments, we have assigned an upper limit to the left-hand boundary of the sequences necessary for autoregulation. We assume that autoregulation is due to the overlap of the initiator protein binding sequences and the promoter sequences. Therefore, the right-hand boundary of the initiator binding sequence assigns a provisional upper limit to the right-hand boundary of the sequences necessary for autoregulation. Hence, the overall *upper limit* of the autoregulatory region is a sequence approximately 100 bp in length.

MATERIALS AND METHODS

Bacterial and Plasmid Strains. These have been described in our previous publications (6, 8).

Purification of RNA Polymerase and the pSC101 Initiator. RNA polymerase of *E. coli* B was a gift from W. Kelley and was purified as described (9), except that at the final step the enzyme was chromatographed in a TSK 4000 (LKB) HPLC gel filtration column. The pSC101 initiator protein was purified as described (6, 8, 10).

***In Vitro* Transcription.** The transcriptions were carried out in a reaction mixture that contained 100 ng of linear template DNA, 0–50 pmol of initiator protein, 25 mM Tris·HCl at pH 7.9, 125 mM NaCl, 3.7 mM MgCl₂, 0.125 mM EDTA, 5% (vol/vol) glycerol, 1.75 mM dithiothreitol, and 5 pmol of RNA polymerase in a final volume of 20 μ l and incubated at 22°C for 5 min. Then 2.5 μ l of heparin (1 mg/ml stock) was added and incubation was continued for 2 min. The synthesis was initiated by adding 5 μ Ci (1 Ci = 3.7×10^{10} becquerels) of [α -³²P]CTP and 2 μ l of a mixture of 2 mM GTP, 2 mM ATP, 2 mM UTP, and 0.1 mM CTP. After 10 min of incubation at 22°C the transcription products in the reaction mixture were precipitated with ethanol, redissolved, and analyzed in denaturing polyacrylamide gels (11).

DNase Protection. DNase protection measurements were performed as described (8, 12, 13).

RESULTS

***In Vitro* Transcription and Localization of the Promoter for the Initiator Cistron.** To localize the promoter of the initiator protein cistron of pSC101 ($P_{pSC101-I}$), run-off transcriptions were carried out *in vitro* using a 935-bp *Sau3A* restriction fragment or a 655-bp *HinfI* restriction fragment from the minimal replicon of pSC101 (4). The *Sau3A*-935 fragment partially overlapped the *HinfI*-655 fragment (Fig. 1). Both of these fragments, on the basis of previous nucleotide sequence analyses (6), were expected to contain $P_{pSC101-I}$. As an internal control, a 1514-bp *HinfI* restriction fragment or a 637-bp *Sau3A* fragment of the plasmid pUC9 (14), both of which contain the promoter of the β -lactamase cistron (P_{amp}), was also transcribed *in vitro*. The *in vitro* transcrip-

FIG. 1. Restriction fragments used as templates in the *in vitro* transcription experiments. The arrows indicate the start point and direction of transcription. H, *Hinf*I site; S, *Sau*3A; □, pSC101 sequence; —, pUC9 sequence. (a) Two overlapping fragments, *Sau*3A-935 and *Hinf*I-655, that contained the initiator cistron promoter ($P_{pSC101-I}$). Δ_I , Right end point of the deletion d387; Δ_{II} , right end point of the deletion d443. (b) Restriction fragments *Hinf*I-1514 and *Sau*3A-637 fragments contained the β -lactamase promoter (P_{amp}).

tion reactions were carried out in the presence of [α - 32 P]CTP and under conditions described. The run-off transcripts were analyzed in 5–8% polyacrylamide/7 M urea gels in the presence of molecular weight markers (Fig. 2).

The 935-bp *Sau*3A fragment of pSC101 and the 637-bp *Sau*3A fragment of pUC9 (Fig. 1) generated an ≈ 700 -nucleotide (nt) and an ≈ 125 -nt transcript (Fig. 2, lane A), respectively. The 655-bp *Hinf*I fragment of pSC101 and the 1514-bp *Hinf*I fragment of pUC9 (Fig. 1) generated an ≈ 270 -nt and an ≈ 800 -nt transcript (Fig. 2, lane D), respectively. On the basis of these experiments, the positions of the $P_{pSC101-I}$ and P_{amp} were approximately localized to the sites indicated in Fig. 1.

To map the initiation site of transcription from $P_{pSC101-I}$ more precisely, we performed nuclease S1 mapping experiments. Unlabeled transcripts initiated from $P_{pSC101-I}$ *in vitro* were hybridized to 5'-end-labeled DNA probes and digested with nuclease S1 to remove single-stranded nucleic acids. The protected labeled DNA was sized in a DNA sequencing gel with a sequence ladder used as molecular weight marker (data not shown). On the basis of these experiments $P_{pSC101-I}$ was mapped at the coordinate 474 (Fig. 5).

Effect of the Addition of Purified Initiator on Transcription Initiated from $P_{pSC101-I}$. Mixtures of either *Sau*3A fragments or *Hinf*I fragments containing $P_{pSC101-I}$ and P_{amp} (Fig. 1) were transcribed *in vitro* with RNA polymerase of *E. coli* in the presence of (i) initiator protein of pSC101, (ii) buffer without the initiator, or (iii) heat-inactivated initiator protein. The labeled reaction products were analyzed in denaturing polyacrylamide gels. The results (Fig. 2, lanes B–G) show that the initiator protein of pSC101 specifically repressed transcription *in vitro* from $P_{pSC101-I}$ but not from P_{amp} . Thus, the ≈ 700 -nt transcript but not the ≈ 125 -nt transcript was repressed by the initiator (Fig. 2, lane C), and the ≈ 270 -nt transcript but not the ≈ 800 -nt transcript was similarly repressed (Fig. 2, lane F). This establishes the promoter specificity of the initiator-mediated repression.

We have also performed the transcription experiments by preincubating the restriction fragments with RNA polymerase in the transcription buffer before adding NTPs and equimolar to 5 times equimolar amounts (with respect to RNA polymerase) of initiator protein. Under these conditions, we obtained identical results; thus, binary complexes of $P_{pSC101-I}$ with RNA polymerase, when challenged by purified β -galactosidase-tagged initiator, repressed transcription from $P_{pSC101-I}$ without affecting the transcription initiated from the heterologous promoter, P_{amp} , under identical conditions (data not shown).

Mechanism of the Initiator Protein-Mediated Repression of Transcription. To investigate the molecular basis of the tran-

FIG. 2. Autoradiogram of analytical denaturing 5% polyacrylamide/7 M urea gels showing the run-off transcripts from the initiator protein promoter and the β -lactamase promoter. Lane A, run-off transcripts from *Sau*3A-935 (initiator cistron promoter $P_{pSC101-I}$) and *Sau*3A-637 (β -lactamase promoter). The ≈ 700 -nucleotide (nt) transcript was initiated from $P_{pSC101-I}$, while the ≈ 125 -nt transcript was initiated from the β -lactamase promoter (P_{amp}). Lane B, same as A except that dilution buffer (10 mM Tris·HCl, pH 7.4/250 mM NaCl) without the initiator protein was added; lane C, initiator protein (0.25 μ g) in dilution buffer was added to the transcription mixture; lane D, ≈ 270 -nt run-off transcript initiated from $P_{pSC101-I}$ located in *Hinf*I-655 and the ≈ 800 -nt transcript initiated from P_{amp} located in *Hinf*I-1514; lane E, similar to D except that initiator dilution buffer was added; lane F, 0.25 μ g of initiator protein added; lane G, 0.25 μ g of heat-inactivated protein added to the transcription reaction. Note the strong repression of the ≈ 700 -nt and ≈ 270 -nt transcripts by exogenously added initiator protein in lanes C and F. In contrast, the transcripts initiated from P_{amp} were not repressed by the initiator protein.

scriptional repression described above at the level of DNA-protein interaction, we have studied DNase I protection patterns of RNA polymerase, initiator protein, or a mixture of RNA polymerase and initiator protein at the $P_{pSC101-I}$ sequence.

A DNA fragment from the pSC101 origin region was 5'-end labeled at the *Alu* I site at coordinate 577 (Fig. 5) and was incubated with either RNA polymerase (0.5–5 μ g) or initiator protein (0.5 μ g) or was incubated first with RNA polymerase and then challenged with the initiator. Under these conditions, DNase I protection patterns were obtained as described in a previous section. The region of $P_{pSC101-I}$ protected by RNA polymerase of *E. coli* was revealed by the

attenuation of a few bands and a very strong enhancement of a residue at coordinate 433 and less pronounced enhancement of some neighboring bands (Fig. 3 *Left*, lanes D and E). On this particular strand of $P_{\text{pSC101-1}}$, over a wide range of concentration of RNA polymerase, the most striking effect was the enhancement of the residue at 433. In contrast, the initiator protein yielded a pattern that was characterized by enhancement at the coordinates 488 and 467 and pronounced attenuation of intensities of other residues between the coordinates 431 and 496 (Fig. 3 *Left*, lane F). When a complex of 2.7 μg of RNA polymerase and 0.1 μg of the restriction frag-

FIG. 3. DNase I fragment patterns of the region of P_{pSC101} in the presence of RNA polymerase of *E. coli* and the pSC101-encoded initiator protein. (*Left*) Autoradiogram of a 5% denaturing polyacrylamide gel. Lane A, molecular weight markers; the numbers refer to the coordinates of the sequence published in ref. 6 (see Fig. 5). Lane B, DNase I ladder of a DNA fragment that was 5'-end labeled at the *Alu* I site located at coordinate 577 (see ref. 6). Lanes C, D, and E, patterns with 0.5, 2.7, and 5 μg of RNA polymerase, respectively; the protected region is shown by the brace (}). Note the strong enhancement of the residue 433. Lane F, pattern with 0.5 μg of initiator protein (without added RNA polymerase), the protected region is demarcated within the vertical arrows. Lane G, pattern with 0.5 μg of initiator and 5 μg of RNA polymerase; note that the pattern is almost identical to that in lane F; the pattern with RNA polymerase, including the strong enhancement at 433 shown in lanes D and E, has been superseded by the pattern typical of the initiator protein. (*Right*) Autoradiogram showing DNase I digest pattern of a DNA fragment that was 5'-end labeled in the strand complementary to that shown in *Left* at the *Sau*3A site at coordinate 252. Lane A, molecular weight markers; the numbers refer to the coordinates shown in Fig. 5. Lane B, DNase-generated ladder, no protein added. Lane C, pattern with 5 μg of RNA polymerase; note the protected region marked by the vertical line between lanes C and D. Lane D, pattern with 0.5 μg of initiator; note the protected region between the two vertical arrows. Lane E, pattern with 0.5 μg of initiator and 5 μg of RNA polymerase; note the dominance of the pattern characteristic of the initiator over that of RNA polymerase. Horizontal arrows indicate enhancement due to the initiator.

ment was performed and then 0.5 μg of the initiator protein was added and the mixture was subjected to the DNase reaction, the pattern generated was similar to that generated by the initiator protein by itself (compare Fig. 3 *Left*, lanes G and F). The addition of the initiator caused the disappearance or pronounced attenuation of the strong enhancement observed at residue 433 (compare Fig. 3 *Left*, lane E with lanes F and G). The residues at 467 and 488 were enhanced both in the initiator pattern (lane F) and in the pattern obtained by the sequential addition of RNA polymerase and then the competing initiator (lane G). These two residues were not enhanced in the RNA polymerase protection pattern (lanes C, D, and E). Thus, in the presence of both proteins the initiator protection pattern appeared to be the dominant one. Our interpretation of the protection experiments are as follows. First, the domain of the $P_{\text{pSC101-1}}$ sequence protected by RNA polymerase of *E. coli* falls within the domain protected by the initiator protein. Second, the initiator protein appears to displace the RNA polymerase from the enzyme-promoter complex.

We have repeated the protection experiments by adding initiator, RNA polymerase, and $P_{\text{pSC101-1}}$ DNA simultaneously, and doing a controlled DNase I digestion. The resulting patterns were almost identical to those generated with the initiator alone (data not shown). Thus, it is very likely that initiator protein inhibited RNA polymerase from binding to $P_{\text{pSC101-1}}$, presumably due to steric hindrance.

We have confirmed the results described above by 5'-end labeling the complementary strand at the *Sau*3A site at coordinate 252 and repeating the DNase protection experiments. In this case the RNA polymerase yielded a clearly recognizable pattern of DNase protection (Fig. 3 *Right*, lane C). When first RNA polymerase and then initiator protein was added to the reaction mixture (Fig. 3 *Right*, lane E), the pattern characteristic of the initiator protein (lane D) was observed. Thus, the initiator again appeared to displace RNA polymerase from the $P_{\text{pSC101-1}}$ sequence.

Sequences Necessary for Transcriptional Autoregulation.

We have previously shown that the initiator protein of pSC101 binds to at least five contiguous sites vicinal to the replication origin (8). To determine which and how many of these binding sites were necessary for transcriptional repression, we used two deletion mutants, d387 and d443 (Fig. 5; ref. 6). These mutants cannot replicate autonomously (6). The mutant d387 has lost two out of the three 19-bp direct repeats, while d443 has lost all three of the direct repeats and a portion of binding site II (Fig. 5). Binding site I, which constitutes the strongest initiator binding sequence (8), remains intact in d443.

The mutant origin sequences were transcribed *in vitro* in the presence and absence of purified initiator protein (Fig. 4). The results of the transcription experiments showed that d387 contained a functional promoter and that transcription initiated *in vitro* from this promoter was still repressed by exogenously added initiator protein. In contrast, the mutant d443 was transcribed very poorly or not at all *in vitro*, presumably because the deletion significantly impaired $P_{\text{pSC101-1}}$.

On the basis of the transcription of the d387 mutant, it is possible to assign an *upper limit* to the left-hand boundary of the sequence necessary for autoregulation to coordinate 387 (Fig. 5). We do not have direct experimental data to assign a right-hand limit. However, it is reasonable to assume from the data presented here that the overlap of the initiator binding sites with $P_{\text{pSC101-1}}$ is the basis of autoregulation. Therefore, the right-hand limit is the right-hand border of the initiator protein binding region, which, on the basis of the results of the previously published exonuclease III protection experiments (see ref. 8), is at coordinate 496. Thus, a provisional *upper limit* to the autoregulatory sequence is from 387 to 496, or just over 100 bp.

FIG. 4. Autoradiogram of denaturing polyacrylamide gels of *in vitro* transcripts initiated from $P_{pSC101-1}$ of the mutants d387 and d443 and from the P_{amp} promoter of *HinfI*-1514 (see Fig. 1). Lanes A and D, transcription of d387 plus control (*HinfI*-1514) and d443 plus control (*HinfI*-1514) templates, respectively, without added initiator. Lanes B and E, transcription of d387 plus control and d443 plus control templates, respectively, with initiator protein. Lanes C and F, d387 plus control and d443 plus control templates, respectively, with initiator protein dilution buffer (without initiator protein). Note that d387 mutant transcription is still repressed by exogenously added initiator protein, whereas d443 is greatly reduced even without added initiator. The d443 promoter is apparently defective.

DISCUSSION

The physical proximity of the putative promoter for the initiator protein cistron of pSC101 and the initiator binding sequences reported in our previous papers (6, 8) had suggested that the protein transcriptionally autoregulates its own synthesis. This suggestion is strongly supported by the *in vitro* transcription experiments reported in this paper. The repression mediated by the initiator protein was promoter specific, as shown by the observation that the $P_{pSC101-1}$ promoter but not the heterologous P_{amp} promoter was repressed by the pSC101 initiator protein *in vitro*.

DNase I protection experiments in which RNA polymerase and initiator were simultaneously added to the reaction mixture have revealed that the initiator protein represses its own gene by preventing RNA polymerase from binding to the promoter. Experiments in which RNA polymerase is allowed to form a binary complex with the promoter sequence before the complex is challenged by adding equivalent amounts of initiator yielded patterns that were substantially similar to the ones generated by the initiator protein in the absence of RNA polymerase. These results are consistent with the suggestion that initiator protein can displace RNA polymerase from the promoter.

Using deletion mutants of the autonomously replicating fragment of pSC101, we have been able to assign an *upper limit* to the left-hand boundary of the sequence necessary for autoregulation. Although deletion data for the right boundary are lacking, one can assign an upper limit to the boundary by noting that the right-hand boundary for the initiator protein binding site is coordinate 496 (8). Since autoregulation appears to be the result of initiator binding that interferes with RNA polymerase binding to $P_{pSC101-1}$, the assignment of the coordinate 496 as the upper limit to the right-hand boundary of the autoregulatory sequence is not unreasonable. Thus the provisional estimate of the maximum sequence necessary for autoregulation is from 387 to 496 or just over 100 bp.

We have examined the transcription of the deletion mutant d443 and found that the promoter of this mutant is severely impaired. Hitherto, we have not found a deletion mutant that transcribes from $P_{pSC101-1}$ but does not autoregulate.

The results from deletion analysis show that binding sites IV and V and a part of site III are unnecessary for autoregulation and that the strongest initiator binding sites, I and II (8), completely overlap the region protected by RNA polymerase (Fig. 5).

It is interesting to note that autoregulation of initiator protein synthesis has also been observed in other systems such as *E. coli* (15) and simian virus 40 (16) and may be a general property of most initiator proteins.

The plasmid-encoded initiator probably negatively controls initiation of replication by maintaining its intracellular concentration below a critical level by autorepression. However, the protein almost certainly positively controls initia-

FIG. 5. The sequence, vicinal to the replication origin of pSC101, recognized by the initiator protein and RNA polymerase of *E. coli*. (a) The five binding sites recognized by the initiator protein are labeled I-V and are indicated as solid bars. The coordinates refer to the sequence previously published (6). Regions III, IV, and V are three tandem repeats. Region I is the strongest binding site (8). The boxed sequences are the putative canonical sequences of $P_{pSC101-1}$. The region (433-490) protected by RNA polymerase is underlined. The broken line and arrow indicate the direction of transcription of the mRNA of the initiator. The initiation point of transcription is the A residue marked by the arrow at coordinate 474. The brace indicates the initiator triplet of the initiation protein. The right-hand end points of deletions 387 and 443 are shown. Note that the initiator binding sites I and II completely overlap the sequences protected by RNA polymerase. Immediately upstream from binding site V is an A+T-rich region that is necessary for a functional origin (ref. 6 and unpublished data). (b) Binding site I, part of binding site II, and a part of the RNA polymerase binding site constitute an inverted repeat. The canonical sequences for the promoter are boxed. The brace shows the initiator triplet of the initiation protein coding sequence.

tion by mechanisms that are not clear at this time. Future experiments will undoubtedly be directed at the latter aspects of the function of the initiator protein.

Besides the strongest binding site for the initiator (coordinates 496–459; see Fig. 5 and ref. 8), which appears to be a part of the autoregulating sequence, the initiator binds to four other adjacent sites located between 460–423, 405–383, 373–351, and 350–330. The last three binding sites contain three 19-bp direct repeats (6, 8), which have been implicated in plasmid incompatibility (7). It is unlikely that the direct repeats control DNA replication simply by passively sequestering initiator protein in a nonproductive manner, thereby reducing its concentration to a critical level. Since the pSC101-encoded initiator is already autoregulated by binding to the region 387–496, it is unlikely that the level of the initiator is controlled by passive adsorption of the protein to the direct repeat sequences.

The experiments reported in this paper have been performed by using the initiator protein that was tagged with β -galactosidase. This fact raises the possibility that the presence of the β -galactosidase moiety might contribute to or alter the protection pattern. However, this is extremely unlikely, because interactions of sequence-specific DNA-binding proteins with DNA sequences do not involve the entire protein surface but are restricted to the DNA-binding domains (8, 13). Furthermore, that the β -galactosidase moiety does not affect the DNA binding properties of an initiator has been conclusively demonstrated in the case of the R6K-encoded 36-kDa protein (8, 10). Thus, we believe that tagging with β -galactosidase is an efficient and attractive way to isolate sequence-specific DNA-binding proteins and to study their interactions with DNA. To study other biological properties of the protein, there might be a need to remove the β -galactosidase moiety. We have invented techniques involving subunit association chromatography (17) or the use of a

specifically hydrolyzable linker peptide (10) to separate a target protein from the β -galactosidase moiety.

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