C1 Repressor-mediated DNA Looping Is Involved in C1 Autoregulation of Bacteriophage P1*

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Bacteriophage P1 can enter two different developmental pathways after infection of a bacterial cell, lytic growth or lysogeny. The critical elements of this molecular switch are located within the immC region of the P1 genome (Fig. 1; for a review see Ref. 1). The c1 gene codes for the main repressor of lytic functions. The C1 protein exists as a monomer in solution and binds to at least 14 operators which are distributed widely over the P1 genome. From these operators a 17-bp asymmetric consensus sequence ATTGCTCTAATMAT° has been derived (2–4). Two classes of operators have been identified: monovalent operators have a single 17-bp repressor-binding site whereas bivalent operators consist of two overlapping repressor-binding sites forming an incomplete palindrome (5). C1 repressor synthesis is autoregulated via the bivalent operator Op99a,b which overlaps the promoter P99a (5). The coi gene, encoding a C1 inhibitor protein, is located immediately upstream of Op99a,b. C1 repressor is required to repress the lytic functions of a P1 prophage in vitro. Transcription of the c1 gene is autoregulated via the C1-controlled operator Op99a,b which overlaps the promoter of the c1 gene. It is negatively affected by Lxc corepressor and the DNA region upstream of c1, which contains the additional operators Op99c, d, and e. We have explored these effects by constructing a set of lacZ reporter plasmids with Op99a,b and varying parts of the upstream DNA region. Transcription levels were measured in vitro with a two-plasmid system containing the lacZ reporter and a cl-repressor plasmid. Compared to the C1+Lxc-repressed lacZ reporter with all operators present, the basal level of β-galactosidase activity increases successively when (i) upstream operators were deleted or inactivated, (ii) Lxc corepressor was removed, and (iii) C1 and Lxc were absent. By that means a 2 x 2 x 15-fold stepwise increase in enzyme activity was found. Using electron microscopy we visualized the interaction of C1 repressor with the operators in vitro, looped DNA molecules were observed. Although all operators can participate in C1-mediated DNA looping, loops between Op99a,b and Op99d occurred predominantly. Lxc is not required but increases drastically the frequency of loop formation. The results indicate that C1-mediated DNA looping may be a second element besides Lxc for fine-tuning the autoregulation of c1 transcription.

MATERIALS AND METHODS
Recombinant Plasmids—The construction of P1 immC plasmids has been described (6). In each of the plasmid series pTH7, pTH5, pTH6 and pTH51, pTH52, pTH43, an increasing part of the P1 immC DNA is deleted (Fig. 1), and the P1 DNA fragments are inserted into pUC19 (Smal-HindIII) and pCB302a (HindIII-Smal), respectively. Plasmids pTH52 and pTH51 are identical to pTH42 and pTH41 (6) with the exception that the coi reading frame was destroyed by insertion of 2 bp at the Accl site. Plasmids pTH52 and pTH51 are derived from pTH2 and pTH51, respectively, with Op99c altered by site-directed mutagenesis (see below). Plasmid pMV150 is based on the vector pKT101 (17). It contains the P1 c1 repressor gene on a 1,018-bp Mac-AuII fragment under control of the tac promoter and the P1 lac gene and promoter on a 498-bp Ddel-DraI fragment (11). Plasmid pMV192 is homologous to pMV190 but carries a P1 lac amber mutation (9). Plasmid DNA was prepared according to Clewell and Helinski (18) and purified twice by CsCl density gradient centrifugation. For the determination of copy numbers, plasmid DNAs were isolated from bacteria using the QIAGEN protocol. The DNA was cleaved with HindIII, subjected to electrophoresis in 0.7% agarose, and stained with ethidium bromide. DNA (fragment) concentration was measured using the FluorImager 575 (Molecular Dynamics).

Site-directed Mutagenesis—Site-directed mutagenesis of CsCl-purified plasmid DNA (19) was performed using the 24-mer oligodeoxy nucleotide GACATTGCCGCGACCGTTCTC to exchange three

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§ The abbreviations used are: bp, base pair(s); kb, kilobase(s); Op, operator; P, promoter.
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RESULTS

Repression of Autoregulated c1 Transcription Is Enhanced by Upstream Operators—To test the effect of the upstream operators Op99c and Op99d on the c1 autoregulation via Op99a,b, a set of reporter plasmids was constructed. Plasmids pTH43, pTH52, and pTH51 contain the lacZ gene under the control of Op99a,b and increasing parts of the upstream regulatory region (Fig. 1). In pTH52 and pTH51 the coi reading frame is destroyed in order to avoid the inactivation of C1 repressor by the coi gene product (6). Two additional plasmids, pTH52c and pTH51c, were constructed, in which the operator Op99c is inactivated by site-directed mutagenesis. β-Galactosidase activity was measured with cells harboring either the reporter plasmid alone (non-repressed conditions) or the latter in combination with the repressor plasmid pMV190 (c1‘Ixc‘) and pMV192 (c1‘Ixc‘), respectively. A repression factor was then determined as described in the legend to Table 1.

In all plasmids the transcriptional activity of promoter P99a was found to be similar under non-repressed conditions. In the presence of C1 repressor, the activity is repressed about 15-fold in plasmid pTH43, which contains only the operator Op99a,b. An additional 2-fold increase in repression (30-fold totally) is observed in plasmid pTH52 (Op99a,b-c) but not in pTH52c (Op99a,b-c). Thus, the critical element for the additional repression is the operator Op99c and not a particular DNA configuration in this part of the immC region. No significant further increase in repression was measured when either plasmid pTH51 (Op99a,b-c-d) or pTH51c (Op99a,b-c-d) instead of pTH52 was tested (Table I). Obviously, C1 repression via the upstream regulatory region does not require a particular operator but can use either Op99c or Op99d. We analyzed whether the corepressor Lxc would modulate the effect of the upstream regulatory region on c1 transcription because Lxc binds to all C1 repressor-occupied operators of the immC region and down-regulates c1 transcription specifically (5). The repression factor increased about 2-fold when a c1‘Ixc‘ instead of a c1‘Ixc‘ repressor plasmid was used. This additional repression was roughly the same irrespective of the presence or absence of upstream operators and/or a functionally active Op99c (Table I).

Quantitative determinations of the plasmid DNAs excluded the possibility that the effect of the upstream regulatory region is due to variations in the plasmid copy number. The number of pTH51 and pTH43 molecules (as measured per pMV190 and pMV192) varied by less than 20% (Table I).

Binding of C1 Repressor to Operator DNA Is Shown by Electron Microscopy—Supercoiled DNA of plasmids pTH5, pTH5, and pTH6, containing two, three, or four C1-controlled operators of the P1 immC region (Fig. 1), was incubated either with C1 alone or with C1 and Lxc. After cleavage of the plasmid with PvuII various protein-DNA complexes besides free DNA molecules were observed. The complexes consisted of (i) single DNA molecules with bound protein, (ii) two or more aggregated DNA molecules with bound protein, and (iii) DNA molecules with loops formed by the joining of operator-bound proteins. All of these complexes were observed when operator DNA was incubated with C1 repressor alone but were far more abundant in the presence of C1 and Lxc. In the latter case, loop formation was strongly favored when supercoiled instead of linear DNA was used. Therefore, and in order to mimic the in vivo situation of the immC region as far as possible, we focused on the experiments with C1 and Lxc using supercoiled DNA. Aggregates were not used for quantifications because it was often impossible to determine the path of DNA molecules unequivocally.

Representative examples of linear complexes of C1- and Lxc-treated pTH7 DNA are shown in Fig. 2. Occupation of a single operator (Fig. 2A) and the simultaneous occupation of two operators (Fig. 2B) are detectable. Even when C1 and Lxc were used in a large molar excess over DNA the repressor molecules did not bind randomly to DNA but almost exclusively to operator sites (data not shown). With C1 alone, the operators Op99a,b and Op99d, which overlap the c1 and the coi gene promoter, respectively, were occupied predominantly (Fig. 3A). Considerably smaller numbers of C1-Op99c and C1-Op99e complexes were found. The reason for the different C1 binding affinities for these operators is not yet understood. A strong binding of C1 to Op99d is indicated by the fact that in an Op99d-Op999 reporter plasmid the transcriptional activity is repressed about 100-fold by C1 repressor (6). Among the monovalent operators the decreasing number of C1-operator complexes in the order Op99d > e > c may be explained by the fact that Op99d, e, and c deviate from the 17-bp operator consensus sequence by 1, 2, and 5 base substitutions, respectively (1, 4). However, this possible correlation contrasts with the fact that C1 repressor binds strongly to the bivalent operator Op99a,b.
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E. coli CB454 bacteria (Δ lacZ lacY* galK recA56) (22) harboring either a reporter plasmid alone (−) or in combination with plasmid pMV190 (cl' lac'c) and pMV192 (cl' lac'c), respectively, were grown to OD600 = 0.6 at 37 °C. β-Galactosidase activity was then determined by the method of Miller (23). The reporter plasmids designated c' carry an inactivated Op99c. The repression factor is defined as P-galactosidase activity of the reporter plasmid under non-repressed conditions, divided by P-galactosidase activity of the reporter plasmid repressed by either cl' or c'.

Data were calculated from three independent experiments.

| Plasmid                  | β-Galactosidase activity | Repression factor |
|--------------------------|--------------------------|-------------------|
|                          | cl' lac'c                | cl' lac'c         |
| pTH51 (Op99a,b, c, d)    | 604 ± 41                 | 16.9 ± 4.2        |
| pTH51c (Op99a,b, c, d)   | 617 ± 49                 | 18.7 ± 5.8        |
| pTH52 (Op99a,b, c)       | 507 ± 43                 | 17.1 ± 3.4        |
| pTH52c (Op99a,b, c')     | 494 ± 37                 | 31.7 ± 5.7        |
| pTH45 (Op99a,b)          | 546 ± 53                 | 35.4 ± 7.8        |

Fig. 2. Electron micrographs of linear C1-Lxc-pTH7 DNA complexes. Supercopied pTH7 DNA (0.4 μg) was incubated with C1 (0.4 μg) and Lxc (0.1 μg) for 15 min at 37 °C and then prepared for electron microscopy. C1 repressor and Lxc corepressor were purified as described (11, 24). The DNA was cleaved with PvuII resulting in a 991-bp fragment containing the P1 DNA insert flanked by pUC19 DNA and a 2,364-bp pUC19 fragment. Examples of linear complexes of C1 and Lxc with the 991-bp PvuII fragment of pTH7 are shown. A, occupation of one operator, B, occupation of two operators. Magnification is approximately × 80,000 (bar = 500 bp). The 991-bp fragment is drawn schematically at the bottom of the figure. Single and double lines represent P1 and pUC19 DNA, respectively. Operators Op99a,b, Op99c, Op99d, and Op99e are shown as filled boxes.

although the binding sites a and b deviate by 3 and 7 base substitutions, respectively (11). Here, cooperativity of two C1 molecules may facilitate repressor binding and thus explain the differences in the binding frequency of mono- and bivalent operators. A drastic change in the distribution of bound proteins is observed when Lxc is present in addition to C1 (Fig. 3B). Lxc directs the majority of C1 molecules toward Op99a,b whereas binding of C1 to the other operators is strongly reduced. As will be shown later, the percentage of C1-mediated looped DNA molecules at the same time rises about 4-fold in the presence of Lxc.

When the concentration of C1 and Lxc is increased about 3-fold/operator, aggregates of two and more DNA molecules are found frequently. An experiment with pTH6 DNA containing only Op99a,b and c is shown as an example (Fig. 4). The most common type consists of two DNA molecules either cross-joined by protein(s) or with bound protein(s) on the verge to interact (Fig. 4A). When two or more molecules tend to aggregate each DNA molecule contains a protein (or proteins) bound to a single position (Fig. 4B). We will return to this point in the discussion. This tethering of one operator to the other increases the protein concentration near the second operator and generates cooperativity in the binding to the two sites (13). Therefore, if the aggregates reflect the functional interaction of operator-bound proteins then looped DNA structures should also be observed easily. This is indeed the case as shown in Fig. 4C. The size of the observed loops roughly corresponds to the distance of Op99a,b to Op99c.

C1 Lxc-mediated DNA Looping between Operators of the P1 immC Region—When plasmid DNAs containing two or more operators of the immC region were incubated with C1 and Lxc, looped structures were observed by electron microscopy. Size measurements revealed all possible combinations of two operators with the intervening DNA being looped out. The exception was the combination of Op99d with Op99e. Loops between these operators with a 83-bp center-to-center distance were not found. They may either be to small to be detected or may not exist because of the limited flexibility of the DNA. Examples of looped structures of pTH5 DNA carrying the operators Op99a,b, c, and d are shown in Fig. 5. The majority of loops was found to be of the Op99a, b → d type with a loop size of about 470 bp (Fig. 5A). In a significant portion of these molecules, a protein bead was observed additionally at the position of Op99a,b. Molecules of the Op99a,b → c (Fig. 5B) and Op99c → d type (Fig. 5C) with a loop size of about 180 and 290 bp, respectively, were observed less frequently. A total of 18% looped molecules were found among 1,150 pTH5 DNA molecules scored (Table II). When linear instead of supercoiled

TABLE I

Effect of the upstream regulatory region on the transcriptional activity of P99a

E. coli CB454 bacteria (Δ lacZ lacY* galK recA56) (22) harboring either a reporter plasmid alone (−) or in combination with plasmid pMV190 (cl' lac'c) and pMV192 (cl' lac'c), respectively, were grown to OD600 = 0.6 at 37 °C. β-Galactosidase activity was then determined by the method of Miller (23). The reporter plasmids designated c' carry an inactivated Op99c. The repression factor is defined as P-galactosidase activity of the reporter plasmid under non-repressed conditions, divided by P-galactosidase activity of the reporter plasmid repressed by either cl' or c'.

Units presented and standard deviations were calculated from the data of at least six independent experiments. Molar ratios of reporter versus repressor plasmid were 1.32 ± 0.10 (pTH51/pMV190), 1.10 ± 0.15 (pTH43/pMV190), 1.03 ± 0.10 (pTH51/pMV192), 1.04 ± 0.18 (pTH43/pMV192).

Data were calculated from three independent experiments.
plexes of C1 and Lxc with the 704-bp fragment are shown. Aggregates DNA and a 2,364-bp pUC19 fragment. Examples of aggregated complexes of C1 and Lxc with the 704-bp fragment containing the P1 DNA insert flanked by pUC19 DNA and a 2,364-bp pUC19 fragment. Examples of aggregated complexes of C1 and Lxc with the 704-bp fragment shown. Aggregates of A, two complexes, B, multiple complexes, and C, looped and linear complexes. Magnification is approximately × 80,000 (bar = 500 bp).

pTH5 DNA was used only 2 molecules out of 900 contained a loop. Even when a large excess of repressor was used (0.03 µg of linear DNA + 0.75 µg of C1 + 0.75 µg of Lxc) not more than 3.5% (86 out of 2,431) looped molecules were observed. These results indicate clearly that loop formation is stimulated by DNA supercoiling.

Among plasmids pTH6, pTH5, and pTH7, the types and frequencies of looped DNA molecules, which were formed upon incubation of the DNAs with C1 and Lxc, were compared quantitatively (Fig. 6). For each molecule the size of long tail, loop, and short tail was determined, and the positions of the DNA molecules that are in contact with the joining protein complex were calculated. These data closely correlate with the location of immC operator sites, and the background of molecules that do not match the prediction of operators joined via C1 and Lxc (0.2 pg) for 15 min at 37 °C. Cleavage with PvuII resulted in a 934-bp fragment containing the P1 DNA insert flanked by pUC19 DNA and a 2,364-bp pUC19 fragment. Looped complexes of C1 and Lxc with the 934-bp fragment are shown. DNA loops are formed between A, Op99a,b and Op99d; B, Op99a,b and Op99c; C, Op99c and Op99d. Magnification is approximately × 80,000 (bar = 500 bp).

Comparison of experiments in which pTH7 DNA was incubated either with C1 repressor alone or with C1 and Lxc. Among all pTH7 DNA molecules with bound proteins (linear complexes and loops) the percentage of looped structures increased 4-fold from 12% in the absence of Lxc, to 48% in the presence of Lxc (Table II, last and second to last column). This is to be expected since Lxc strengthens the binding of C1 to the operators so that more repressor-occupied operators are available for loop formation. However, the fact that the addition of Lxc causes far more C1 molecules to bind to Op99a,b than to any other operator (Fig. 3) reveals the preferential effect of Lxc on the occupation of Op99a,b by C1 repressor. This is in agreement with the finding that Lxc further down-regulates the C1-controlled promoter P99a (5).

**DISCUSSION**

C1 repressor mediates looping between operators of the immC region in vivo. Loop formation is favored in the presence of the corepressor Lxc. Although looping can occur between monovalent operators, it is most frequently observed between the bivalent operator Op99a,b and any of the monovalent operators, especially Op99c and d (Fig. 6 and Table II). Moreover, it can occur independently of the orientation of the operator, as shown by loop formation between Op99c → d and Op99c → e. The multitude of looped structures observed suggests that DNA looping may also occur among the numerous C1-controlled operators of P1 outside of immC.

The araC, lac, and deoR repressors of the corresponding operons of *Escherichia coli*, which have been shown to loop DNA exists as dimers, tetramers, and octamers, respectively (25–27). These repressor molecules can bind to at least two separate operators simultaneously. On the other hand, the C1 repressor of P1 exists as a monomer in solution (5). Therefore, each of the two operators which participate in the looping reaction would have to be occupied by one C1 monomer first. We...
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TABLE II
Summary of C1-Lxc-DNA complexes

| Structure                          | pTH6 (Op99a, b, c) | pTH5 (Op99a, b, c, d) | pTH7 (Op99a, b, c, d, e) | pTH7* (Op99a, b, c, d, e) |
|-----------------------------------|--------------------|----------------------|-------------------------|--------------------------|
| Free DNA                          | 27.5               | 55.7                 | 37.5                    | 73.5                     |
| Linear complex                    | 65.0               | 26.3                 | 32.6                    | 23.2                     |
| Loops                             | 7.5                | 0.9                  | 2.0                     | 0.33                     |
|                                  | a,b --> c          | a,b --> d            | c --> d                 | 1.4                      |
|                                  |                   | a,b --> e            | c --> e                 | 3.1                      |
|                                  |                   |                      |                         | 0.8                      |
|                                  | No. of molecules scored | 774             | 1,150                  | 864                     |
|                                  |                    |                      |                         | 1,523                    |

*a Structures observed in the absence of Lxc protein.

FIG. 6. Measurements of looped C1-Lxc-DNA complexes. Histograms represent the positions of the DNA molecule that are in contact with the protein bead. For this reason, there are two data points for each molecule. The PvuII DNA fragments are drawn as described in the legend to Fig. 2. Looped complexes were measured in A, 56 molecules of the 704-bp PvuII fragment of pTH6; B, 295 molecules of the 934-bp PvuII fragment of pTH5; and C, 173 molecules of the 991-bp PvuII fragment of pTH7.

expect that upon binding to the operator the repressor undergoes a conformational change which enables the molecule to interact with a second operator-bound C1 monomer. Alternatively, a C1 monomer may bind first to an operator-bound repressor molecule and then contact the second operator. The finding that all the DNA molecules which are in the process to interact with each other contain repressor protein (or proteins) bound to a single position (Fig. 4) supports the former hypothesis. In that case looping may be restricted to the interaction of two monomeric C1 molecules bound to two different operators.

The Lxc corepressor stimulates DNA loop formation in vitro (Table II). However, the enhancement of repression by Lxc in vitro is roughly the same in the presence or absence of upstream operators (Table I). This discrepancy is not yet understood. The ratio of Lxc molecules to C1 occupied operators may be lower in vitro than in vivo under the experimental conditions. If this is the reason for the discrepancy then overexpression of Lxc in vivo should yield more looped immC DNA molecules which in turn should increase the negative effect of upstream operators on the transcriptional activity of P99a. It remains to be shown whether the corepressor molecule remains bound if the C1-repressor complex is part of a looped DNA molecule or whether it is released again perhaps for steric reasons. A rearrangement of the bound repressor upon loop formation has been observed with the AraC repressor. Here a single dimer of AraC protein generates the loop although a dimer molecule is bound to each of the two interacting operators on a linear molecule (28).

The presence of one or more C1 repressor-occupied operators upstream of Op99a,b appears to down-regulate the basal level of c1 transcription from P99a about 2-fold, irrespective of the presence or absence of Lxc (Table I). We suggest that this is due to a C1-mediated loop formation between Op99a,b and Op99c or Op99d. The effect is small when compared to other repressor-controlled prokaryotic systems in which DNA looping was shown to be a regulatory element (13). For example, the uninduced, basal level of the promoter of the arabaBAD genes increases 10-fold when the upstream araO, site is deleted (29). In the lac operon the specific destruction of an operator downstream of the primary operator rises the basal level of the corresponding promoter 5-fold (30). Depending on which of the three operators is inactivated in the deo operon the basal level of transcription rises up to 12-fold (31). But provided that our plasmid system mimics the in vivo situation of the P1 immC region the peculiarities of the regulatory circuit of this system immediately become obvious: repression of c1 transcription is arranged in three steps, namely by C1 alone (about 15-fold), by C1 and Lxc (about 2-fold additionally), and by DNA looping (about 2-fold additionally), amounting to about a 60-fold repression totally (Table I). The latter value is similar to those of the prokaryotic operons mentioned above if totally repressed and derepressed states are compared.

We suggest that in the P1 immC region both Lxc binding and DNA looping serve for a fine-tuning of repression of c1 transcription. Upon P1 infection of a bacterial cell, c1 and coi are transcribed simultaneously from the promoters P99a and P99d, respectively. Whenever C1 synthesis prevails, coi transcription is completely shut off by binding of C1 to Op99d (6). On the other hand, down-regulation of c1 transcription by Lxc binding and DNA looping may be a slow process because the lac gene is transcribed from a weak promoter (11). At the end a level of C1 repressor synthesis is maintained which on the one side is sufficiently high to keep the P1 prophage repressed by saturating the numerous operators in the genome, and which on the other side is sufficiently low to enable the prophage to reverse the repression if the cellular conditions become unfavorable for its continued maintenance.

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