Possible ideal *lac* operator: *Escherichia coli* *lac* operator-like sequences from eukaryotic genomes lack the central G·C pair

(*lac* repressor/protein–DNA recognition/symmetry/molecular cloning)

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ABSTRACT Five DNA fragments have been cloned from yeast, chicken, and mouse DNA that titrate *lac* repressor in an *Escherichia coli* *lac*⁺ *I*⁺ *Z*⁺ wild-type strain when on a multicopy plasmid. The five repressor-binding sequences have been identified by DNA sequence determinations and DNase cleavage-inhibition patterns. They share the 14-base-pair symmetrical consensus sequence 5' T-G-T-G-A-G-C-G-C-T-C-A-C-A 3' (the colon represents the center of symmetry), which is an inverted repeat of 7 base pairs of the left half of the *E. coli* *lac* operator. A similar perfect palindromic DNA fragment—an 11-base-pair inverted repeat of the left half of the *lac* operator—was synthesized. The cloned synthetic DNA 5' G-A-A-T-T-G-T-G-A-G-C-G-C-T-C-A-C-A-A-T-T-C 3' binds *lac* repressor 8-fold more tightly than does wild-type *E. coli* *lac* operator DNA.

The *lac* operon of *Escherichia coli* is under negative control (1). *lac* repressor binds to *lac* operator and, thereby, inhibits the start of transcription (2). *lac* operator was first defined genetically (3). Later, the sequence of several mutations in the *lac* operator has been determined (4). Within the *lac Z* gene, another sequence has been found that also binds *lac* repressor tightly (5, 6). Both sequences show a similar symmetry. A dyad axis cuts through a central G·C pair. Repressor has been used to shield operator DNA against modification by various reagents (6). The sequence shielded shows only little if any symmetry (6). From this it was concluded that repressor operator recognition may be completely asymmetrical. On the other hand, evidence exists that is in favor of two subunits of *lac* repressor being necessary and sufficient for recognition of one operator (7–9).

Attempts have been made to understand *lac* operator by synthesizing various operator analogues (10). One conclusion from this type of work was that 17 base pairs (bp) are necessary for recognition (11). All analogues had a similar symmetry as wild-type *lac* operator. To exclude all preconceptions with regard to the structure of DNA that could be recognized by *lac* repressor, we screened cloned DNA from eukaryotic sources (yeast, chicken, mouse) for the specific binding of *lac* repressor. We found five clones that had *lac* operator activity. They share a 14-bp consensus sequence with a dyad axis of symmetry between two central base pairs.

MATERIALS AND METHODS

Enzymes and Chemicals. Restriction endonucleases *Bam*HI, *Eco*RI, *Hpa*II, *Hind*III, *Pst*I, *Xba*I, and DNA polymerase I, large fragment, were purchased from Boehringer Mannheim; *Acc*I was from Biolabs (Bad Schwalbach, F.R.G.); *Sau*3A, *Taq*I, and T4 DNA ligase were from Bethesda Research Laboratories (Neu-Isenburg, F.R.G.); terminal transferase was from P-L Biochemicals GmbH (St.

Goar, F.R.G.); and DNase I (bovine pancreas grade I) was from Sigma (München). 5-Bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) and isopropylthiogalactoside were obtained from Bachem Fine Chemicals (Torrance, CA).

The chemicals used for sequence analysis by the method of Maxam and Gilbert (12) are listed in ref. 13. S.M. 113 nitrocellulose filters were bought from Sartorius (Göttingen, F.R.G.), and the chemicals for automated DNA synthesis on an Applied Biosystems 380 A synthesizer were from Applied Biosystems (Pfungstadt, F.R.G.). Chicken blood DNA was bought from Calbiochem, purified mouse DNA was provided by U. Krawinkel, and yeast (*saccharomyces cerevisiae*) DNA was prepared as described in refs. 14 and 15. Purified *lac* repressor was a gift of K. Beyreuther.

Strains and Media. *E. coli* K-12 CSH52 [*lac-pro*]_Δ (ϕ 80*dlac*⁺) *F'**pro*⁺ and MPS51 [*lac-pro*]_Δ *F'**pro*⁺ *lacI*⁺ *Z*⁺ *Y*⁺ are described in refs. 16 and 17, and media are described in ref. 16.

Plasmids. pBR322 (18) was used as a vector for shot-gun cloning of DNA fragments (chicken and mouse) produced by *Pst*I. For the subcloning of smaller fragments, we constructed plasmid pAS8 (Fig. 1), which combines the possibility to detect operator-like sequences on inserted fragments and the advantages of DNA sequence analysis described for pUR250 (19) (for details, see the legend of Fig. 1). *Bam*HI-cleaved pAS8 also was used for the cloning of *Sau*3A-digested yeast DNA. pGR21 is described in ref. 20.

Procedures for Cloning and Identification of DNA Fragments That Titrate *lac* Repressor. Our strategy to isolate *lac* operator-like fragments from eukaryotic DNA was simple in principle. We used the fact that *lac* operator on a multicopy plasmid is capable of titrating the 15 copies of *lac* repressor produced from the *lac I* gene with a wild-type promoter. If the *lac I* gene is wild type, one can isolate the resulting constitutive colonies; if the *lac I* gene carries an *I*⁺ mutation, which makes the repressor noninducible by lactose, one can use the resulting *lac*⁺ phenotype for the selection. Two technical difficulties had to be overcome. In order to avoid contamination by *lac* operator-containing plasmid DNA used in the laboratory, only new plastic tubes and disposable pipettes were used for cloning procedures. Glassware was rinsed extensively with chromic/sulphuric acid in order to exclude contamination by *lac* operator-carrying plasmids that might have escaped routine cleaning.

The strategy to cope with spontaneous *lac* constitutive mutations arising at a frequency about 2 orders of magnitude above the frequency of cloned *lac* operator-like sequences was as follows. Mouse DNA (50 μ g) and pBR322 DNA (12.5 μ g) (18), both cleaved with *Pst*I, were incubated with 30 units of T4 DNA ligase in a total volume of 2.5 ml at 12°C overnight. The mixture was then used to transform about 10¹¹ MPS51 cells suspended in 6 ml of 50 mM CaCl₂ (21) in five independent vials containing 1.2 ml each. Each sample

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Abbreviations: bp, base pair(s); X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside.

FIG. 1. Physical map of plasmid pAS8. Plasmid pAS8 was derived from pBR322 (18) by deleting the 1875 bp between the *Bam*HI site at position 375 and the *Acc* I site at position 2247 of pBR322. Into the resulting *Bam*HI/*Acc* I-resistant, tetracycline-sensitive plasmid, the small *Hind*III/*Eco*RI polylinker fragment from pUR 250 (19) was inserted between the *Eco*RI and *Hind*III sites of pBR322 to yield pAS8, which offers the facilities of rapid DNA sequence analysis as described for pUR250 (19) but does not contain the *lac* operator sequences present on pUR 250.

was allowed to recover in 200 ml of dYT medium (16) at 37°C for 2 hr. The efficiency of transformation was determined, and half of each culture was diluted further into 500 ml of dYT medium. Tetracycline was added to a final concentration of 15 µg/ml, and the culture was allowed to grow to saturation overnight. One milliliter of the saturated culture was washed twice with saline and finally suspended in the same volume of saline; 0.1 ml was spread onto a minimal lactose plate containing tetracycline and incubated at 37°C for 2 days. dYT medium (20 ml) was then inoculated with 100

single colonies from the lactose plate and aerated overnight. Plasmid DNA was prepared from the cells (22, 23) and used to transform fresh MPS51 cells. The transformants were plated onto minimal glucose plates containing X-Gal and tetracycline. They were analyzed further when the blue colonies appearing on this plate outnumbered the blue colonies on the control plate prepared with another sample of MPS51 transformed with the vector pBR322. In this case, small cultures were grown from 12 single blue colonies in order to prepare plasmid DNA. Aliquots of the DNAs were used to transform once more MPS51 cells in order to check whether the *lac*⁺ phenotype was plasmid linked. Finally, the inserted mouse DNA fragment that titrated the noninducible *lac* repressor was visualized by agarose gel electrophoresis of *Pst* I-digested plasmid DNA.

The DNase I protection experiment was performed essentially as described (24). The rate of dissociation of repressor-operator complexes was monitored by filter-binding as described (25).

RESULTS

Cloning and Identification of *lac* Operator-Carrying Fragments. *Sau*3A-digested DNA of *Saccharomyces cerevisiae* was ligated to *Bam*HI-cleaved pAS8 DNA (Fig. 1). About 3×10^5 transformants, 6×10^4 of which contained hybrid plasmids, were screened for constitutive β -galactosidase activity on minimal glucose plates containing X-Gal. Constitutive synthesis of β -galactosidase indicates that the multicopy-plasmid may carry a *lac* operator-like sequence that titrates the 15 copies of *lac* repressor of the host. The inserts that were thus inspected correspond to 1.5 haploid yeast genomes (10^7 bp). One of them contained a *lac* repressor binding sequence. The complete nucleotide sequence of this 200-bp fragment was determined from both strands and screened for homologies to *E. coli lac* operator. There was only one operator-like sequence close to one end of this fragment, which is present in Fig. 2.

source of DNA		center of symmetry	
		↓	
<i>E. coli</i> K12	O _I	g T g G A A T T G T G A G C g	G a T a A C A A T T t c A c
	O _{II}	C a t t A A a T G T G A G C g	a g T a A C A A c c C g t c
	O _{III}	a g c G g g c a G T G A G C g	c a a C g C A A T T a a t G
yeast	O _I	g T A t A t a T G T G A G C	G C T C A C g t a T t c t t
chicken	O _I	t T c c c c T G T G A G C	G g T C A C A A c a g a g a
mouse	O _I	g a A t g g T T G T G A G C	G g T C A C A t g a g t g c
	O _{II}	. . . G g A a T G T G A G C	G C T C A C A t c T C a a a
	O _{III}	a T g t g t a T G T G A G C	G C T C A C A A a a g T c c
synthetic operator		C T A G A A T T G T G A G C	G C T C A C A A T T C T A G

FIG. 2. Comparison of *lac* repressor binding sites from *E. coli*, yeast, chicken, and mouse to the synthetic, possibly ideal operator. The center of symmetry is shown by a dotted line. Identical bases are represented by capital letters, and palindromes are boxed.

Chicken blood DNA was digested with *Pst* I and ligated to *Pst* I-cleaved pBR322 DNA; 2×10^6 transformants were screened as above. The frequency of hybrid plasmids as determined by insertional inactivation of the ampicillin resistance gene was 5%. Among the 10^5 inserts corresponding to about one-fourth of the haploid chicken genome (1.5×10^9 bp), one was found that rendered the cells constitutive for β -galactosidase. The cloned chicken DNA fragment was about 5000 bp. The *Sau*3A fragments of this plasmid were subcloned into the *Bam*HI site of pAS8, and the *lac* repressor binding activity was found to reside on a 340-bp fragment. The complete nucleotide sequence of the fragment was determined from one strand and compared to *E. coli lac* operator. A sequence similar to *lac* operator was located in the middle of the fragment. This sequence (Fig. 2) was confirmed by analysis of the complementary strand. The same fragment also was used in a DNase I cleavage-inhibition assay (24) (Fig. 3) that demonstrated that *lac* repressor does indeed recognize the homologous sequence and binds tightly to it. The protection was symmetrical in both strands but differed in length by two nucleotides (Fig. 4). The protected sequence is shorter than wild-type *lac* operator (Fig. 4). The *in vitro* binding of *lac* repressor to the eukaryotic sequences was completely abolished in the presence of 1 mM isopro-

FIG. 3. DNase I cleavage-inhibition patterns. Autoradiographs show the areas on the two strands protected by *lac* repressor and the previously determined DNA sequence of the cloned chicken DNA (A and C) and the effect of inducer isopropylthiogalactoside (B). Lanes: d, g, and h, 5–10 ng of 3' end-labeled DNA were degraded by DNase I in the presence of 1 μ g of sonified calf thymus DNA and 70 μ g of bovine serum albumin per ml; c, f, i, and k, purified *lac* repressor was added (1–5 μ g/ml) prior to degradation [the activity of *lac* repressor was about 60% as determined by filter binding assay (23, 24)]; a, b, l, and m, sequence-specific degradation (11) was performed on the same fragments; e, 1 mM isopropylthiogalactoside was added to the *lac* repressor DNA mixture prior to degradation by DNase I. Similar results were obtained with cloned mouse operators.

FIG. 4. Protected areas of wild-type *E. coli lac* operator (a) and cloned chicken DNA (b) (data from ref. 25 and Fig. 3). The protected areas are indicated by brackets.

pylthiogalactoside (Fig. 3). In a competition experiment (26, 27), the cloned chicken operator DNA was about 1/20th as effective in competition for *lac* repressor as *E. coli lac* operator (data not shown).

With mouse DNA we carried out nine experiments as described in *Materials and Methods*. Each yielded 10^6 transformants containing 5×10^4 hybrid plasmids. Thus, we estimate that we screened half the mouse genome. We isolated five hybrid plasmids carrying four different inserts of approximately 4, 3, 1.4, and 0.8 kilobase(s), respectively (the 1.4-kilobase fragment turned up twice in two independent experiments). From three of these, subclones were derived that carry 50- to 200-bp fragments produced by either restriction enzymes or limited DNase I digestion and subsequent G-C-tailing. The *lac* operator-like sequences as determined by the method of Maxam and Gilbert (12) are given in Fig. 2. Their *lac* repressor binding activity was confirmed by DNase cleavage inhibition analysis (ref. 24; data not shown; see also the legend to Fig. 4).

The most striking common feature of all the sequences presented in Fig. 2 is the absence of the central G-C pair of the natural *E. coli lac* operator. Their limited size and their higher degree of symmetry as compared to *E. coli lac* operator prompted us to synthesize the 24-base oligonucleotide 5' C-T-A-G-A-A-T-T-G-T-G-A-G-C-G-C-T-C-A-C-A-A-T-T-3', which can hybridize to yield an 11-bp inverted repeat of the left half of the *E. coli lac* operator with protruding 5' single-stranded ends that fit into an open *Xba* I restriction site:

The synthetic DNA was deprotected and ligated to *Xba* I-cleaved pAS8 DNA without further purification. Hybrid plasmids were detected by their blue phenotype on plates containing X-Gal. The presence of the desired 22-bp perfectly palindromic operator sequence was confirmed by DNA sequence analysis (12). Purified plasmid DNA and *lac* repressor were used to determine the rate of dissociation of preformed repressor-operator complexes (25). The results presented in Fig. 5 show that the new synthetic *lac* operator binds *lac* repressor 8 times more tightly than does the natural *lac* operator under the same conditions. Preformed complexes between *lac* repressor and the synthetic tight-binding *lac* operator can be induced to dissociate by the addition of isopropylthiogalactoside (see the legend to Fig. 5).

DISCUSSION

Frequency of Eukaryotic *lac* Operator Clones. The five fragments of eukaryotic DNA that titrate *lac* repressor share at least 13 bp of a consensus sequence of 14-bp. If we assume that any substitution in one of these 14-bp would leave an active operator, one would predict finding one such structure among 6×10^6 random base pairs. The assumption of randomness does not strictly apply to the DNAs we screened. In exonic regions the differential codon use disturbs random-

FIG. 5. Rates of dissociation of preformed *lac* repressor-*lac* operator complexes. pgR21 DNA (20) served as a source of wild-type *lac* operator. This 4-kb plasmid carries *lac* operator opposite to its unique *Eco*RI site. The latter was cut and labeled with α - 32 P]dATP. The synthetic perfectly palindromic operator cloned into the *Xba*I site of pAS8 was also labeled at the unique *Eco*RI site of pAS8 (see Fig. 1). Here the operator is situated close to one end of the linear molecule. Repressor-operator complexes were allowed to form at room temperature for 10–20 min. Aliquots were then filtered in order to determine the ratio of binding. Then a 100-fold excess of the same unlabeled DNA was added at time zero. The decrease of the preexisting complexes was monitored by filtering 20- to 60- μ l samples at the times indicated. Values from three independent experiments at slightly different concentrations of labeled operator and repressor (see below) have been used for each curve. ●, Measurements with wild-type *lac* operator (the half-life of the complexes is about 1.5 min); ▲, experiments with the perfectly palindromic operator (the half-life of these complexes is about 12 min). The radioactive input was 3,000 to 12,000 cpm per sample corresponding to 10–60 pM operator. Purified *lac* repressor (about 60% active) was added in sufficient amounts to retain 40–60% of the input DNA. All values have been corrected for a background of 3–7% of radioactivity retained by the filter in the presence of 1–10 mM isopropylthiogalactoside.

ness. In intronic regions slippage errors may lead to further deviations. There may be a selection for or against palindromes. And finally, our procedure selected for short DNA fragments produced by the restriction enzymes we used.

We found no additional operator-like structure in *E. coli* (data not shown) and one positive clone among the 10^7 bp of yeast DNA. In chicken DNA we had to screen about 4×10^8 bp to get one clone, and in mouse DNA, about 1.5×10^9 bp to find five clones, two of them being identical. Three different mouse clones have been analyzed in detail. In yeast DNA the clone occurred with about the expected frequency; in chicken and mouse DNA, they were almost 2 orders of magnitude more infrequent.

We cannot say whether this low frequency is due to the fact that not any one of the 14 bp may be exchanged without abolishing the operator activity of the remaining 13 or to the nonrandomness of the screened DNA. We also do not know whether these *lac* operator-like sequences have any function in yeast, chicken, or mouse.

Recognition of *lac* Operator by *lac* Repressor. The existence of the class of operators described in Fig. 2 has attractive implications for the mechanisms of recognition of *lac* operator by *lac* repressor. The dyad axis of symmetry between the two central base pairs allows the recognition of half of the operator by one subunit of *lac* repressor. This is in line with several older experiments (7–9). Furthermore, the dyad axis between the two central base pairs positions the symmetrical sequences 5' G-T-G 3' and 3' C-A-C 5' at a distance of exactly 34 Å. Thus, the alpha-helical region postulated to exist between residues 16 and 25 of *lac* repressor (28–30) could bind to these sequences in two adjacent major

grooves. The symmetry is imperfect in *E. coli* wild-type *lac* operator. Furthermore, the extra G·C pair introduced at the center of symmetry disturbs operator recognition. This explains the pronounced nonsymmetrical behavior of the *E. coli lac* operator in various protecting experiments (6)—as opposed to the more symmetrical protection of the cloned eukaryotic sequences (Fig. 4).

These results indicate that the NH₂-terminal arms of *lac* repressor are not rigorously fixed in space but can move appreciably. This is in line with the findings that 72 NH₂-terminal residues of *gal* repressor suffice for repression of the *gal* operon when fused to β -galactosidase (31). Residues 50–75 probably serve as a hinge or transmitter region for induction (32). They allow *gal* repressor to recognize *lac* operator, weakly (20) in spite of its extra G·C pair at the center of symmetry (33).

Inspection of the data reveals a paradox: natural *lac* operator binds to *lac* repressor 1/8 as tightly as the synthetic perfectly palindromic operator. The two operators actually differ in three respects. The natural *lac* operator contains an additional central G·C pair and two different base pairs in its right half (Fig. 2). One may come to the conclusion that *lac* repressor can specifically bind to DNA in two different modes, in a symmetric one (with the synthetic palindromic DNA) or in a nonsymmetric one (with natural *lac* operator). The paradox might be solved if one considers the possibility that one *lac* repressor subunit recognizes one half of the operator with two separate subdomains. Probably *lac* repressor recognizes the sequence 5' T-G-T-G 3' on the left side of the center of symmetry with an α helix formed by residues 16–25. Perhaps some of the more NH₂-terminally situated residues of *lac* repressor are involved in recognition of base pairs further to the left of the symmetry center of *lac* operator. One may assume that the proper spacing of the α -helical arms functions considerably better with the natural *lac* operator, where the additional base pair forces the subunits further apart. One might consider that some kind of induced fit is necessary for the binding of *lac* repressor to the shorter palindromic operator. The base pairs 5' A-G-C 3' directly to the left of the center of symmetry are possibly recognized by residues 50–60 of *lac* repressor (33). Depending on whether recognition in this region involves fitting an α helix into the deep groove or binding an antiparallel or parallel β -sheet to the minor groove, or any other way of interaction, a partially nonsymmetric recognition may also occur just around the center of symmetry.

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