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gal4 transcription activator protein of yeast can function as a repressor in *Escherichia coli*

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The chromosomal *lac* operator of *Escherichia coli* was replaced by a 22 bp oligonucleotide containing the binding site of the yeast *gal4* protein. Induction of *gal4* protein synthesis in these bacteria repressed β-galactosidase synthesis at least 30-fold. These results show that it is possible to detect in bacteria with a simple assay the DNA binding activity of a eukaryotic protein with a defined sequence specificity. This opens new avenues for the isolation in *E. coli* of mutants of DNA binding proteins unable to bind to their DNA targets, and for direct cloning in bacteria of cDNA coding for DNA binding proteins with defined sequence specificity.

Key words: *gal4* transcription activator of yeast/*lac* system of *E. coli*/repressor of transcription

Introduction

The expression of many prokaryotic genes is regulated by repressors which bind to their DNA target sites, called operators. In the *lac* operon, the centre of symmetry of the *lac* operator is positioned 9 bp downstream from the transcription start site (Reznikoff and Abelson, 1978). When bound to its operator, *lac* repressor inferferes with RNA polymerase access to the *lac* promoter, and thereby prevents initiation of transcription. In the present work we examined whether the *lac* operator–repressor couple can be replaced by a eukaryotic DNA binding protein and its DNA target site.

Many of the eukaryotic DNA-binding proteins recently characterized act as positive transcriptional regulatory factors (Dynan and Tjian, 1985). This is the case of the *gal4* protein from *Saccharomyces cerevisiae* which activates the expression of the yeast *gal1* and *gal10* genes by increasing the rate of their transcription (Hopper *et al.*, 1978; St. John and Davis, 1981). The induction of these genes depends on a *cis*-acting DNA element, the *gal* upstream activating sequence (UAS_G) which is located midway between these divergently transcribed genes (Johnson and Davis, 1984; West *et al.*, 1984). *gal4* protein binds directly to four related 17-bp sites in the UAS_G to turn on transcription of both genes (Giniger *et al.*, 1985). It was shown that a single consensus synthetic 17-bp oligonucleotide is sufficient to mediate significant *gal4*-dependent activation of a downstream gene. In addition Giniger *et al.* (1985) showed by DMS protection that the *gal4* protein expressed in *E. coli* was able to bind the 17-bp oligonucleotide *in vivo*.

We show here that the *gal4* protein is able to repress the *lacZ* gene expression in *E. coli* more than 30-fold when the *lac* operator site is replaced by the *gal4* binding site. This opens the way for direct genetic selection of mutants of eukaryotic proteins such as *gal4*.

Results

Replacement of the lac operator with the gal4 binding site on the E. coli chromosome

We took advantage of plasmid pWB300 (Lehming *et al.*, manuscript in preparation) which carries the *lac* promoter and *lacZ* coding sequences. The *lac* operator is deleted and replaced by a *Xba*I linker as in pK060 (Besse *et al.*, 1986). We subcloned the 5′ portion of this operatorless *lacZ* gene in a pBR322 derivative. A 22-bp synthetic oligonucleotide containing the 17-bp consensus binding site of *gal4* with *Xba*I cohesive ends was inserted into the *Xba*I site of this plasmid (pBMN) to give plasmid pBMG (see Figure 1).

In order to introduce the modified operator regions into the chromosome plasmids, pBMN and pBMG were transformed into strain HfrG6Δ15 of *E. coli*. This lac^- strain (constructed by M.Dreyfus) bears a short deletion of the *lac* operon from position −14 to position +57 relative to the transcription-initiation site which removes part of the promoter and the 5′ end of the *lacZ* gene. Plasmids pBMN and pBMG bear homology with HfrG6Δ15 in two segments: (i) with the 5′ side of the *lacZ* promoter and (ii) with the beginning of the *lacZ* coding sequence.

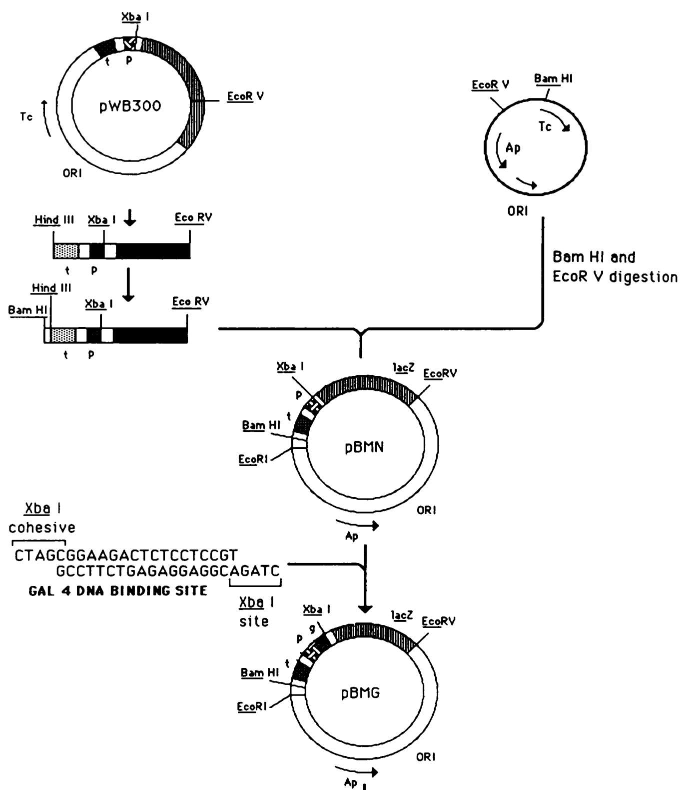

Fig. 1. Construction of plasmids containing modified *lac* operator regions.

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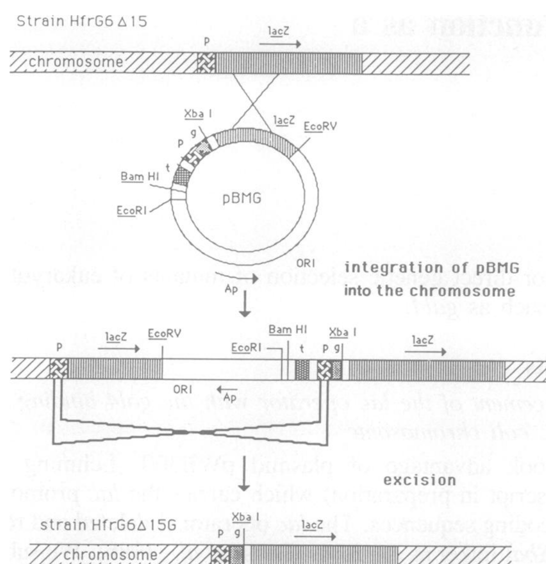

Fig. 2. Integration of modified *lac* operator regions into the *E. coli* chromosome.

The transformed HfrG6Δ15 colonies were plated on MacConkey lactose plates supplemented with ampicillin and gave white *lac* colonies. After incubation for two or three days at 37°C, some red colonies appeared. We reasoned that at first the white colonies exhausted from the medium all carbon sources other than lactose. Only recombinants that gained a functional *lacZ* gene were able to utilize lactose and grow and were easily identified because of the red color. These colonies were still ampicillin-resistant. We suppose that plasmids pBMN and PBMG were integrated into the chromosome by a single recombination event. To induce plasmid loss we grew the bacteria for successive passages in medium devoid of ampicillin. During this time a second recombination event probably occurred leading to the integration of the operator deletion or of the 22-bp oligonucleotide in the chromosome, excision of the plasmid derivative and loss of free plasmid (Figure 2). From such cultures we isolated HfrG6Δ15N and HfrG6Δ15G strains (resulting from recombination with pBMN and pBMG respectively) which had an ampicillin-sensitive constitutive lac^+ phenotype. The integration of the *gal4* binding site into the *lac* operator region was confirmed by Southern blot analysis of genomic DNA with radioactive oligonucleotides or DNA fragments as probes (data not shown). The resulting Hfr strains were conjugated with $TGIF^-$ (deleted for the *lac* region) to transfer our constructions to an F^- strain. Finally we introduced into the recombined F^- strains an F' episome carrying an overproducing *lacI* gene. The new strains were named TΔ15N and TΔ15G, originating from HfrG6Δ15N and HfrG6Δ15G respectively.

Repression of β-galactosidase synthesis by the gal4 protein

Plasmid pLPK76-7 carries the *gal4* gene under the control of a strong *lac* promoter derivative in which the −35 region of the *lac* UV5 promoter was replaced by the corresponding sequences from the *trp* promoter (Amann *et al.*, 1983; Keegan *et al.*, 1986). Such a promoter is repressed by the *lac* repressor and is inducible by the addition of isopropyl-β-D-thiogalactoside (IPTG). This plasmid and pBR322 as a control were introduced into TΔ15G and TΔ15N. To look for possible repression of our chimeric operon we measured β-galactosidase activity in extracts from liquid cultures grown in the presence or absence of IPTG (Table I). The constitutive level of β-galactosidase in strains TΔ15N and TΔ15G not harbouring the *gal4* gene was slightly different. This difference could be due to different translational efficiency because the leader of the mRNA is different in both cases. Introduction of pBR322 has no effect on the levels of β-galactosidase in these strains. Furthermore the expression of *gal4* protein in TΔ15N did not affect significantly the expression of *lacZ* either in the presence or absence of IPTG. On the contrary introduction of pLPK76-7 in TΔ15G showed a 5-fold repression without addition of IPTG. The *lac* promoter responsible for *gal4* protein synthesis is very strong, and the level of *lacI* expression might not be high enough to totally shut off the *gal4* gene. When IPTG was added to induce synthesis of *gal4* protein, we observed a further 7-fold decrease in the β-galactosidase activity or a 30-fold repression relative to the strain lacking *gal4* protein.

Table I. β-galactosidase synthesis in the different strains

Strains and plasmids	Spec. act. of β-gal −IPTG	Repression rate	Spec. act. of β-gal +IPTG	Repression rate
TΔ15N	430	1	440	0.98
TΔ15N + pBR322	400	1.07	392	1.1
TΔ15N + pLPK76-7	350	1.23	330	1.3
TΔ15G	305	1	300	1.02
TΔ15G + pBR322	263	1.16	258	1.2
TΔ15G + pLPK76-7	63	4.8	9	33

Strains TΔ15N and TΔ15G contain an *XbaI* restriction site in place of the *lac* operator sequences. In TΔ15G a 22-bp oligonucleotide carrying a *gal4* DNA-binding site was inserted into the *XbaI* site. Plasmid pLPK76-7 carries the yeast *gal4* gene under the control of the *lac* promoter (Keegan *et al.*, 1986). Overnight cultures in L.Broth were diluted 100-fold and synthesis of *gal4* was induced by addition of 10^{-3} M IPTG when the cultures reached a density of 0.1 at OD_{600}. β-galactosidase activities were determined when the cultures reached a density of 0.7. R is a measure of repression, it equals the specific activity of β-galactosidase of the uninduced strains (lacking *gal4* or pBR322 plasmids) divided by the specific activity of β-galactosidase in strains expressing *gal4*.

When plated on Luria medium (ML) containing X-gal, IPTG and ampicillin, TΔ15G colonies with pLPK76-7 plasmid were unambiguously white whereas TΔ15G colonies with pBR322 were blue. Even though β-galactosidase synthesis was partially repressed in TΔ15G + pLPK76-7 without IPTG, this 5-fold repression is not sufficient to render the colonies white on these plates.

TΔ15G + pLPK76-7 colonies were smaller when grown on ML + IPTG than when grown without IPTG. In liquid medium their generation time was 60 min with IPTG and 40 min without. The generation time of TΔ15G without any plasmid was 40 min. We interpret this change in growth rate as an indication that induction of *gal4* protein in *E. coli* interferes somehow with cell growth.

Evidence for specific gal4 target site binding activities in E. coli

To confirm that this repression is due to direct interaction of *gal4* protein with the 17-bp *gal4* target site, we used the gel retardation assay of Garner and Revzin (1981), which is based on the slower migration of DNA−protein complexes with respect to free DNA on polyacrylamide gels. Extracts were prepared from cultures of strains TΔ15G and TΔ15G containing pLPK76-7 in the presence or absence of IPTG. As a control the protein extracts were also tested with another 30-bp oligonucleotide which has no relation to any *gal4* DNA binding site. As shown in Figure 3, no genuine *E coli* protein bound to the *gal4* target oligonucleotide. When pLPK76-7 is introduced in TΔ15G and no IPTG is added, there is a low binding activity specific for this

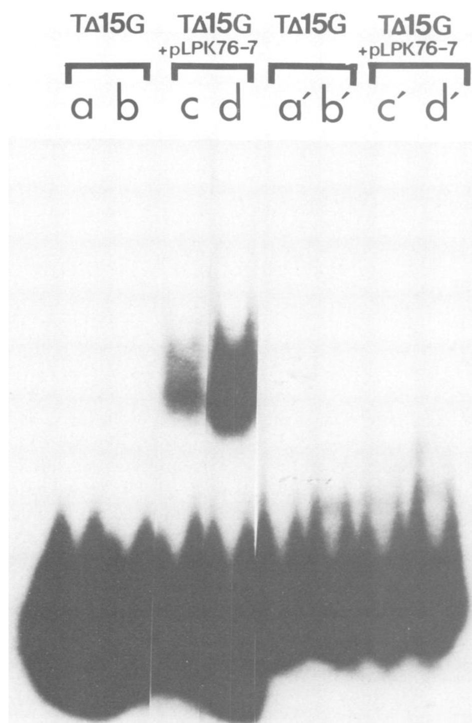

Fig. 3. Detection of *gal4* activity in bacterial extracts. Crude extracts were prepared as described in Materials and methods. The comparative studies of DNA-binding activities were done with 1.5 μg of protein in the binding assay. **a**: TΔ15G−IPTG extract with 22-mer; **b**: TΔ15G + IPTG extract with 22-mer; **c**: TΔ15G + pLPK76-7−IPTG with 22-mer; **d**: TΔ15G + pLPK76-7 + IPTG with 22-mer; **a′**, **b′**, **c′**, **d′**, extracts with the non-related 30-bp oligonucleotides respectively.

oligonucleotide. We interpret this as reflecting the basal expression of *gal4* gene despite the presence of *lac* repressor as mentioned above. Finally when synthesis of *gal4* was induced by addition of IPTG, we observed a more abundant specific complex.

Discussion

The present experiments show that the *lac* repressor operator system can be replaced functionally by a eukaryotic DNA-binding protein and its DNA recognition site. In agreement with the experiments of Giniger *et al.* (1985) the *gal4* protein made in *E. coli* acquires a conformation that permits rather specific DNA recognition on the chromosome of *E. coli*. Even though in yeast this protein is a positive transcription activator, in *E. coli* under these circumstances it can act as a repressor. We assume that the binding of a *gal4* protein dimer to the target DNA that overlaps the transcription initiation site will inhibit transcription by a mechanism similar to that of the *lac* repressor. We did not try to measure accurately the number of molecules of *gal4* protein made in the bacteria, and hence it is impossible to compare the relative affinities of *gal4* protein and *lac* represor for their respective targets. However it is clear that the affinity of the *gal4* protein is high enough to achieve a repression rate of at least 30-fold after the induction of *gal4* synthesis. The inhibition of bacterial growth that we observed after induction suggests that other sequences on the chromosome may be recognized by the *gal4* protein, which thereby interferes with the expression of one or more essential bacterial genes. The repression level we obtain after induction of *gal4* protein is sufficient to render these bacteria phenotypically *lacZ* negative or white on X-gal plates containing IPTG, whereas the initial bacteria expressing *lacZ* constitutively were blue on X-gal plates lacking IPTG.

Our results open a new prospect for the study of eukaryotic DNA-binding proteins with tools of classical bacterial genetics. It should be easy to isolate spontaneous or mutagen-induced lac^- derivatives that still retain the plasmid carrying *gal4*. These mutants would appear blue among white colonies on plates containing X-gal and IPTG. At least a class of these mutants should contain mutations in the DNA-binding domain of the *gal4* gene. The same approach can be used with other cloned DNA-binding proteins after introduction of their DNA target in place of the *lac* operator.

A similar approach can be attempted for the cloning of eukaryotic proteins with defined sequence specificity. In many cases the DNA targets of eukaryotic transcription regulatory proteins were identified by DNase I footprinting, dimethylsulfate protection or interference or by gel retardation assays with synthetic double-stranded oligonucleotides. After cloning of the specific target sequence in place of the *lac* operator, expression cDNA libraries in plasmid or phage can be introduced into these bacteria followed by screening for the few lac^- descendants that can be expected. It is plausible that even short cDNA or yeast DNA fragments carrying only the DNA-binding domain of a protein will permit repression and hence easier identification of genes coding for transcription factors.

Materials and methods

Strains and media

Standard bacterial cloning was performed in the strain TG1: *E. coli* K12Δ (*lac−pro*) SupE *thi hsd* D5/F′ *tra* D36 *pro* A^+B^+ *lacI*^{Q} *lacZ* M15. HfrG6Δ15 is a lac^- derivative of HfrG6 (Hofnung *et al.*, 1976) constructed by M.Dreyfus (personal communication). Bacteria were grown in L.Broth media described by Miller (1972) and were transformed in 100 mM $CaCl_2$ (Mandel and Higa, 1970).

Plasmids

Plasmid pLPK76-7 directs synthesis of *gal4* protein from the *tac* promoter in *E. coli*. The second codon of *gal4* is joined to the *E. coli lacZ* translation initiation codon (Keegan *et al.*, 1986). Synthesis of *gal4* protein is induced by addition of 10^{-3} M IPTG. The F′ episome used has the genotype *pro*A^+B^+, *lacZ* M15, *lacI*^{Q}. Preparation, purification, restriction enzyme digestion and gel electrophoresis of DNA were performed according to standard methods (Maniatis *et al.*, 1981).

Chemical DNA synthesis

Two 22-bp long oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer. They were purified on a 20% denaturing acrylamide gel followed by filtration on a G10 Sephadex column. These two complementary oligonucleotides reconstitute the 17-bp consensus *gal4* DNA-binding site. The 5′ end contained a *Xba*I cohesive end whereas the 3′ side reconstitutes the *Xba*I site after cloning into pWB300. The synthetic DNA fragments were phosphorylated with polynucleotide kinase, and complementary strands were annealed. A fragment containing the inserted oligonucleotides was subcloned in M13 phage, and the sequence of the synthetic operator and its orientation were verified by dideoxy sequencing with the standard M13 primer.

β-galactosidase measurements

The specific activity of β-galactosidase was determined as described by Miller (1972). Specific activity was calculated as follows:

$$A = \frac{1000 \times \text{OD } 420 \text{ nm}}{v \times t \times \text{OD } 600 \text{ nm}}$$

where v is the volume of bacterial culture in ml and t the time of reaction expressed in min.

Preparation of E. coli extracts

Cells grown to an OD_{600} of 0.7 were centrifuged at 4°C. The cell pellet was resuspended with the extraction buffer (200 mM Tris HCl pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 7 mM β-mercaptoethanol, 400 mM $(NH_4)_2SO_4$, 10% glycerol, 1 mM PMSF) and an equal volume of glass beads (0.45 mm diameter). The cells were disrupted at 0°C by agitation for 1 min with a vortex, and left on ice for 2 min. This operation was repeated 5 times. The extracts were cleared by cen-

trifugation for 1 h at 100 000 *g*. The final extract contained 5–15 mg of protein/ml and could be stored frozen at −70°C for several months without loss of binding activity.

Electrophoresis binding assay

Binding tests were carried out in 20 μl of Buffer O (20 mM Hepes pH 8, 5 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol) containing 0.5 ng ^{32}P-labelled double-stranded oligonucleotide, 0.4 μg of sonicated double-stranded salmon sperm DNA as carrier and 2–10 μg of protein. The reaction mixtures were incubated at 30°C for 20 min and immediately loaded on a 4% vertical polyacrylamide gel and electrophoresed at 120 V in TBE Buffer (90 mM Tris, 90 mM H_3BO_3, 2.5 mM EDTA) at room temperature. After migration for 2 h the gels were treated for 10 min in 10% acetic acid/10% ethanol mixture, dried and autoradiographed.

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