Dual mechanism of repression at a distance in the lac operon
(lac transcription/cooperativity/remote operators)

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ABSTRACT The mechanism by which the internal lacZ gene sequence O₂ influences lac repression was investigated by using in vivo footprinting of operon mutants. Quantitative in vivo binding curves show that O₂ strengthens by ~3-fold repressor binding to O₁ that is located 400 base pairs upstream at the transcription start site. The internal O₂ sequence also contributes to repression by a second mechanism: repressor bound internally blocks elongation of β-galactosidase gene expression. This second mechanism of repression involves base-pairing between the lacZ gene and O₂, which is facilitated by the remote O₁ operator that strengthens binding to O₂ by ~12-fold. Thus, lac repression involves two mechanisms, both of which involve cooperation between remote operator elements. During mild repression only the initiation mechanism applies, but more severe repression favors formation of the presumptive O₁–O₂ repression loop that allows both mechanisms to act simultaneously.

The lac operon contains a strong operator, O₁, that overlaps the start of transcription and two other sequences that bind lac repressor (1–9). The more important of these secondary elements is the lac O₂ site that is located within the lacZ gene, 402 base pairs downstream from O₁. Inactivation of O₂ by site-directed mutagenesis leads to a modest increase in expression under severe repression conditions (10). Therefore, O₂ is a functional operator element. The third operator O₃, located 93 base pairs upstream of O₁, is not detectably bound by repressor when O₁ and O₂ are present (6, 9, 11).

The mechanism by which the downstream O₂ operator contributes to lac repression is not known. The existing data are consistent with two possibilities. First, O₂ could bind repressor tightly and thereby block the progress of RNA polymerase transcribing within the lacZ gene. This mechanism is supported by several experiments that show that the lac O₂ operator can block expression when placed in a transcribed region (refs. 12 and 13, but see ref. 14). It is not known, however, whether the much weaker O₂ operator can do this at its natural position within the lacZ gene. The second mechanism involves O₂ strengthening repressor binding to O₁, thereby assisting blockage of the initiation of transcription near O₁. This mechanism receives support from experiments that show cooperative binding between remote operators in constructs in vitro (see below) and cooperative repressibility in similar artificial constructs in vivo (15, 16). Cooperative repressibility of O₂ and O₁ has also been tested, either in vitro or in vivo. In this paper we use in vivo footprinting and expression assays, on lac operon DNA lacking O₂ or O₁, to probe the in vivo mechanism of repression.

MATERIALS AND METHODS

In Vitro Mutagenesis of O₂. The entire lacZ gene with the L8 lac promoter carried on fl phage (constructed by and a kind gift of Claire Cupples and J. Miller, University of California–Los Angeles) served as a template for in vitro mutagenesis. The mutagenesis was done according to the standard two-primer method (17) using two 21-mer primers, one of which is complementary to the phage fl DNA sequence. The mutagenizing primer AAATGGTAGTAGAAGAACC carries two nucleotide substitutions compared to the wild-type sequence. These are changes in the third position of codons, a guanosine for adenosine in the serine codon and a cytidine for adenosine in the leucine codon. Thus, the amino acid sequence of β-galactosidase will be unchanged. Based on known lac repressor-operator interactions, these combined changes should reduce repressor affinity for O₂ by >200-fold (18). The screening of plaques was done by plaque-lift, hybridization to the mutagenizing primer, and washes with tetramethylammonium chloride solution to identify clones with DNA forming high-melting hybrids with the altered DNA sequences (17, 19). The mutagenized sequence was confirmed by direct DNA sequence analysis by using the lac O₂ oligonucleotide probe as primer in a dieoxyx sequence protocol. The mutagenized lacZ gene was isolated as an EcoRI fragment from double-stranded fl DNA. This was inserted into the EcoRI site of plasmid pAS21 (20) to obtain plasmid pAYZ21. In parallel, an EcoRI fragment carrying the unmutagenized lacZ gene was also ligated to the EcoRI fragment of pAS21 to pAYZ2. Both plasmids are pBR322 derivatives in which the lacZ gene and its control region has replaced the tet promoter. pAYO2, the plasmid that carries lac O₂ but not O₁, was constructed as follows: phage M13mp18 replicative form DNA was cut with EcoRI and Bgl II to yield a fragment containing lac O₂ but not O₁. This fragment was used to ligate the EcoRI-BamHI fragment of pAS21 to obtain pAYO2. The lacZ gene sequences in pAYO2 are from nucleotide 59 to nucleotide 464. The three constructs, pAYZ21, pAYZ2, and pAYO2, have the same pBR origin of replication.

Plasmid pMC4, a source of unmutagenized lacZ DNA (21), was a kind gift of J. Miller, as was the Escherichia coli host strain strain P90C: ara- thi-Δ(lac pro)RecA-. Strains P90C-L8 and P90C-M15 each contain a lac I′, lacZ episome bearing the L8 and M15 mutations, respectively. In Vitro and in Vivo lac Repressor Binding Determination. Typical conditions for in vitro binding reaction were as follows. Reactions were normally done in 25 μl containing 30 mM Tris·HCl (pH 8), 100 mM KCl, 3 mM MgCl₂, 0.2 mM dithiothreitol, and 0.1 mM EDTA. The DNA concentration was usually 7.6 nM; the concentrations of repressor are given in the text and refer to active repressor as measured by titration (6). Complexes were incubated for 30 min at 37°C before dimethyl sulfate was added to 10 mM for 5 min.

Abbreviation: IPTG, isopropyl β-D-thiogalactoside.

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Dimethyl sulfate protection experiments were done according to primer extension procedures described (6, 22, 23). The level of operator occupancy was determined by gel scanning and analysis as described, except that a Bio-Rad videodensitometer was used (6). Protection of the guanosine at position 409 was used to determine $O_2$ occupancy; $O_1$ occupancy determinations were as described (6). The $O_2$ test and reference bands used in quantitation are indicated in Fig. 1. The uncertainty in the subsequent quantitative occupancy experiments is approximately ±5%. The uncertainty is slightly greater in in vivo experiments. The sequence of the oligonucleotide that was hybridized and extended through this region is GATGCACGGTTACGATGGGCC. β-Galactosidase activity was measured according to Miller (24).

RESULTS

$O_1$ and $O_2$ Bind Repressor Cooperatively in vivo. To address the issue of cooperative binding involving $O_2$ in vivo, repressor occupancy of $O_2$ was measured in vivo by using plasmids that either do or do not contain the remote $O_1$ primary operator site. Plasmid pAYO2 contains $lac$ operon sequences between nucleotides 59 and 464 and bears only $O_2$, whereas plasmid pAYZ2 contains all $lac$ operator sequences. Each plasmid was transformed into E. coli strain P90C-M15. This strain contains the lac $i^8$ episome that overproduces lac repressor; this was included to compensate for the multicyclop nature of these operator-bearing plasmids. Operator occupancy in vivo was determined as described in previous reports (6, 9, 23). Briefly, transformed cells were treated for several minutes with dimethyl sulfate and the methylated plasmid DNA was purified. The DNA was then broken chemically at the methylated guanines. The precise methylation/breakage sites within each operator were then analyzed by in vitro extension of a hybridized end-labeled oligonucleotide designed to read through the $O_2$ operator.

Fig. 1 shows the in vivo methylation pattern at the $O_2$ operator both in the presence in cis and in the absence of the $O_1$ operator. The experiment was done using various concentrations of the $lac$ inducer isopropyl β-d-thiogalactosidase (IPTG) so that partial occupancies accompanying partial expression might be probed. The extent of repressor binding can be seen qualitatively by referring to the bottom band in the $O_2$ region of Fig. 1. Under full-induction conditions (lane 1), this $O_2$ marker band has an intensity roughly similar to both the band just above it and the upper of the reference bands. As the inducer concentration is lowered in lanes 2–5, this $O_2$ band is selectively and progressively protected by repressor, which is increasing in activity in vivo. By analogy with previous experiments (6, 9) probing both $O_1$ and $O_2$, the extent of this protection is taken as a measure of the extent of repressor binding to $O_2$ in vivo. This same protection pattern can be reproduced in a purified system containing operator and repressor (data not shown, but see below).

When, instead, the protection is followed in the construct lacking $O_1$, the $O_2$ marker band remains equally as intense as the other two bands at high-to-moderate inducer concentration (lanes 6–8). Recall that over this same range of inducer in the presence of $O_1$, specific protection occurred. Only at low inducer concentration does the $O_2$ marker band begin to lighten compared to the others (lanes 9 and 10). That is, $O_2$ protection only occurs when the intracellular repressor activity is very high. Thus, the presence of the primary $lac$ operator stimulates binding in vivo to the internal $O_2$ operator; that is, the two remote operators bind repressor cooperatively in vivo.

 Autoradiographs from in vivo protection experiments of this type were scanned with a densitometer to quantify the extent of $O_2$ occupancy by repressor. We have previously (9) that the changing occupancy of an operator site, caused by changing inducer concentration, can be analyzed to yield relative in vivo binding constants for repressor-operator interactions. In this analysis the binding constant is inversely proportional to the slope of a plot of $(1 - Y)/Y$ vs. [IPTG]$^2$ (9, 25), where $Y$ is the occupancy determined from the in vivo protection experiment. Fig. 2A shows such an analysis for repressor binding to the $O_2$ sequence, both in the presence and absence of the remote $O_1$ operator. The steeper slope for the construct lacking $O_1$ indicates weaker binding. This analysis confirms that $O_1$ assists repressor binding 400 base pairs away at $O_2$; quantitatively, the consequence is to strengthen binding to $O_2$ 12-fold.

An analogous analysis was done comparing repressor binding to $O_1$ on plasmids with and without functional $O_2$. The parent plasmid for these experiments was pAYZ2, which contains all three $lac$ operator elements and the intact lacZ gene. A derivative lacking functional $O_2$ was constructed (pAYZ21) in which two third-position codon changes were introduced into $O_2$ by site-directed mutagenesis. That these strongly inhibited repressor binding was confirmed by in vitro footprinting (data not shown). Repressor binding to $O_1$ in vivo was measured as described (6, 9), which is precisely analogous to the $O_2$ probing just described. The result of the analysis is shown in Fig. 2B (note the different range of inducer needed to remove repressor from $O_1$ compared to $O_2$ in Fig. 2A).

Qualitatively, Fig. 2B shows that at every inducer concentration there is more repressor bound to $O_1$ in the presence of $O_2$ compared to its absence. Quantitatively, the data are not as accurate as for $O_2$, but they clearly demonstrate that binding to $O_1$ is strengthened slightly >2-fold (2- to 4-fold is the range) by the presence of $O_2$.

This estimate for strengthening of $O_1$ is a slight underestimate. When $O_2$ is inactivated, the very weak $O_1$ operator can now participate in a cooperative interaction with $O_1$, strengthening it slightly (9). This effect, however, is rather small (9), and taken together with the current data suggests that $O_1$ is 3-fold stronger when other operators are present. Thus, $O_1$ and $O_2$ participate in a cooperative interaction in viva.
vivo where $O_1$ is strengthened 3-fold and $O_2$ is strengthened 12-fold.

The $O_1$-$O_2$ Complex Is Not Always Stable. Despite these very strong cooperative effects, the \textit{in vivo} cooperative complex, presumably involving a DNA loop, is not the predominant complex under all conditions. This can be seen in Table 1, where the amount of repressor binding to $O_1$ and $O_2$ is compared by tabulating the $O_2/O_1$ occupancy ratio. As the IPTG concentration decreases, this ratio approaches unity, as would be expected if a stable loop predominates under these more severe repression conditions. This does represent a cooperative interaction, as implied by Fig. 2 and as seen directly at 20 $\mu$M IPTG where deletion of $O_1$ leads to a loss of binding at $O_2$ (see Fig. 1). By contrast, when repression is partly relieved at higher concentrations of IPTG, the $O_2/O_1$ ratio drops from near one to near one-third (Table 1). Obviously, there is much more repressor bound to $O_1$ than $O_2$ under these less severe repression conditions; on the average, only up to one-third of the complexes can involve simultaneous binding to $O_1$ and $O_2$.

For purpose of comparison, we performed \textit{in vitro} protection experiments and quantified the ratio of repressor occupancy at $O_2$ versus $O_1$. These involved cooperative complexes since restriction cleavage between $O_1$ and $O_2$ eliminated binding to $O_2$ (data not shown). Table 1 shows that at all these moderately high \textit{in vitro} concentrations the $O_2/O_1$ ratio is close to one. Thus, under these solution conditions the presumptive loop appears to be stable. Several-fold dilution, within the technical range of these experiments, did not lead to less binding. The experiment also shows that a \textit{lac} $O_1$/$O_2$ cooperative complex can form on linear DNA. This is in contrast to the weak \textit{lac} $O_1$/$O_2$ loop that formed only on supercoiled DNA (6, 26).

$O_2$ Can Act as an Internal Block to \textit{lacZ} Gene Expression. Müller-Hill and colleagues (10) have shown that inactivation of $O_2$ by \textit{in vitro} mutagenesis leads to less effective repression of $\beta$-galactosidase expression. As discussed above, we have also inactivated $O_2$ by site-directed mutagenesis. The two related plasmids were constructed and introduced into \textit{E. coli} strain P90C-L8. Recall that plasmid pAYZ2 contains the wild-type \textit{lacZ} gene with associated control elements and plasmid pAYZ21 is identical except that the $O_2$ internal operator has been mutagenized using two third-position point mutations. Both contain the entire $\beta$-galactosidase gene and the upstream \textit{lac} L8 regulatory region. The cells also carry an $\lambda$ episome so that a wider range of induction conditions can be explored \textit{in vivo}.

Table 2 compares $\beta$-galactosidase levels from the $O_2$ (pAYZ2) and $O_2$ (pAYZ21) plasmids. First, it can be seen that under full-induction conditions (500 $\mu$M IPTG) the expression levels are essentially identical from the two plasmids (expression ratio of 0.99). This confirms that the introduced mutations and associated processing during plasmid constructions did not change the intrinsic activity of the $\beta$-galactosidase enzyme, as expected. However, when expression was measured over a wide range of partial induction conditions, repression of $\beta$-galactosidase expression is greater in pAYZ2 ($O_2^*$) than in pAYZ21 ($O_2$). Table 2 shows that in the absence of inducer, expression from the wild-type plasmid is less than in the mutant (0 $\mu$M IPTG in P90C-L8). This confirms the related observation of Müller-Hill and colleagues (10), although the effect is smaller, possibly due to our use of plasmid constructs.

This effect of $O_2$ is not surprising in view of the above results that show that $O_2$ facilitates repressor binding to $O_1$ that overlaps the transcription start site. However, part of this $O_2$-mediated repression could be due to blocking the progress of elongating RNA polymerase that escaped repression by initiation at $O_1$ (see refs. 12 and 13). To test this possibility, \textit{E. coli} P90C-M15 cells were also transformed.
Table 2. β-Galactosidase expression ratio ± O2

<table>
<thead>
<tr>
<th>IPTG, μM</th>
<th>P90C-L8</th>
<th>P90C-M15</th>
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<tr>
<td>0</td>
<td>0.70</td>
<td>0.87</td>
</tr>
<tr>
<td>20</td>
<td>0.72</td>
<td>0.86</td>
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<tr>
<td>50</td>
<td>0.77</td>
<td>0.85</td>
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<tr>
<td>75</td>
<td>0.86</td>
<td>0.86</td>
</tr>
<tr>
<td>100</td>
<td>0.82</td>
<td>0.84</td>
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<tr>
<td>500</td>
<td>0.99</td>
<td>0.97</td>
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The indicated concentrations of IPTG were used to induce lac expression. In each strain the β-galactosidase levels expressed from wild-type pAYZ2 and O2 pAYZ2 plasmids were measured. The table shows the ratio of levels in a wild-type plasmid to the one with O2 inactivated. The total amount of β-galactosidase was ~1000 units in both strains. In P90C-M15 a complementation is allowed. The background in nontransformed cells was negligible for strain P90C-M15 and was ~30 units in strain P90C-L8. The absolute levels of β-galactosidase varied somewhat from experiment to experiment, presumably due to changes in plasmid copy number. In general, half repression, or 500 units of β-galactosidase, was achieved near 40 μM IPTG. The ratios were much more reliable >10% or less since they were obtained from multiple experiments, each involving transformed cultures grown in parallel and harvested at identical phases of growth. These plasmids contain the lac L8 promoter, which is a low level lac promoter mutant.

Either with pAYZ2 or pAYZ2. This strain has on its episome the p gene and the M15lacZ gene, which allows a complementation (27), while the closely related episome in P90C-L8 does not. That is, short RNAs that might be formed as the result of RNA polymerase is blocked by repressor-bound O2 could contribute to expression in strain P90C-M15, but not in strain P90C-L8. Therefore, if such blocking of RNA polymerase at O2 occurs, the loss of O2 should have the largest effect in strain P90C-L8; that is because artificial expression by a complementation is very unlikely in this strain.

Table 2 shows that this expression difference between the two strains is indeed observed; the 0.7 expression ratio in P90C-L8 increases to 0.87 in P90C-M15 due to this artificial expression of short RNAs. This occurs only when the IPTG concentration is low, implying that the repressor concentration is high. The observed effect must be considered the minimum consequence of blocking since shortened RNAs may not all survive to be translated, and all translated peptides may not find a lac fragment and associate to form functional enzyme. Thus, blocking the progress of the transcribing RNA polymerase contributes to natural lac operon repression in vivo.

DISCUSSION

Previous results have shown that inactivation of either lac O2 (2, 28) or O2 (ref. 10 and see above) leads to defective repression. Inactivation of O1, which overlaps the transcription start site, is much more deleterious than inactivation of O2, which is located 0.4 kilobases downstream in the lacZ gene. Previous experiments did not address the issue of whether the two natural operators functioned independently or cooperatively. In the former case, repressor bound to O1 would first inhibit initiation and then any repressor bound to O2 could block elongation. In the latter case, a single repression complex involving both O1 and O2 could act as the repression apparatus. The current results, by using a combination of in vivo protection and expression assays, show that the mechanism of natural repression involves a cooperative repression complex.

The extent to which this long-range cooperativity affects repressibility was shown to depend on the amount of inducer present in cells. When O2 is inactivated, the defect in repression is greatest under severe repression conditions (Table 2, 0–50 μM IPTG in P90C-L8). This incremental defect is solely due to the inability to block expression within the lacZ gene, as evidenced by its restoration when α complementation is allowed (Table 2). That is, the internal blockage mechanism applies only when the induction environment requires severe repression and the internal active repressor concentration is very high. This secondary repression mechanism involving the O2 element appears to be a fail-safe mechanism to block polymerases that escaped repression at O1, which otherwise is totally repressed.

Nevertheless, both the blockage and initiation control mechanisms involve long-range cooperativity between the two operator sites. The in vivo binding curves (Fig. 2) can be used to calculate the amount of repressor that is bound to either operator. Consider first the situation that applies using 20 μM IPTG, moderately severe repression conditions. Here the loss of O1 reduces occupancy of O2 from 70% to 20%. The loss of O2, however, has only a minor effect on occupancy of O1 since during severe repression the active repressor concentration is high enough to completely fill O1 even without the cooperation of O2. Thus, under severe repression conditions the major consequence of cooperativity is to fill O2 with repressor that can block elongation.

When repression is less severe, the situation is rather different. For example, at 100 μM IPTG, O2 is only slightly occupied; this is apparently why the blockage mechanism does not apply over mild-to-moderate repression conditions. O2 does have an effect, however, since its loss reduces repressor binding to O1 from 60% to 40%. This occurs under these conditions because the concentration of active repressor is low enough so that O1 needs the cooperation of O2 to minimize the loss of DNA-bound repressor to free solution. Thus, under less severe repression conditions the major consequence of cooperativity is to help fill O1 with repressor at the transcription start site. These results show that cooperativity has consequences for both mechanisms of repression. Each mechanism functions primarily under a different physiological condition.

By analogy with previous studies, the cooperative lac complex (6, 9, 26, 29) is expected to involve a DNA loop (6, 7, 29, 30). Since deletion of either operator affects occupancy of the other, the predominant equilibrium state in vivo is a cooperative repression complex involving both O1 and O2. At high intracellular repressor activity (low IPTG), the occupancy of O2 and O1 is not so large as predicted for stable loop formation (Table 1, 0.9 ratio at 20 μM) although both operators could be bound separately at very high repressor concentrations. When the active repressor concentration is lowered (higher IPTG), then the amount of repressor bound to O2 decreases to well below that bound to O1 (Table 1, 0.3 ratio at 100 μM). Under these conditions the predominant equilibrium state is repressor bound to O1 alone, with a lesser contribution from the looped state. This in vivo behavior at high inducer concentration contrasts to that observed in vitro (Table 1), where the occupancies of O1 and O2 are always equal, as expected for strongly cooperative loop formation. Thus, whether or not loops constitute the predominant form of the repression complex depends on the in vivo inducer concentration. The source of this unexpected in vivo effect is not known, and it could have interesting implications for the control of loop formation.

There are now many examples of transcriptional control at a distance (for example, see reviews by Ptashne (31) and Schleif (32)). The lac operon case involves a repressor tetramer with two DNA-binding sites (6). Other bacterial cases such as the ara operon, where DNA looping was first indicated (33, 34), the gal operon (35), and the deo operon (36, 37) could also involve analogous homotypic multimeric
plexes, while the glnA operon (38) and many eukaryotic examples may involve heterotypic multiprotein complexes. In either case, the concepts developed from the lac O₁–O₂ system should contribute to understanding the mechanism of transcriptional control at a distance.

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