The EMBO Journal vol.5 no.1 pp.167-173, 1986

The upstream operator of the *Escherichia coli* galactose operon is sufficient for repression of transcription initiated at the cyclic AMP-stimulated promoter

Günter Kuhnke, Andrea Krause, Carin Heibach, Ursula Gieske, Hans-Joachim Fritz[1] and Ruth Ehring

Institut für Genetik der Universität zu Köln, Weyertal 121, D-5000 Köln 41, and [1]Max-Planck-Institut für Biochemie, Abteilung Zellbiologie, Am Klopferspitz, D-8033 Martinsried bei München, FRG

Communicated by B.Müller-Hill

Two operators are known to bind *Escherichia coli* galactose repressor with roughly equal affinity. A study of the control these two operators exert on the two overlapping *gal* promoters is reported. The experiments rest on a set of mutations specifically constructed to inactivate individual control units of the *gal* operon and on quantitation of *gal* promoter activities. Messenger RNAs initiated at one or other of the promoters in a cell-free transcription-translation system were determined by a primer extension assay with synthetic deoxyoligonucleotide primers. The main conclusions are: (i) the classical galactose operator O_1, located upstream with respect to the two overlapping promoters is sufficient for negative control of the cAMP activated promoter P_1; (ii) complete repression of the second promoter P_2, on the other hand, needs the presence of both intact operators O_1 and O_2. Thus, the two overlapping *gal* promoters (with only 5 bp separating their respective transcriptional start sites) are both subject to negative control by the galactose repressor. This regulation, however, is exerted by two different mechanisms.

Key words: DNA-protein recognition/galactose operon/oligonucleotide-directed mutation construction/regulation of gene expression

Introduction

The individual elements of gene regulation in procaryotes are comparatively simple (cf. recent reviews by von Hippel *et al.*, 1984; Sauer and Pabo, 1984). These elements, however, can be components of complex regulatory circuits where certain genes or operons are simultaneously subject to several controls, such as repression, transcription activation, attenuation or translational repression. Combinations of these permit regulation of gene expression in response to qualitatively different environmental stimuli and can also quantitatively increase the range over which this control is exerted.

The *Escherichia coli* galactose operon is a prototype example of such a complex system of superimposed positive and negative controls which differentially regulate the expression of the structural genes from two overlapping promoters (recently reviewed by de Crombrugghe *et al.*, 1984). In this system, the cAMP-CRP (cAMP receptor protein) complex acts as a positive and as a negative control element to activate transcription from one promoter P_1 and, acting from the same binding site, to repress the other promoter P_2 (cf. Busby *et al.*, 1982 and references cited therein).

Both promoters are subject to control by the *gal* repressor. Although this repressor exhibits a high degree of sequence homology with the *lac* repressor, particularly within the DNA-binding domain, and although the two repressors are evidently evolutionarily related (von Wilcken-Bergmann and Müller-Hill, 1982; von Wilcken-Bergmann *et al.*, 1983), they must act in different ways: whereas the *lac* repressor is known to prevent transcription by competing with RNA polymerase for overlapping binding sites (see review by Siebenlist *et al.*, 1980 and references cited therein), this mechanism is very unlikely to apply in the same straightforward way to the action of the *gal* repressor. The classical *gal* operator located upstream of both promoters as defined by O^c mutations (diLauro *et al.*, 1979; Adhya and Miller, 1979) and by repressor binding (von Wilcken-Bergmann *et al.*, 1983) does not overlap with any position implicated in the binding of RNA polymerase.

The discovery of a second *gal* operator (O_2) located within the first structural gene *galE* opened up various mechanistic possibilities to resolve the seemingly paradoxical situation (Irani *et al.*, 1983; Fritz *et al.*, 1983). Unlike the second operator of the *lac* operon, which exhibits a 5- to 10-fold lower affinity for *lac* repressor as compared with the major operator (Gilbert *et al.*, 1976), the second operator of the galactose operon binds *gal* repressor with essentially the same affinity as does the first operator. Plasmids containing both operators are twice as efficient in titrating repressor as those containing only one of the operators. The two operators act in a plainly additive way *in vitro* in a coupled transcription-translation system; a cooperative effect *in vivo* has neither been proven nor ruled out (Fritz *et al.*, 1983). Using purified galactose repressor, Majumdar and Adhya (1984) have also shown simultaneous occupation of the two *gal* operators located on the same DNA fragment.

While participation of the second operator O_2 in repression *in vivo* has been shown by a partially constitutive phenotype caused by a mutation located in that sequence (Irani *et al.*, 1983), there is no direct evidence so far for an obligatory interaction between repressors occupying both operators, as suggested by these authors.

On the contrary, our results presented here show that efficient repression of the cAMP-stimulated promoter P_1 can be achieved even though the downstream operator O_2 located within the *galE* gene has either been inactivated by a point mutation or been removed by *in vitro* DNA manipulations. We demonstrate here that the mutational inactivation of O_2 preferentially affects the regulation of the promoter P_2 which is also subject to repression by the cAMP-CRP complex.

Results

Phenotypes of single point mutations inactivating individual gal promoters P_1 or P_2 revealed in primer extension assays

To study the function of *gal* repressor in the control of the two overlapping promoters of the galactose operon, we have used a series of single point mutations specifically constructed to inactivate one or other promoter or either one of the two operators with minimal effect on the other regulatory elements. Details of their construction by oligonucleotide-directed mutagenesis or by

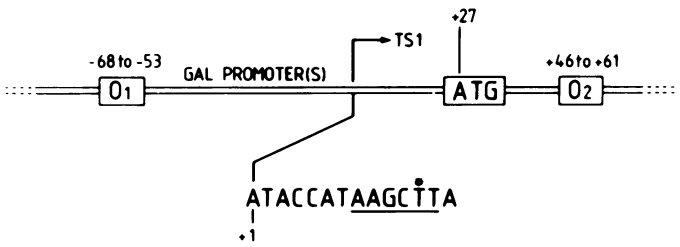

Fig. 1. Schematic representation of regulatory region of the *E. coli* galactose operon (taken from Fritz *et al.*, 1983). Location of the two *gal* operators O_1 and O_2 relative to the transcriptional start at position +1 of the cyclic AMP-stimulated promoter (TS1) is indicated. The *Hind*III recognition site in the *gal* leader DNA created by a single point mutation (C to T transition at the place marked by an asterisk) permits easy construction of plasmids carrying different combinations of wild-type and mutant *gal* operators.

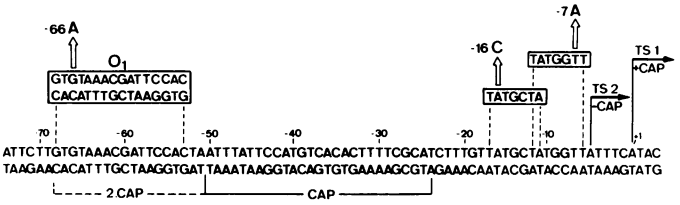

Fig. 2. Positions of structurally pre-determined mutations introduced into control elements of the galactose operon by oligonucleotide-directed mutation construction. Derivatives of plasmid pLF101 (see Figure 8) were constructed which differ from the parent plasmid only by one of the single point mutations indicated. In addition, a promoter mutation (A→T at position −11), originally characterized by Musso *et al.* (1977), has been reconstructed by the same method in this background (not indicated in this figure). The replacement G→A in position −66 (identified by diLauro *et al.*, 1979) in the O^c mutation $gal O^c_{81}$) was combined with either the wild-type configurations of both *gal* promoters or with the P_1 and P_2 mutations, also by oligonucleotide-directed mutation construction. The O^c_2 mutation (+59T) employed likewise has been described previously. It was isolated after hydroxylamine treatment of plasmid DNA *in vitro* and has been shown to inactivate the *gal* operator O_2 (Fritz *et al.*, 1983). In all cases the designation of the mutation indicates the newly introduced nucleotide at the site of the mutation; numbering is relative to the position of the cAMP-activated transcription initiation (TS1) at +1. In addition, in the figures the phenotype (P^- and/or O^c) is marked for clarity.

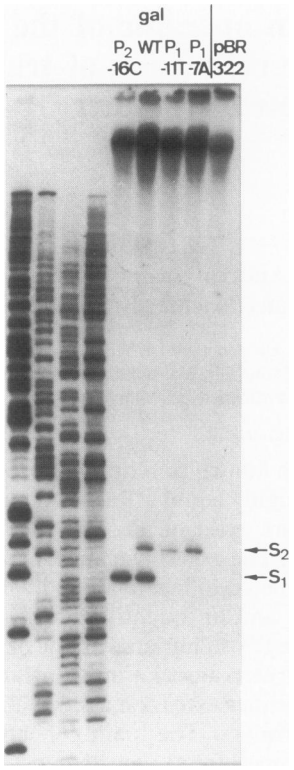

Fig. 3. Promoter-down phenotypes exhibited by newly constructed *gal* promoter mutations. Total mRNA was extracted from growing *E. coli* cells which differ from each other only with respect to the *gal* wild-type (WT) or mutant promoter alleles present on otherwise identical plasmids (pLF101 type, compare Figure 8). The bacterial chromosome carries a *gal* deletion. Cells harboring the vector plasmid pBR322 without the *gal* insert were analyzed in parallel as a control. *gal* mRNA was measured by the synthesis of cDNAs on these mRNA templates using a synthetic oligonucleotide primer (cf. Materials and methods). The autoradiograph shows these cDNAs after electrophoretic separation. As a size marker, products of chain degradation reactions performed on a known DNA fragment (Maxam and Gilbert, 1980) were applied to the first four lanes of the polyacrylamide gel.

other *in vitro* DNA manipulations will be presented elsewhere. The location of the regulatory sites and the mutational exchanges involved are summarized in Figures 1 and 2.

The effect of the different promoter mutations on the expression of the galactose operon *in vivo* is demonstrated by a primer extension assay (McKnight and Kingsbury, 1982), as described in the legend to Figure 3. Under the conditions used here, the wild-type galactose operon is predominantly expressed from the cAMP-activated promoter P_1. This yields the reverse transcript band labeled 'S1' in Figure 3, lane 'WT'; transcripts originating from the second promoter P_2 located 5 bp upstream contribute a minority band (labeled 'S2') as expected from studies by Aiba *et al.* (1981) who determined by S1 mapping the synthesis of both mRNA species under similar growth conditions.

The newly constructed promoter mutations *gal*(−16C) and *gal* (−7A) (see Figure 2) inactivate P_2 or P_1, respectively, with no detectable effect on the other promoter (Figure 3). A very low residual P_1 activity of the mutation *gal*(−7A) can be visualized on longer exposures (see also *in vitro* assays described below). An additional P_1 mutation was reconstructed by oligonucleotide-directed mutagenesis in this background for comparison. It carries the exchange of an AT to a TA base pair in position −11 which was originally identified by Musso *et al.* (1977) as the mutational exchange in two independently isolated promoter mutations *gal* p-211 and *gal* p-8-3. No residual activity of P_1 has been detected by the primer extension assay for this mutation *gal*(−11T). As seen in Figure 3 and confirmed in other experiments, the level of expression of promoter P_2 is also reduced by this mutation, as might be expected from the fact that the replacement of the AT base pair in position −11 which inactivates P_1 also represents an exchange of the moderately conserved purine nucleoside in the seventh position of the 'Pribnow box' of promoter P_2 (see Figure 2).

Differential function of the two gal operators in repression of the two promoters P_1 and P_2

To study the regulation of the two galactose promoters by the galactose repressor, we have applied the coupled transcription-translation system described previously for the analysis of the two operators (Fritz *et al.*, 1983). Here we test the function of *gal* repressor bound to either a single operator or to both operators to repress the structural genes of the galactose operon (see Materials and methods). The expression of the galactose operon under the control of wild-type or mutant promoters and/or operators was determined by primer extension assays as described above. Cell-free protein synthesis was programmed in each case by the addition of the appropriate plasmids and the resulting mix-

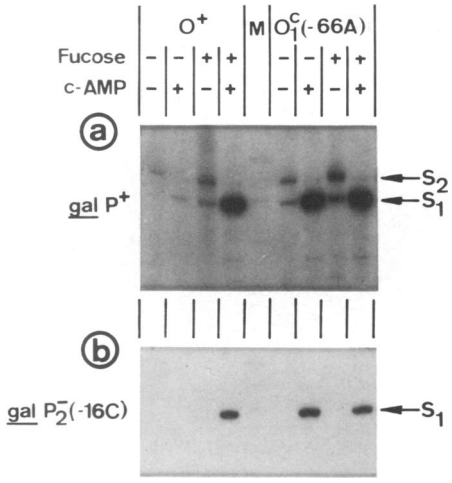

Fig. 4. Regulation by *gal* repressor of promoters P_1 and P_2 in a coupled transcription-translation system. *gal* mRNA transcribed in the presence of excess *gal* repressor with or without addition of cAMP and/or the inducer D-fucose, as indicated, was determined by the primer extension assay (cf. Figure 3 and Materials and methods). The DNA templates used in **(a)** contain both *gal* promoters P_1 and P_2 in wild-type configuration; in the experiment shown in **(b)** *gal* expression was limited to transcription from operator P_1 by use of the P_2 mutation. The last four lanes of **a** and **b** represent transcription reactions from the corresponding DNA templates in which repressor binding was limited to operator O_2 due to the O_1^c mutation. Products of a DNA sequencing reaction mixture applied as size marker to **lane 'M'** are visualized only by longer exposures. It cannot be decided at present whether the small increase of P_2 expression upon addition of D-fucose in the O_1^c background is significant (see also Figure 5).

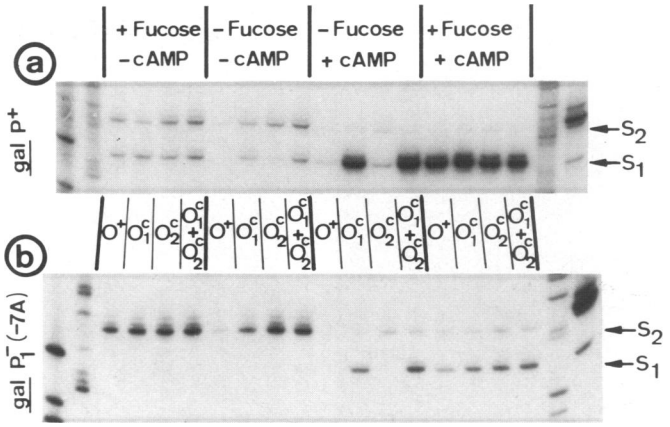

Fig. 5. Different role of the two *gal* operators in repression of the two promoters P_1 and P_2. Templates containing both *gal* operators in wild-type configuration (O^+) or with either one (O_1^c or O_2^c) or both (O_1^c + O_2^c) operators mutationally inactivated were used for transcription with or without cAMP and/or inducer and analyzed as described in the legend to Figure 4. In **(a)** these operator alleles were present in combination with both *gal* promoters in wild-type configuration, in **(b)** with the *gal*(−7A) promoter mutation (phenotype: normal transcription initiated at S_2, low level residual transcription from S_1). Products of DNA sequencing reactions were applied as size standards to the first two lanes and the last two lanes.

ture was incubated with or without the addition of cAMP and inducer (D-fucose) as indicated.

Transcription from the wild-type galactose operon is demonstrated in the first four lanes of Figure 4a. Under most conditions *in vivo*, each promoter contributes a fraction of the total number of transcripts (see Figure 3 and cf. Aiba *et al.*, 1981). Under the conditions used in our cell-free system, expression of the two promoters is almost mutually exclusive, dependent on the presence or absence of cyclic AMP. Initiation of transcription at promoter P_1 is practically completely dependent on the presence of cAMP and it is repressed unless inducer is added (see cDNA band in position 'S1' in lanes 3 and 4 of Figure 4a). *gal* mRNA transcribed from promoter P_2, on the other hand, is observed only in the absence of cAMP (see cDNA band in position 'S2' in lanes 1 and 3 of Figure 4a). As is expected (Musso *et al.*, 1977), it is repressed by the cAMP-CRP complex.

Furthermore, expression of P_2 is also repressed by *gal* repressor (compare band in position 'S2' in lanes 1 and 3 of Figure 4a). Thus, the cell-free system used here faithfully reflects the *in vivo* situation with respect to repressor control Tof both promoters, P_1 and P_2.

As seen in Figure 4b, inactivation of promoter P_2 does not change the expression of the galactose operon from P_1. Whereas the latter is completely repressed, when both operators are present in wild-type configuration ('O^+'), inactivation of the upstream operator O_1 results in a fully constitutive expression of P_1 (see last four lanes of Figure 4a and b). Conversely, it will be demonstrated below that inactivation of the downstream operator O_2 has very little effect on the expression from the cAMP-activated promoter P_1 (see Figure 5).

Inactivation of the second promoter P_2 does not change the extent of cAMP dependence of promoter P_1 (compare expression of P_1 in Figure 4a and b). This result is important with respect to a model proposed by Malan and McClure (1984) for the mechanism of positive control of the *gal* promoter P_1 by cAMP (see Discussion).

Additional mutant forms of the galactose operon have been included in this analysis to test the relative contribution of the downstream operator O_2 to the repression of the two *gal* promoters. Figure 5a summarizes tests performed with a series of plasmids carrying both promoters in wild-type configuration and, in addition, mutations which inactivate either one or both operators as indicated. Transcripts synthesized *in vitro* under different conditions were determined by the primer extension assay as described above.

The expression of the galactose genes from promoter P_2 is seen in the left half of Figure 5 (transcription in the absence of cAMP). The upper band ('S2') represents the *gal* mRNA initiated under the control of P_2. With both operators in wild-type configuration, its synthesis is repressed unless the inducer, D-fucose, is present. Mutations in either O_1 or O_2 result in an at least partially constitutive synthesis of this *gal* mRNA species in the absence of D-fucose.

Thus, both operators have to be intact for complete repression of transcription from promoter P_2. These results are supported by the analysis of a corresponding series of mutant *gal* operons which carry the promoter mutation *gal*(−7A) in addition to the different combinations of O_1 and O_2 alleles (see Figure 5b). As seen in the previous experiment, mRNA initiated at S_2 is efficiently repressed only when both operators are active, this time in the complete absence of transcription from P_1. Inactivation of either operator results in a constitutive expression from promoter P_2. Further experiments are required to test whether small differences in the levels of constitutivity exhibited by the mutations inactivating one or both operators are significant (see Figure 5, lanes under -fucose, -cAMP).

Essentially the same response to repressor bound either exclusively at O_1 or O_2 or to both operators was also observed for the reduced level of P_2 expression in tests with a series of plasmids carrying the promoter mutation *gal*(−11T) (data not shown).

Negative control of the cAMP-activated promoter P_1 tested in

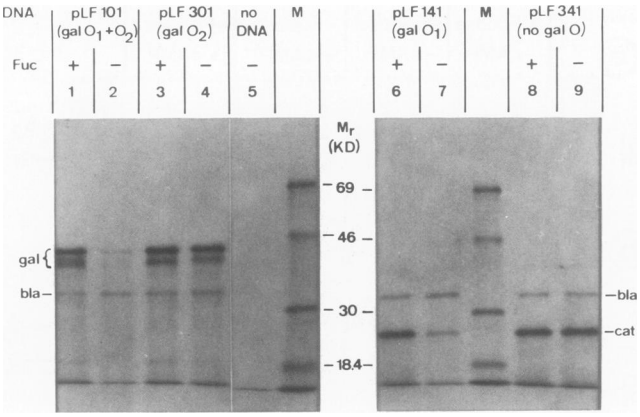

Fig. 6. Regulation of cAMP-activated *gal* transcription in the presence and absence of *gal* operator O_2. The autoradiograph shows the analysis of protein products synthesized from different genes under the control of *gal* regulatory elements. Different templates were used to direct the synthesis of either the galactose enzymes (gal) or of the chloramphenicol acetyl transferase (cat) in the coupled transcription-translation system. Under these conditions (+cAMP) there is practically no transcription from *gal* promoter P_2 (see Figure 7). The presence of both operators in wild-type configuration (gal O_1+O_2) or of either O_1 (gal O_1) or O_2 (gal O_2) on the plasmids is indicated in the figure as is the addition of D-fucose. The structure of plasmids used as templates is shown in Figure 8. The precursor of the β-lactamase (bla) encoded by the ampicillin resistance gene is transcribed from its own promoter on all plasmids. For conditions for protein synthesis and electrophoretic separation see Materials and methods.

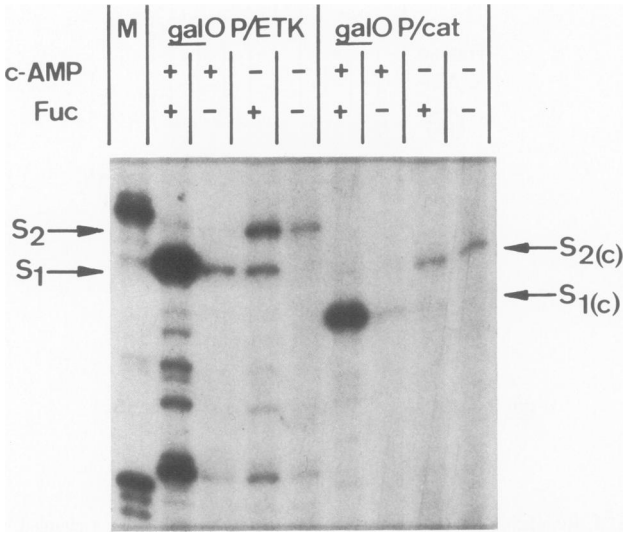

Fig. 7. Transcription from cAMP-activated *gal* promoter P_1 can be normally repressed despite the absence of *gal* operator O_2. *gal* mRNA and *cat* mRNA levels were determined under conditions similar to those shown in Figure 6. Expression of wild-type *gal* operon (four lanes labeled *gal* OP/ETK) and of *cat* structural gene expressed from *gal* promoters under control of operator O_1 in the absence of operator O_2 (four lanes labeled *gal* OP/cat) were studied in the cell-free transcription-translation system programmed by plasmids pLF101 and pLF141, respectively (cf. Figure 6). Addition of cAMP and D-fucose was as indicated. *gal* mRNA initiated at S_1 and S_2 and *cat* mRNA initiated at $S_{1(c)}$ and $S_{2(c)}$ were determined by primer extension assays as described for Figures 3–5, with the exception that an oligonucleotide primer complementary to *cat* mRNA was used for the analysis of *cat* mRNA transcribed from plasmid pLF141. For details see Materials and methods.

parallel is seen on the right of Figure 5 (transcription carried out in the presence of cAMP). It is evident that repression of this promoter is not affected by mutational inactivation of the operator O_2 (compare lanes 'O_2^c' and 'O^+'), provided the upstream operator O_1 is present in a functional state. Inactivation of O_1, on the other hand, results in a completely constitutive P_1 expression. This also holds true for the regulation of the low level P_1 expression seen in Figure 5b. In this experiment, plasmids have been used which carry the *gal*(−7A) allele and, in addition, the operator mutations indicated. Taken together, the results shown in Figures 4 and 5 demonstrate that the second operator is not required for the control of the cAMP-stimulated transcription from P_1. In contrast, it clearly has a function in regulating P_2 which is also at least partially controlled by repressor bound to O_1.

Repression of P_1-initiated transcription in the absence of a second operator O_2

A possible argument against the above conclusion that repression of P_1 is solely controlled by O_1 could be to assume that even the mutated form of O_2 in *gal*(+59T) (indicated as O_2^c in Figure 5) contributes some residual function which might be required for the repression of P_1. This argument was ruled out as follows: the *cat* gene of transposon Tn9 (Alton and Vapnek, 1979) was placed under the control of the *gal* promoters replacing the structural genes of the galactose operon and hence removing the downstream operator O_2 from the resulting plasmids (see Materials and methods). DNA sequence comparison and *in vitro* competition assays were used to rule out the fortuitous presence of a functional *gal* operator in the *cat* gene (see Materials and methods).

Despite the absence of operator O_2, synthesis of chloramphenicol acetyltransferase in the cell-free system programmed by this plasmid is repressed, unless the inducer D-fucose is added (compare lanes 6 and 7 in Figure 6), as is the synthesis of the galactose enzymes encoded by the parent *gal* plasmid which contains O_2 and which was tested in parallel reaction mixtures (lanes 1 and 2 in Figure 6). Repression of the *cat* gene under these conditions has also been demonstrated by measurements of the chloramphenicol acetyltransferase activity synthesized *de novo* (data not shown). These results also show that the mRNA molecules determined by the primer extension assay had been elongated to yield functional translation templates giving rise to complete gene products.

Tests of the proteins synthesized, as shown in Figure 6, actually underestimate the extent of repression of P_1 transcription. In the absence of inducer, there is only a very low concentration of mRNA initiated at S_1 (see Figure 7). The protein products observed in the absence of inducer for both the *gal* enzymes as well as for the chloramphenicol acetyltransferase mainly result from readthrough transcription initiated within pBR322 sequences located counterclockwise from the *Eco*RI site (data not shown).

It should be pointed out that the cell-free protein synthesis illustrated in Figure 6 proceeded in the presence of cAMP and thus does not reflect any transcription initiated at the promoter P_2.

Lanes 3 and 4 of Figure 6 show the results of experiments in which transcription was initiated at the modified tRNA promoter P′ (Ryan *et al.*, 1979). They are in agreement with the notion that, under these conditions, *gal* repressor bound exclusively to operator O_2 in the absence of the upstream operator O_1 does not affect significantly the regulation of the galactose operon (see Figure 8 for the structure of the plasmids). These findings correspond to those shown in Figures 4 and 5, where a completely constitutive expression from promoter P_1 has been demonstrated after inactivation of the upstream operator O_1 as a result of a point mutation.

The analysis of the protein products encoded by the *gal* wild-

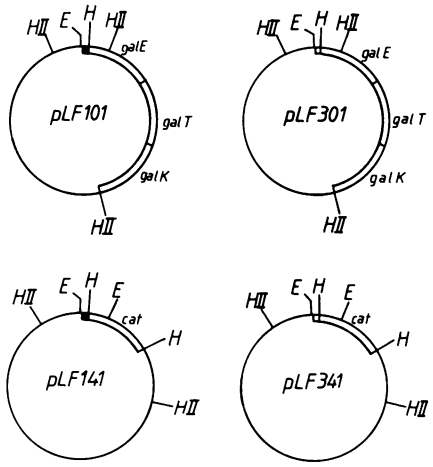

Fig. 8. Structure of plasmids used in this study. The diagram (not drawn to scale) shows plasmids derived from pLF101. This is a *gal* derivative of pBR322 described by Fritz *et al.* (1983). In pLF301, the *gal* control region is replaced by a modified promoter of the tyrosine tRNA gene, leaving only wild-type operator O_2 in the *galE* gene. pLF141 and pLF341 correspond to pLF101 and pLF301, but the structural genes of *gal* operon are replaced by the *cat* structural gene. pLF141 carries *gal* operator O_1 and both *gal* promoters in wild-type configuration, but is devoid of operator O_2. pLF341 does not contain any *gal* control element. All point mutations used in this study are carried on plasmids otherwise identical to pLF101. Abbreviations E, H and HII denote recognition sequences for restriction endonucleases *Eco*RI, *Hind*III and *Hinc*II, respectively.

type plasmid (pLF101) and by the *cat*-containing plasmid (pLF-141) is complemented and extended by the analysis of the corresponding mRNAs (Figure 7). P_1 transcripts initiated in the presence of cAMP (bands labeled 'S_1' and '$S_{1(c)}$', respectively) are repressed to the same extent despite the fact that the plasmid expressing the *cat* gene under control of the *gal* promoters does not contain the second operator sequence, O_2.

In agreement with the results presented in Figures 4 and 5, the expression of the structural *cat* gene from P_2 is at least partially constitutive in the absence of O_2, confirming the requirement of this operator for full repression of P_2.

Discussion

Two operators have been assumed to be involved in the repression of the two overlapping *gal* promoters of *E. coli* on the basis of DNA sequence homology as well as the equal affinity for *gal* repressor (Irani *et al.*, 1983; Fritz *et al.*, 1983). In particular, participation of the second operator in repression *in vivo* has been inferred from the fact that a mutation within this operator (located within the *galE* gene) results in a partially constitutive phenotype (Irani *et al.*, 1983). A model suggested to explain this effect involves simultaneous interaction between one repressor molecule and two operators or between two repressor molecules bound to the two operators (Irani *et al.*, 1983; Majumdar and Adhya, 1984) thus holding together a loop of DNA. This supposed loop would contain the two promoters and transcription was thought to be inhibited due to this configuration.

Contrary to this assumption, our results show that an intact upstream operator O_1 suffices for repression of the cAMP-activated promoter P_1. Furthermore, they show that repression of the two overlapping promoters must be achieved by two different mechanisms. The downstream operator O_2 is indispensable for complete repression of promoter P_2, but this promoter is also affected by *gal* repressor bound at the upstream operator O_1. Findings with the wild-type form of both promoters were confirmed by the analysis of mutants in which either P_1 or P_2 had selectively been inactivated. Finally, it was shown that, even in the complete absence of the second operator, initiation at S_1 can be completely repressed by *gal* repressor bound to O_1. Expression from promoter P_2, however, is at least partially constitutive under these conditions.

The reduced level of residual P_1 activity observed with the promoter mutation *gal*(−7A) responds to negative control by repressor in the same way as wild-type P_1 (no influence of O_2). This rules out the possibility that it is only the low transcriptional activity of promoter P_2 that enables repressor bound at the downstream operator O_2 to block transcription initiated at S_2.

At present, repression of the cAMP-activated promoter P_1 can best be explained by a model considered by diLauro *et al.* (1979) and modified by Shanblatt and Revzin (1983): *gal* repressor bound to O_1 may interfere with the binding of the cAMP-CRP complex and thus prevent transcription from promoter P_1. Initially this appeared unlikely as the sequences protected by these proteins do not overlap (diLauro *et al.*, 1979; Taniguchi and de Crombrugghe, 1983). Recent findings of Shanblatt and Revzin (1983), however, support such a model. According to these authors two molecules of CRP are required for activation of transcription from the *gal* promoter P_1. The DNA sequence occupied by the complex of cAMP and CRP together with bound RNA polymerase extends into the upstream operator O_1. Thus, competition between *gal* repressor and this complex for overlapping binding sites could account for repression of promoter P_1.

No such model appears adequate to explain repression of P_2. This promoter is at least partially constitutive in the absence of the downstream operator; repressor bound exclusively to this site, however, does not result in complete repression. Although this operator titrates repressor with essentially the same efficiency as does the upstream operator (Fritz *et al.*, 1983), *gal* repressor bound to this site does not completely block the progress of transcription initiated at S_2 and it has no influence on transcription from S_1. This corresponds to earlier reports by Eron *et al.* (1971) of a failure of *lac* repressor to interfere with transcription initiated further upstream.

Complete repression of *gal* promoter P_2 is only observed when both operators, O_1 and O_2, are intact. The results of Majumdar and Adhya (1984) are in agreement with titration experiments by Fritz *et al.* (1983) which revealed that DNA fragments comprising two *gal* operator sequences can bind twice as much *gal* repressor as fragments containing only one operator sequence. However, there is no evidence available to date which proves simultaneous occupation of both operators to be required for repression. Alternatively, a possible passive contribution of a second binding site could consist of more efficient locating of the functional operator by repressor in the process of linear diffusion (Winter *et al.*, 1981).

A variant of the model proposed by Adhya and co-workers was suggested by Dunn *et al.* (1984) to explain the regulation of the *ara* BAD operon. In this case, however, the situation is different in that the promoter is not located between the two operators.

An additional feature important to the regulation of the galactose operon may be the mutual effects exerted by the two promoters on each other. In analogy to results on the regulation of the lactose operon, Malan and McClure (1984) proposed promoter selection as an important control mechanism also for the *E. coli* galactose operon. According to this model, the stimulatory effect of the cAMP-CRP complex on *gal* promoter P_1 is at-

tributed, at least to a certain extent, to the repression of the competing promoter P_2 by the cAMP-CRP complex. In this context, it should be pointed out that in our experiments neither the expression of the *gal* promoter P_1 nor its dependence on cAMP have been affected by mutation *gal*(−16C) which completely inactivates promoter P_2. Thus, our data are in agreement with the conclusion put forward by de Crombrugghe *et al.* (1984) who, on the basis of different evidence, suggested that activation of promoter P_1 cannot be merely the result of repression of promoter P_2 by the cAMP-CRP complex.

Our results show that the two *gal* promoters are not only differentially affected by the cAMP-CRP complex, but they also differ in their response to repressor bound to O_1 and/or O_2. The DNA loop model of Adhya and co-workers is clearly ruled out for repression of the cAMP-activated transcription from promoter P_1. For P_2 the evidence available to date does not allow a decision for or against any particular mechanism.

Materials and methods

Materials

The materials used were from the following sources: 2′-deoxycytidine 5′-α-[^{32}P]triphosphate and the mixture of uniformly ^{14}C-labeled L-amino acids from Amersham Buchler (Braunschweig, FRG); 2′-deoxyriboside 5′-triphosphates from Boehringer (Mannheim, FRG); substances for polyacrylamide gel electrophoresis from BioRad (München, FRG); avian myeloblastosis virus reverse transcriptase ('Super RT') from Stehelin and Cie AG (Basel, Switzerland). Substrates for cell-free protein biosynthesis and other chemicals were from the sources indicated previously (Fritz *et al.*, 1983; Wetekam *et al.*, 1971, 1972).

Bacteria and plasmids

E. coli K12 strain RE739 carrying plasmid pgR6 (von Wilcken-Bergmann, 1983) was used to prepare protein-synthesizing cell extracts, as has been described (Fritz *et al.*, 1983). The *galR* gene on the multicopy plasmid (von Wilcken-Bergmann and Müller-Hill, 1982) results in increased levels of *gal* repressor.

All plasmids mentioned below were introduced by transformation into *E. coli* K12 strain F165, a T1-resistant derivative of Fi 165 which carries a deletion of most of the galactose operon (Fiethen and Starlinger, 1970). Cultures harboring the different plasmids were used for isolation of *gal* mRNA as well as for preparation of plasmid DNA.

The construction of plasmid pLF101 (see Figure 8) has been described (Fritz *et al.*, 1983). This pBR322 derivative contains an *E. coli* galactose operon which differs from the wild-type by elimination of the *Hind*III restriction site originally present within the *galE* structural gene and by the introduction of a new unique *Hind*III site into the leader sequence (cf. Figure 1). The 83-bp *Eco*RI-*Hind*III fragment containing the upstream operator O_1 and both *gal* promoters has been replaced in plasmid pLF301 by a 55-bp *Eco*RI-*Hind*III fragment containing the modified synthetic promoter of the tyrosine suppressor tRNA gene (Ryan *et al.*, 1979). Plasmids pLF141 and 341 resemble pLF101 and 301 (see Figure 8) with the exception that instead of the galactose structural genes the chloramphenicol acetyltransferase gene (*cat*) has been placed under the transcriptional control of the two promoter regions. The 785-bp *Hind*III fragment contains the 773-bp *Taq*I fragment from transposon Tn9 (Alton and Vapnek, 1979) on which the *cat* gene, including its ribosomal binding site, is located. Control experiments (data not shown) have confirmed that a plasmid with the *cat* insert but lacking the *gal* control region fails to titrate the *gal* repressor in the *in vitro* test applied by Fritz *et al.* (1983). A computer search for a sequence resembling that of the *gal* operators did not reveal any significant homology within the *cat* insert.

Derivatives of plasmid pLF101 containing single point mutations inactivating one or other *gal* promoter and the upstream operator O_1 have been obtained by oligonucleotide-directed mutation construction essentially as described by B.Kramer *et al.* (1984) and by W.Kramer *et al.* (1984); details will be described elsewhere (Fritz *et al.*, in preparation). Double mutants carrying the *gal*(−66A) mutation in O_1 in combination with either one of the promoter mutations were prepared by a second cycle of oligonucleotide-directed mutation construction. The unique *Hind*III site was utilized to prepare, by *in vitro* recombination, the derivatives carrying mutation *gal*(+59T). This mutation, previously referred to as allele 110-2, has been shown to inactivate the downstream operator O_2 (Fritz *et al.*, 1983). It has been verified by recloning and sequence analysis that the mutant galactose operons differ from that on pLF101 only by the exchange(s) indicated in their designation (see Figure 2).

It should be stressed that in all cases the designations O_1^c and O_2^c in Figures 4 and 5 denote the same −66A and +59T mutational exchange, respectively.

Conditions for transformation, DNA preparation and restriction analysis have been described (Fritz *et al.*, 1983).

Cell-free protein biosynthesis

Cell-free protein biosynthesis in a repressor-containing cell extract was as previously described (Fritz *et al.*, 1983) with the following modifications: *gal* plasmid DNA was added at 15 μg/ml final concentration, the plasmids containing the *cat* gene were used at the same molarity. Incubation was for 20 min at 37°C without polyethylene glycol. A mixture of L-amino acids uniformly labeled with ^{14}C was added to 50 μCi/ml (= 1.85 MBq/ml) final concentration. In addition, all 20 L-amino acids were present at 0.22 mM final concentration, as indicated by Zubay *et al.* (1970). The reaction was terminated by the addition of DNase and RNase and 5 μl aliquots of each reaction mixture were separated on polyacrylamide gels containing 15% acrylamide/0.087% bisacrylamide in the presence of 0.1 M SDS, as previously described (Ehring *et al.*, 1980). The relative molecular mass of [^{14}C]methylated marker proteins (NEN) applied to lanes marked 'M' is indicated in Figure 6.

RNA isolation

RNA from growing cultures of strain F165 carrying different *gal* mutant and wild-type plasmids as indicated in the figures was isolated as described by Aiba *et al.* (1981). M9 medium glycerol (1%) as carbon source was supplemented with Difco 'casamino acids' (0.1%), L-tryptophan (40 μg/ml) and ampicillin (35 μg/ml). Cells were harvested at an optical density of 0.2−0.3 after growth for ~3−4 generations at 37°C in the presence of the inducer D-fucose (5 mM). All concentrations are final concentrations present in the culture or incubation medium.

RNA was also prepared from cell-free protein synthesis mixtures directed by the different plasmid DNAs. Reaction conditions with an excess of *gal* repressor were as indicated above for cell-free protein synthesis, with the following modifications. Polyethylene glycol 6000 was present at 2.5% except for the experiment shown in Figure 7. Radioactive amino acids were omitted. cAMP and D-fucose, if added as indicated in the figures, were present at 0.5 mM and 20 mM final concentration. After an incubation of 10 min at 37°C in the presence of 5 μg/ml of plasmid DNA (total volume 50 μl), the reaction was terminated by a 1:5 dilution into 0.02 M sodium acetate, pH 5.5, containing DNase I (Worthington DFP) at a final concentration of 25 μg/ml. After 1 min at 37°C, the solution was extracted once with phenol saturated with 0.02 M sodium acetate (pH 5.5). RNA was precipitated in the presence of 0.3 M sodium acetate by the addition of three volumes of ethanol.

Primer extension assay of gal transcripts

Primer extension assays of mRNA transcribed either in plasmid-bearing *E. coli* cells or *in vitro* in a cell-free protein synthesizing system were essentially performed as described by McKnight and Kingsbury (1982). The two oligodeoxynucleotides (a) and (b) served as primers for the synthesis of cDNA on *gal* mRNA and *cat* mRNA templates, respectively, initiated at the two *gal* promoters. The 19-mer (a) 5′-d[CGCTACCACCGGTAACCAG] is complementary to the sequence of *gal* mRNA comprising nucleotides +36 to +54, position +1 being the first nucleotide of the cAMP-stimulated transcription. The 21-mer (b) 5′-d [CCATTTTAGCTTCCTTAGCTC] is complementary to the sequence comprising nucleotides +27 to +48 of the recombinant transcription unit of pLF141, when the *cat* gene is transcribed from *gal* promoter P_1. These oligonucleotide primers were synthesized using a DNA-Synthesizer (Applied Biosystems 380A) tuned to phosphoramidite chemistry essentially as described by Caruthers (1982).

The RNA isolates described above were dissolved in water and 15 μg of total RNA was used for each reaction. These measurements are based on the determination of u.v. absorbance at 260 nm using a nominal absorbance of A_{260} = 0.025 for a solution of 1 μg/ml of RNA (1 cm light path). After an ethanol precipitation the RNA was dissolved and incubated with 300 fmol of the synthetic oligonucleotide (a) needed to prime the reverse transcriptase reaction on *gal* mRNA. The hybridization mixture contained 71 mM Tris-HCl (pH 8.3), 14 mM $MgCl_2$, 14 mM dithiothreitol in a total volume of 14 μl. After 5 min at 65°C the mixture was cooled to room temperature and brought to a final volume of 20 μl by the addition of dATP, dGTP and dTTP (final concentrations of 125 μM each) and of dCTP to 12.5 μM and α-^{32}P-labeled dCTP to a specific radioactivity of ~15 Ci/mmol. After incubation with two units of avian myeloblastosis reverse transcriptase for 30 min at 42°C, the reaction was terminated by ethanol precipitation. The pellet was washed once with 70% ethanol and dissolved in alkaline formamide solution for electrophoresis on 10% polyacrylamide gels containing 7 mM urea. Complete elongation of primer (a) on *gal* mRNA by reverse transcriptase yields cDNAs 54 and 59 nucleotides long for the *gal* transcripts initiated at S_1 and at S_2, respectively (see Figure 2).

cat mRNA transcribed from the *gal* promoters with plasmid pLF141 as template (see Figure 7) was determined by the same procedure with the exception that the synthetic oligonucleotide (b) was used as a primer which is elongated into cDNAs 48 and 53 nucleotides in length, respectively.

Controls were performed to show that production of these cDNAs is completely

dependent on the presence of plamids containing a functional *gal* promoter and on the addition of the oligonucleotide primer. Under the conditions used, the primer was present in excess. cDNA bands of lower mol. wt. were seen in relatively small amounts, but reflecting the different intensities of the major bands. They are attributed to premature termination of the reverse transcriptase reaction at discrete sequences of the mRNA template, as has been noted previously (McKnight and Kingsbury, 1982).

Acknowledgements

We thank B.Müller-Hill and P.Starlinger for critical reading of the manuscript. This work was supported in part by Deutsche Forschungsgemeinschaft through Sonderforschungsbereich SFB 74.

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Received on 22 October 1985