The *ban* Operon of Bacteriophage P1

LOCALIZATION OF THE PROMOTER CONTROLLED BY P1 REPRESSOR*

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Repression of a strong promoter localized 5' to the P1 *ban* gene is a prerequisite for cloning the *ban* operon in the multicopy plasmid pBR325. Repression is brought about by the binding of P1 repressor to the operator of the *ban* operon (Heisig, A., Severin, I., Seefluth, A. K., and Schuster, H. (1987) Mol. Gen. Genet. 206, 368-376). Binding of RNA polymerase in vitro overlaps with the operator and is inhibited by P1 repressor as shown by electron microscopy. The mutant P1 *bac*, which renders *ban* expression constitutive, contains a single base pair exchange within the operator. As a consequence, more repressor is required for the inhibition of binding of RNA polymerase, and for the electrophoretic retardation of a P1 *ban* transcript involving a 4-base pair deletion close to the operator still allows binding of repressor but not of RNA polymerase. By that means, a repressible promoter is located at the P1 map position 72 in a distance of about 2.5 kilobase pairs to the beginning of the *ban* gene.

Phage P1 codes for a dnaB analog (*ban*) protein (2, 3). Expression of *ban* in the P1 prophage is repressed by P1 repressor and is constitutive in the regulatory mutant P1 *bac* (2). An additional P1 regulatory mutation *crr* leads to an overproduction of gpb*ban* in P1 *bac crr* in comparison to P1 *bac* lysogens (4, 5). From genetic data the two regulatory mutations were found to be closely linked to the *ban* structural gene, the most probable order being *bac-crr-ban* in a hypothetical *ban* operon (4). The *ban* operon was found to be located on the P1 EcoRI fragment 3 (P1:3) and the expression of *ban* in a lambda-P1:3 hybrid phage is repressible by P1 repressor (6, 7). In order to obtain a more detailed insight into the regulation of *ban*, P1:3 and P1:3 subfragments from P1 wild-type and P1 *bac* regulatory mutant DNAs were cloned into multicopy plasmid vectors. Dissection of the *ban* operon by that means confirmed the order *bac-crr-ban* found genetically and a P1 repressor binding site was located 5' to the *crr* mutation (1). Cloning of the *ban* operon in multicopy plasmids requires the presence of P1 repressor in the cell, indicating the existence of a repressible strong promoter in the *ban* operon (1). A detailed analysis of that region now reveals an overlap of an RNA polymerase- and P1 repressor binding site. Two mutations within this site were analyzed. One of these is *bac-1* (2) which is characterized as an operator-

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*The abbreviations used are: bp, base pair(s); kb, kilobase pair(s).

Bacterial Strains, Phage, and Plasmids—Escherichia coli NY586bndA107pcoA58 and NY586(pSS7) were used as recipient strains (1). Phage stocks of lambda-P1 (obtained from N. Sternberg, E. I. du Pont de Nemours & Co.) and P1 *bac crr* (obtained from D. Touati-Schwartz) were prepared by the confluent lysis technique using E. coli C600. P1*Cam* was prepared by heat inactivation of C600-lysogen (6). Phage were purified as described (1) and phage DNA extracted by repeated phenol treatment at 60°C. pBR325 ampicillin resistance (12) was used as cloning vector. Plasmid pSS7 contains P1::7 carrying the P1*1* repressor gene (6) inserted into the single EcoRI site of plasmid pKT101 km+ (1, 10).

Binding of RNA Polymerase to DNA—Binding of E. coli RNA polymerase (Boehringer Mannheim) to plasmids or isolated DNA fragments for electron microscopic analysis was done essentially as described (11). Recombinant DNAs were linearized by restriction enzymes followed by incubation with RNA polymerase. In experiments testing the effect of P1 repressor on the binding of RNA polymerase, DNA and repressor were first incubated in RNA polymerase binding buffer (50 mM triethanolamine hydrochloride (pH 7.9), 50 mM KCl, 10 mM MgCl2) for 15 min at 37°C. RNA polymerase (0.1 units, approximately 200 ng) was added, and after 5-min incubation, the complexes were fixed for 15 min in 0.1% glutaraldehyde. Unbound repressor and RNA polymerase molecules were separated on Sepharose CL-4B (Pharmacia Biotechnology, Inc.) and the DNA-protein complexes were prepared for electron microscopy by absorption to mica as described (12). Between 100 and 150 DNA molecules were examined in each experiment by a computer program (11). In general, the results of three different restriction enzyme cuts of one DNA probe were compared in order to prove the reproducibility of the RNA polymerase binding sites.

P1 Repressor—Highly purified repressor protein (about 90% pure, 1-2 mg/ml) in RI buffer (20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM diethiothreitol, 0.1 mM EDTA, 10% (v/v) glycerol) was used. The cloning, overproduction, and purification of P1 repressor will be described elsewhere.

M13mp Cloning and DNA Sequencing—The 310-bp HincII fragment of P1:3 containing the P1 repressor binding site (see Fig. 3) was cloned in M13mp8/9 in two ways: (i) insertion into the *HincII* site of M13mp8 and M13mp9 directly (13); (ii) first, insertion into the *Smal* site of the *HincII* fragment by P1*SalI* (14), followed by excision with EcoRI and HindIII and insertion into the EcoRI- and HindIII-treated M13mp9. Cloning of the *HincII* fragment from plasmids of the pSS series (Table I) required the presence of plasmid pSS7(P1*1*) in the recipient bacteria used for transformation, that of the pAD series (Table I) did not. The complementary strand of M13mp8/mp9 recombinant DNAs were sequenced by the chain-termination method (15) using the M13 17-mer primer +20. The DNase protection ("footprinting") analysis will be described elsewhere (16).

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TABLE I

The different cloning procedures of the PI EcoRI3 DNA fragment lead to recombinant plasmids with different properties

| Cloning of PI EcoRI3 in pBR325 | Recombinant plasmid structure | Thermoresistance | Repressor requirement |
|---------------------------------|-------------------------------|-----------------|----------------------|
| via X-P1:3 hybrid phage and selection of clones in E. coli (SphI) dualts at 40°C | pAI2 | + | - |
| | pAI3 | + | + |
| | pH51 | - | - |
| | pHS2 | - | - |
| Cloning in E. coli (SphI) dualts at 30°C; PI repressor-carrying strain | pSS1 (pSS1-12) | - | (+) |
| | pSS2 (pSS2-12) | - | (+) |

Other Methods—Cloning of P1:3 in pBR325, restriction enzyme treatments, transformation of bacteria by recombinant plasmids, and DNA fragment analysis by gel electrophoresis was done as described previously (1).

RESULTS

Cloning of the ban Operon of Phage P1

We have cloned P1:3 in pBR325, restriction enzyme treatments, transformation of bacteria by recombinant plasmids, and DNA fragment analysis by gel electrophoresis was done as described previously (1).

Second Approach—A λ-P1:3 hybrid phage had been selected directly at 42°C in an E. coli dnaBts(λ) strain (6, 7). P1:3 was isolated from λ-P1:3 and inserted into the EcoRI-linearized pBR325. Upon transformation of NY58 amp' colonies were isolated directly at 40°C. Plasmid pAJ1 isolated from such transformants conferred thermoresistance to NY58 and did not require P1 repressor in the cell (Table I). Plasmid pAJ2, obtained from pAJ1 by Sphi treatment and religation (see above), retained the properties of the latter (Table I). The ability to confer thermoresistance was not due to a bac-type mutation but rather to a promoter activity which arose by an (unknown) mutation in the vector. This was shown by replacing the vector DNA in pAJ1 by new, EcoRI-linearized pBR325 DNA. The resulting plasmids pH51 and pH52, isolated at 30°C, no longer conferred thermoresistance to NY58 (Table I). However, thermoresistant mutants of NY58 (pHS1) again were found at an appreciably higher frequency than with the plasmid-free strain.

These results suggest that plasmids of the pSS series contain a P1-specific strong promoter which has to be repressed by P1 repressor. The same promoter has become inactive by mutation in plasmids of the pAJ- and pH5 series. Confirmation comes from RNA polymerase-binding studies and DNA sequence analysis as described in the next sections.

Mapping of a RNA Polymerase Binding Site Controlled by P1 Repressor

A strong, P1-specific RNA polymerase binding site was identified by electron microscopy when PstI-linearized pSS2 DNA was incubated with RNA polymerase (Fig. 1). Binding sites on the vector DNA belong to promoters of the bla and cat gene (left) and of the origin of replication (right, Fig. 1) (17, 18). Binding of RNA polymerase to the P1-specific site is strongly and selectively inhibited when the DNA is preincubated with P1 repressor (Fig. 1). The DNA-repressor complex itself is not visible under the experimental conditions. In contrast to pSS2, the strong, P1-specific RNA polymerase
P1 Repressor-controlled Operator

FIG. 1. Localization of a P1-specific RNA polymerase binding site controlled by P1 repressor. Plasmid DNAs (0.2 μg each) were linearized by PstI, and RNA polymerase binding sites were determined as described under "Materials and Methods." The histogram is divided into 100 segments, each comprising 95 bp. Dotted peak = RNA polymerase binding site controlled by repressor. The overall structure of the plasmid is shown at the bottom. Numbers in between two restriction enzyme sites represent the fragment size in kilobase pairs. B = BssHII. For the explanation of other symbols and abbreviations see the legend to Table I.

binding site does not exist in pAJ2 (Fig. 1).

When the same procedure is repeated using the EcoRI-linearized pSS2-12(bac cerr) DNA, a similar pattern of RNA polymerase binding sites emerges. Again a P1- and three vector-specific RNA polymerase binding sites are resolved (Fig. 2), and the relative strength of the different binding sites of pSS2-12 and pSS2 is nearly identical. It demonstrates that neither the bac- nor the cerr mutation changes the pattern of the RNA polymerase binding sites. However, in order to inhibit the binding of RNA polymerase to the P1-specific site in pSS2-12, about twice as much repressor is required than with pSS2 (compare Figs. 1 and 2). Similar results were obtained when the DNA was linearized with PstI (data not shown). In all plasmids tested, and irrespective of the presence or absence of P1 repressor, a minor RNA polymerase binding site about 2.6 kb downstream of the repressor-controlled binding site is observed (Figs. 1 and 2). It is not yet known whether it is relevant for the transcription of ban.

Differences in the efficiency of binding of P1 repressor to pSS2 and pSS2-12(bac cerr) were also observed by the retardation of DNA-repressor complexes during gel electrophoresis (19, 20). When the plasmid DNAs were treated with HincII and titrated with increasing amounts of repressor, a 310-bp HincII fragment disappeared and one or two bands of decreasing mobility appeared instead. Conversion to these bands of the HincII fragment from pSS2-12 requires more repressor than that of pSS2 (Fig. 3).

Correlation of RNA Polymerase- and P1 Repressor Binding Site

In order to localize the P1-specific RNA polymerase binding site more accurately, the 1.3-kb BssHII fragment of pSS2-12 was isolated and incubated with RNA polymerase. A strong binding site was identified (Fig. 4). As a control, the isolated BssHII fragment of pSS2 was incubated with repressor with and without subfragmentation by HincII to verify binding of the repressor to the 260-bp BssHII-HincII fragment (Fig. 4, inset). In parallel, the 310-bp HincII fragments of pSS2, pSS2-12 (Fig. 3), and pAJ2 were cloned into M13mp8/9 and sequenced. (It was not possible to isolate stable M13mp8 clones containing the HincII fragment of pSS2-12.) Moreover, the 32P 5’-end-labeled complementary strand of M13mp9(pAJ2) was synthesized and the double-stranded recombinant DNA
subjected to a DNase protection (footprinting) analysis in the presence of repressor as described (16, 21).

The P1 repressor protects an AT-rich region in pAJ2 comprising at least 18 bp from DNase digestion (Fig. 4). The sequence of this region coincides with a 17-bp consensus sequence found for different repressor binding sites in the genome of P1 (16, 24). The repressor- and RNA polymerase binding site are located approximately in the center of the 260-bp BssHI-HincII fragment. Both these sites and potential promoter sequences overlap (Fig. 4). The DNA sequences of pSS2, pSS2-12 (bac crr), and pAJ2 are for the most part identical, but nevertheless have characteristic differences. pAJ2, which has lost the ability to bind RNA polymerase (Fig. 1) contains a 4-bp deletion (Fig. 4) within one of the two major contact regions of the RNA polymerase molecule [22] indicating again the importance of this sequence for binding of the enzyme. In pSS2-12 (bac crr), a G → T base exchange in the sense strand (22) is located within the repressor binding site (Fig. 4), thus classifying P1 bac as an operator-constitutive mutation. Moreover, bac may be considered as a promoter-up mutation moving towards the consensus sequence of a potential -35 region (Fig. 4 and reference 22). (Transcription of the sense strand shown in Fig. 4 should proceed from left to right. The crr mutation and the ban gene are located downstream of the right HincII site as shown previously (1).)

DISCUSSION

The ban operon comprises, in clockwise direction on the P1 map, a P1 repressor binding site or operator overlapping with an RNA polymerase binding site, an unknown gene coding for a 14-kDa protein, and the ban gene (1). It is about 4.1 kb in length. The operator of the ban operon, abbreviated Op72 according to its map position (23), is one out of 11 operators so far identified in the P1 genome. The striking feature of these operators is the asymmetry of its 17-bp consensus sequence ATTCGCTCTAATAAATTT (16, 24). In order to clone the ban operon in multicopy plasmids, the operator Op72 has to be occupied by P1 repressor. Obviously, the repressor-controlled promoter itself and/or the gene product of this promoter is deleterious to the plasmid and/or the bacterial cell. (It is not yet known whether the ban operon still contains a gene(s) in between Op72 and the gene coding for a 14-kDa protein.) Although P1 repressor still binds to the operator in pAJ2, its presence is dispensable because of the loss of the RNA polymerase binding site due to a 4-bp deletion mutation. The latter presumably arose by selecting the P1:3 recombinant plasmid in NY58 at 40 °C in the absence of P1 repressor. On the other hand, the bac mutation weakens

\[ \text{J. L. Eliason and N. Sternberg, personal communication.} \]
the binding of repressor to the operator, Op72. This allows transcription of the ban gene, sufficient to suppress the dnaBts character of the host without impairing the recombinant plasmid itself. It is noteworthy that the bac mutation affects a highly conserved base pair within the operator (16), as it is known for numerous operator-constitutive mutants of phage X (25).

The increase in the mutation rate to thermoresistance of NY58(pSS7) cells harboring ban recombinant plasmids allows the isolation of P1 bac-type mutants. A sequence analysis of these mutant plasmids may lead to a better understanding of the P1 repressor-operator interaction.

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