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Unusual properties of promoter-up mutations in the *Escherichia coli* galactose operon and evidence suggesting RNA polymerase-induced DNA bending

Günter Kuhnke, Hans-Joachim Fritz[1] and Ruth Ehring

Institut für Genetik, Universität zu Köln, Weyertal 121, D-5000 Köln 41, and [1]Max-Planck-Institut für Biochemie, Abteilung Zellbiologie, Am Klopferspitz 18, D-8033 Martinsried bei München, FRG

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Two mutations are described, each of which renders the Pribnow box sequence of one of the two overlapping promoters of the *Escherichia coli* galactose operon identical to the consensus sequence TATAAT. Both double exchanges were specifically introduced into the original context by oligonucleotide-directed mutation construction. Each of the mutant promoters exhibits a greatly enhanced capacity to form stable complexes with RNA polymerase, as judged by nuclease protection experiments and by assaying shifts of electrophoretic mobility. On the other hand, the effect of the same mutations on the rates of transcription from the two *gal* promoters is strikingly different. Unexpectedly, when complexed with RNA polymerase, DNA fragments carrying one of the two double exchanges were found to differ from each other as well as from the corresponding wild-type fragment with respect to their electrophoretic mobilities. These observations are indicative of different three-dimensional structures of these complexes which may reflect different forms of DNA bending induced in these otherwise identical fragments by complex formation with RNA polymerase.

Key words: DNA bending/DNA-protein recognition/galactose operon/promoter selection/RNA-polymerase

Introduction

Organization of regulatory regions for gene expression as complex arrays of several mutually interacting control elements seems to be a frequent phenomenon in both prokaryotic and eukaryotic organisms [see reviews by Dynan and Tjian (1985), Ptashne (1986) and references cited therein]. The *Escherichia coli* galactose operon is a prototype example of that sort. Two physically overlapping promoters P_1 and P_2 are controlled by two regulatory proteins, the cAMP–CRP (cyclic AMP receptor protein) complex and the *gal* repressor. The former stimulates transcription from P_1 and — acting from the same binding site — represses transcription from P_2 (Musso *et al.*, 1977; Aiba *et al.*, 1981; Taniguchi and deCrombrugghe, 1983; Herbert *et al.*, 1986; references cited by these authors). The *gal* repressor exerts negative control on both promoters (Adhya and Miller, 1979), yet via different mechanisms (Kuhnke *et al.*, 1986). In that process, the *gal* repressor utilizes two unusually located operators O_1 and O_2 (Irani *et al.*, 1983; Fritz *et al.*, 1983).

We have started to use oligonucleotide-directed construction of mutations to dissect the regulatory circuitry of the *gal* operon. In a previous analysis (Kuhnke *et al.*, 1986), we identified different contributions of operators O_1 and O_2 to negative control of the individual promoters P_1 and P_2. Moreover, we demonstrated that cAMP–CRP dependent stimulation of transcription from P_1 cannot be explained as a secondary effect of the repression of P_2 by the same protein as has been suggested (Malan and McClure, 1984).

Here we describe properties of two newly constructed mutations *gal* (-8A, -9A) and *gal* (-13A, -14A) which render the 'Pribnow box' regions of P_1 and P_2, respectively, identical to the consensus sequence TATAAT (Hawley and McClure, 1983). Our analysis addresses the following problems associated with promoter structure/function: (i) competition for RNA polymerase of two physically overlapping promoters; (ii) differential effects of promoter mutations on binding of RNA polymerase and on rate of transcription; and (iii) gross geometry of RNA polymerase/DNA complexes, e.g. possibly different DNA bending in these complexes with different mutations of the same control region.

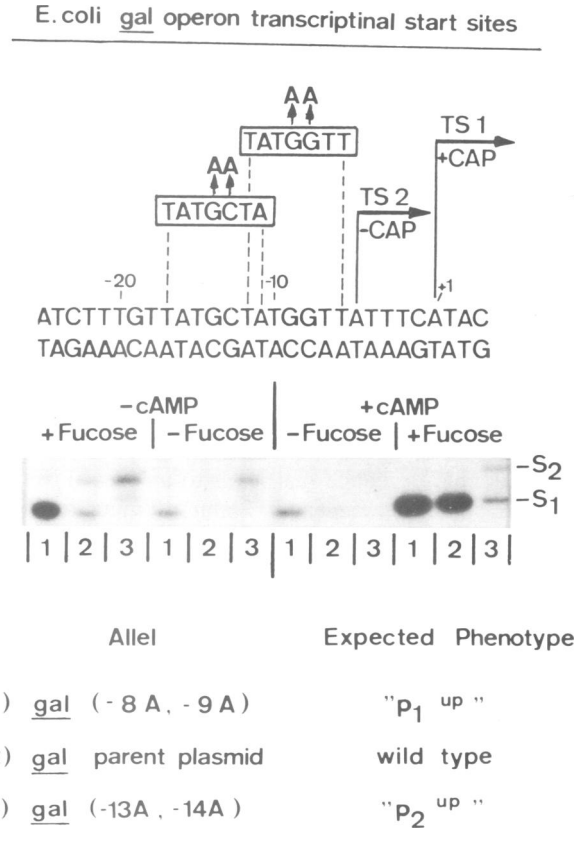

Fig. 1. Mutational exchanges and phenotypes of two newly constructed *gal* promoter mutations. Two mutant plasmids differing from pLF101 in the positions indicated were used as templates for mRNA synthesis in a coupled transcription–translation system. *gal* mRNAs produced in the presence or absence of inducer (D-fucose) and cAMP as indicated were quantitated by a primer extension assay. Positions of the cDNAs corresponding to mRNA initiated at either P_1 or P_2 are marked as S_1 and S_2, respectively. For designation of the mutations and for details of the procedure cf. Materials and methods.

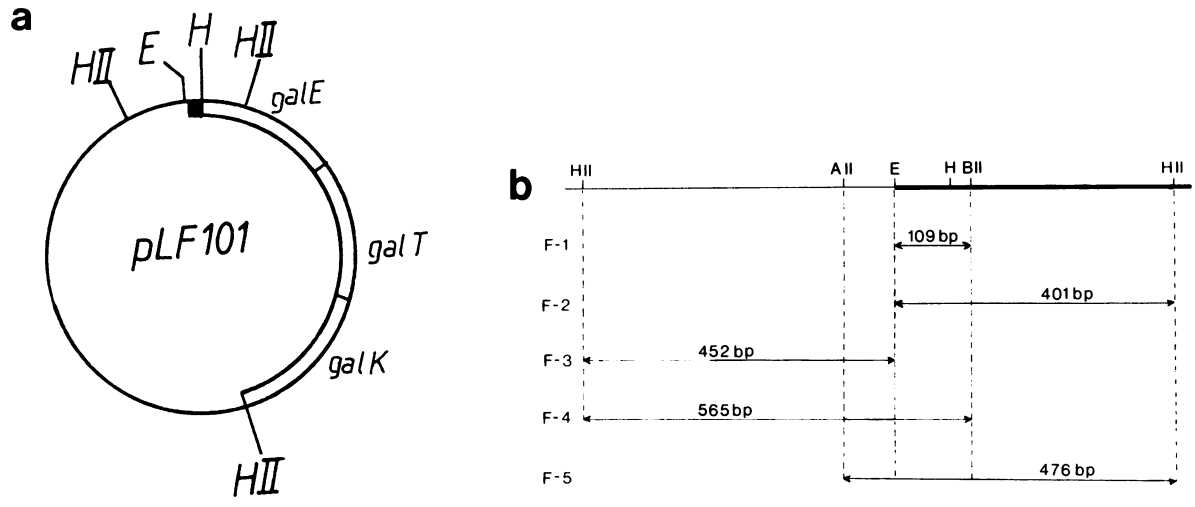

Fig. 2. Diagrammatic representation of plasmid pLF101 and of DNA fragments derived from it. **(a)** The *gal* mutations used in this study have been constructed in the parent plasmid pLF101 (Fritz *et al.*, 1983), a pBR322 derivative which contains the *E. coli* galactose operon. Only those restriction sites relevant for mutation construction or for the preparation of DNA fragments have been indicated in the figure taken from Kuhnke *et al.* (1986). **(b)** DNA fragments F-1 to F-5 to be used in the RNA polymerase binding studies (see below) comprising wild-type or mutant *gal* promoter regions differ only by the mutational exchanges specifically indicated in each case. The heavy line represents *E. coli* DNA, the thin line pBR322 DNA. Fragment length in base pairs (bp) refers to the length of the double-stranded segment. Recognition sites for restriction enzymes are denoted as follows: E, *Eco*RI;H, *Hin*dIII; HII, *Hin*dII; AII, *Aat*II; BII, *Bst*EII.

Results

Construction and phenotypes of mutations gal (-8A, -9A) and gal (-13A, -14A)

In a previous study (Kuhnke *et al.*, 1986) we separated the two *gal* promoters P_1 and P_2 by structurally pre-determined mutations which individually and alternatively deactivate one or the other of these physically overlapping promoters. On the other hand, up-mutations of P_1 and P_2 are of equal interest in the study of possible competition between the two promoters. Moreover, it seemed of particular importance to obtain a mutant of the *gal* regulatory region, which would guide initiation of transcription from promoter P_1 in a cAMP−CRP independent fashion. Precedents for such mutations are known from other regulatory DNA regions. In particular, the *lac*UV5 mutation leads to increased and cAMP−CRP independent transcription of the *lac* operon (see review by Reznikoff and Abelson, 1978). This mutation is a double exchange that leads to an ideal Pribnow box (TATAAT consensus sequence). So far, all mutations of *E. coli* promoters described, that increase the similarity with the Pribnow consensus sequence, had a promoter-up phenotype (Hawley and McClure, 1983; McClure, 1985).

We therefore set out to construct the mutations *gal* (-8A, -9A) and *gal*(-13A, -14A) which render the respective Pribnow box of promoters P_1 and P_2 identical to the consensus sequence TATAAT. As in our previous study (Kuhnke *et al.*, 1986), the gapped duplex DNA approach to oligonucleotide-directed mutation construction (Kramer *et al.*, 1984) was used. Relevant DNA sequences are illustrated in Figure 1. The mutations were moved to plasmid pLF101 (Figure 2a) by replacement of the 83-bp DNA fragment bordered by an *Eco*RI and a *Hin*dIII restriction site.

The resultant plasmids (differing from the parent plasmid only by the promoter mutations indicated) served as templates in a coupled transcription−translation system. As shown in Figure 1, the *gal* transcripts initiated at TS1 and TS2 were quantitated by a primer extension assay (see Kuhnke *et al.*, 1986).

The most conspicuous effect of mutation *gal* (-8A, -9A) was anticipated from the phenotype of the corresponding *lac*UV5 mutation (see Reznikoff and Abelson, 1978; deCrombrugghe *et al.*, 1984): synthesis of mRNA from *gal* promoter P_1 has become largely independent from transcriptional activation by the cAMP−CRP complex. Only a small further increase of initiation at TS1 is observed upon addition of cAMP to the reaction mixture. The transcriptional start sites do not differ from those used in the wild-type P_1 promoter and the expression is controlled by the *gal* repressor [see lanes labeled (1) in Figure 1].

The mutant plasmid carrying the double-exchange *gal* (-13A, -14A) exhibits a moderate increase of transcriptional activity from promoter P_2 as compared to the parent plasmid. This transcription is still repressed by the cAMP−CRP complex, as is known for the wild-type *gal* P_2 promoter and it is also repressed by *gal* repressor [see lanes labeled (3) in Figure 3]. Weak additional starts seen with this mutant promoter at positions −2 and −3 also represent P_2 activity, as judged by their repression in the presence of the cAMP−CRP complex.

Unexpectedly, however, transcription from P_1 is almost completely abolished in the reaction with this mutant *gal* (-13A, -14A) DNA. Even in the presence of the cAMP−CRP complex, only weak residual transcription is initiated at TS1 [see lanes labeled (3) in Figure 1]. Similarly, severe reduction of P_1 promoter activity concomitant with a relatively small increase of transcription initiated at TS2 was also observed for this mutant *in vivo*, when mRNA accumulated in plasmid-bearing cells was analysed by the primer extension method (unpublished experiments by G.K.).

One possible interpretation of this unusual combination of properties is offered by the assumption that mutation *gal* (-13A, -14A) may be an exceptionally strong binder of RNA polymerase at P_2 and a relatively sluggish initiator. Thus, initiation from P_1 could be abolished by diverting most or virtually all RNA polymerase molecules into this strong binding site from which only P_2-type initiation could (rather slowly) take place. A test of this hypothesis clearly exceeds the scope of a simple transcription assay and direct measurements of DNA/protein binding are required (see following paragraph).

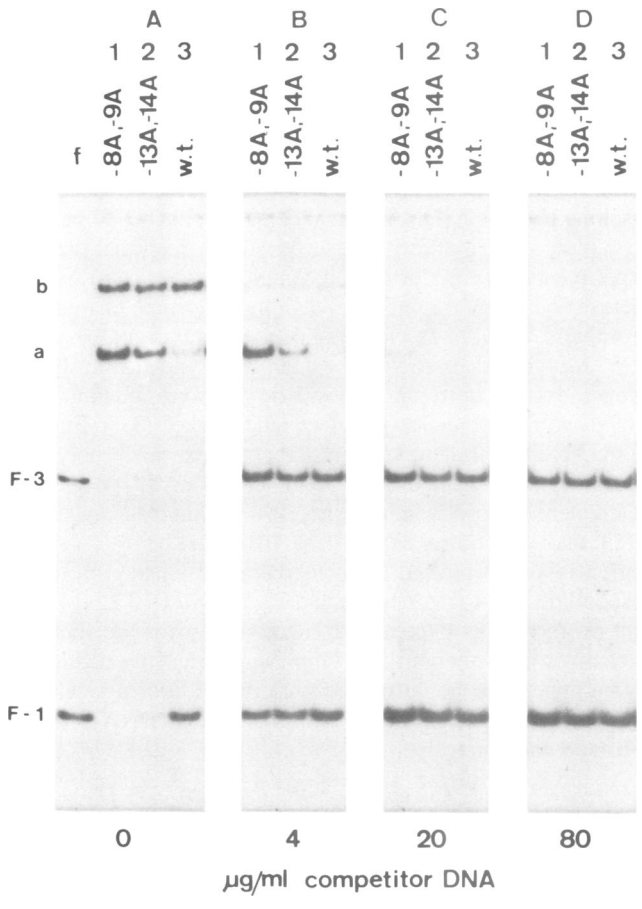

Fig. 3. Comparison of mutant and wild-type *gal* promoters with respect to their capacity to form a stable complex with RNA polymerase. Elecrophoresis through nondenaturing polyacrylamide gels was used to separate free DNA fragments F-1 and F-3 from the corresponding fragments complexed with RNA polymerase (positions of the latter are marked as a and b in the margin). F-1 and F-3 were generated by cleavage with *Eco*RI from fragment F-4 (see Figure 2b). It had been ^{32}P-labeled at both ends with T4 polynucleotide kinase to yield an equimolar mixture of end-labeled fragments carrying either the two *gal* promoters (F-1) or the promoter of the pBR322 β-lactamase gene (F-3). Incubation with RNA polymerase (0.25 U) under conditions described in Materials and methods was in a total volume of 12.5 μl in the absence or in the presence of sonicated calf-thymus DNA as indicated. For each series **(A–D)**, lanes denoted by **1**, **2** and **3** contain the corresponding fragments prepared from mutant or parent plasmid pLF101 (the latter with both *gal* promoters in wild-type configuration). DNA fragments without addition of RNA polymerase were separated in **lane f**.

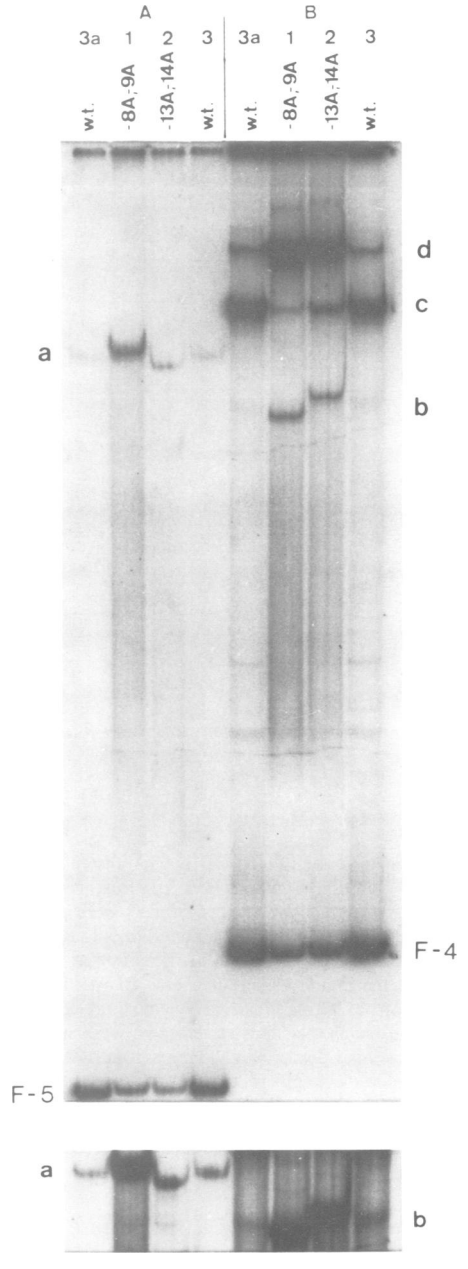

Fig. 4. Complexes formed between RNA polymerase and either mutant or wild-type *gal* promoter fragments can be resolved by polyacrylamide gel electrophoresis. Complex formation of DNA fragments containing mutant or wild-type promoters with RNA polymerase was tested as described in the legend to Figure 3 with the exception that the electrophoretic separation was carried out for a longer time at a reduced voltage. For each series, lanes denoted **1** and **2** contain the corresponding fragments F-5 **(lanes A)** or F-4 **(lanes B)** prepared from mutant plasmids as indicated, **lanes 3a** and **3** the fragment from pLF101 (both *gal* promoters in wild-type configuration). Fragments were ^{32}P-labeled at both ends. Competitor DNA was omitted. Positions of these fragments with RNA polymerase bound to one of the *gal* promoters are marked with a and b. Fragment F-4 carries in addition the promoter of the pBR322 β-lactamase gene (see Figure 2b). RNA polymerase complexes bound to that promoter migrate in the position marked c. The reduced amounts of this complex in **lanes 1** and **2** as compared with **lanes 3a** and **3** is due to the more efficient competition exerted by the mutant *gal* promoters. Fragments in which both the pBR- and one of the *gal* promoters are occupied are seen in the position marked d. The lower part of the figure shows a segment of an autoradiograph of the same gel. The film was overexposed in order to visualize the RNA polymerase complex formed with *gal* wild-type DNA.

The two mutant promoters show enhanced complex formation with RNA polymerase

Escherichia coli RNA polymerase and ^{32}P-labeled DNA fragments carrying either wild-type or mutant *gal* promoters were allowed to form a complex *in vitro* and the assay mixture was analysed in each case by electrophoresis through non-denaturing polyacrylamide gels essentially according to Shanblatt and Revzin (1984). Results are displayed in Figure 3. For each of the two *gal* promoters, conversion of their respective Pribnow box to the consensus sequence results in a considerably increased affinity for RNA polymerase.

Under conditions where RNA polymerase binding in the absence of the cAMP–CRP complex is barely detectable with the fragment containing both *gal* promoters in their wild-type

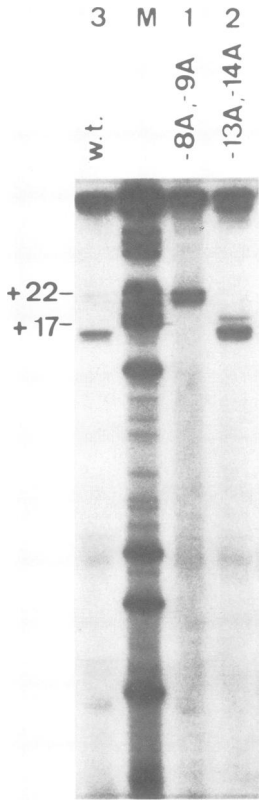

Fig. 5. Protection of mutant and wild-type *gal* promoter DNAs by RNA polymerase against digestion by exonuclease III. Fragments F-1 (see Figure 2b) labeled only at the *Eco*RI site ('upper strand') were prepared from mutant and parent plasmids. After incubation with 5 U RNA polymerase for 10 min at 37°C in a total volume of 50 μl, heparin was added to 80 μg/ml final concentration. Incubation with exonuclease III was for 5 min at 37°C at a concentration of 625 U/ml. The reaction mixtures resulting from the parent and from the mutant plasmids as indicated were separated side by side. (Numbering of lanes conforms to Figures 3 and 4.) It is important to note that for the wild-type fragment an ~10× larger fraction of the reaction mixture was applied to the gel. A G-specific chain degradation reaction according to Maxam and Gilbert (1980) was carried out with the same fragment and the products were applied to **lane M** as size marker.

configuration [see lanes marked (3) in Figure 3], both mutant promoters do exhibit efficient complex formation [see lanes marked (1) and (2), respectively, in Figure 3]. A DNA fragment carrying the promoter of the pBR322 β-lactamase gene was present in each case at an equimolar concentration. RNA polymerase binding to this fragment provides an internal standard.

In experiments similar to that shown in Figure 3, the same mutant DNAs were compared with the two series of longer DNA fragments (see Figure 4). The electrophoretic mobility of the free DNA in each case was decreased to different extents: the complexes containing the shorter 476-bp fragments F-5 (cf. Figure 2b) are migrating more slowly in the gel than those containing the longer 565-bp fragments F-4. Moreover, the DNA−RNA polymerase complexes containing the two mutant promoters differ significantly from each other as well as from the wild-type complex with respect to their electrophoretic mobility. When subjected to electrophoresis as a mixture, they are separated equally well (data not shown). Thus, a trivial experimental artifact can be ruled out. Note that the relative position of the two mutant complexes with *gal* (-8A, -9A) DNA and *gal* (-13A, -14A) DNA is reversed in the two series of fragments.

Nuclease protection studies reveal patterns of RNA polymerase binding to wild-type and mutant gal promoters

The experiments described in the preceding section unambiguously demonstrate increased stability of RNA polymerase binding to DNA fragments containing a *gal* promoter region with either P_1 or P_2 mutated such that the respective promoter has an ideal Pribnow box. The actual region of binding in each case and the resulting patterns of DNA/protein contacts remained to be analysed separately. To address these questions, nuclease protection experiments were performed on complexes of *E. coli* RNA polymerase and DNA fragments containing wild-type or mutant *gal* promoters. Results are illustrated in Figures 5 and 6.

With both *gal* promoters in their wild-type form, DNA sequences located upstream of position +15 were found to be protected against exonuclease III [see lane marked (3) in Figure 5]. In the absence of the cAMP−CRP complex, RNA polymerase is known to bind to the P_2 promoter on wild-type *gal* DNA (Taniguchi and deCrombrugghe, 1983; Shanblatt and Revzin, 1983; also see control experiments discussed below). The same region is also protected at an increased efficiency on the corresponding DNA fragment containing the *gal* (-13A, -14A) mutant promoter, as expected from the nature of the mutational exchanges [see lane marked (2) in Figure 5]. A weak additional signal appears to be correlated with the additional weak transcriptional initiation observed with this mutant DNA at position −3 (see above).

As the result of the mutation *gal* (-8A, -9A), the border of the protected sequence is shifted downstream by 5-bp as compared to the wild type [see lane marked (1) in Figure 5]. Thus, on the basis of the exonuclease protection data, the RNA polymerase binding site on DNA carrying the *gal* (-8A, -9A) mutation corresponds to the location of the *gal* P_1 promoter.

Protection afforded by RNA polymerase against digestion with DNase I was tested by DNA footprint analyses according to Galas and Schmitz (1978). In addition to the mutant *gal* promoters described above, two other mutants — *gal* (-16C) and *gal* (-35G) — were included in this test as controls (see below). The parent plasmid containing both *gal* promoters in their wild-type configuration exhibits the pattern of protection against cleavage and of enhancement of cleavages characteristic of the interaction between RNA polymerase and *gal* promoter P_2 (Taniguchi and deCrombrugghe, 1983; Shanblatt and Revzin, 1983). Qualitatively, the same pattern is observed with the fragment containing the double mutation *gal* (-13A, -14A); most notable is the striking enhancement of cleavage at position −28 in both cases (see Figure 6, experiments A and B). In contrast to the wild-type promoter, however, the characteristic footprint pattern is apparent at a much lower concentration of RNA polymerase (cf. corresponding lanes under A and B in Figure 6), indicating a considerably increased affinity of the mutant P_2 for RNA polymerase.

An increased affinity for the enzyme is also evident from this test for the other mutant promoter carrying the double exchange *gal* (-8A, -9A). Here, the pattern of protection and enhancement clearly differs from that seen with the wild-type (note e.g. the enhancement of cleavage at position −24 rather than at position −28). As stated above, under the conditions used in our experiments, RNA polymerase binds predominantly to promoter P_2 on *gal* wild-type DNA. This interpretation is confirmed by the parallel analysis of control DNAs in lanes D. Binding of RNA polymerase is completely abolished by the mutational exchange to *gal* (-16C), a mutation constructed and shown to selectively

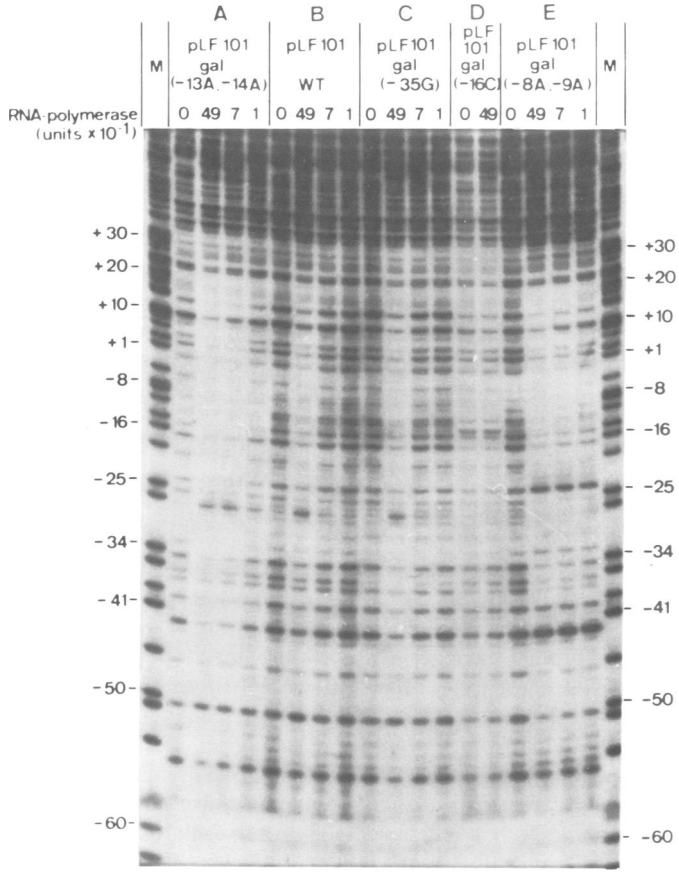

Fig. 6. Protection of mutant and wild-type promoter DNA against digestion by DNase I. Fragments F-2 labeled uniquely at the *Eco*RI site were prepared from mutant and parental plasmids. They were incubated with different concentrations of RNA polymerase as indicated, each in a total volume of 50 μl. After 10 min at 37°C, heparin was added to 80 μg/ml. DNase I was added to 3 μg/ml (final concentration) 30 s later and incubated for 30 s at 37°C. Products of a purine-specific chain degradation reaction of the same fragment (Maxam and Gilbert, 1980) were applied to **lane M.**

inactivate *gal* promoter P_2 (Kuhnke *et al.*, 1986). Even at the highest concentration used in this experiment the reaction of DNaseI with the fragment containing this P_2 promoter mutation was not significantly affected by RNA polymerase (cf. lanes D and B in Figure 6). It may be added that this mutant promoter also fails to yield stable complexes with RNA polymerase in tests similar to those shown in Figure 3. Under conditions where complex formation with wild-type *gal* DNA could be detected, only a very weak residual binding of RNA polymerase to the fragment carrying the *gal* (-16C) mutation was observed which was clearly more sensitive to competition with unrelated (calf thymus) competitor DNA (unpublished experiments by G.K.). On the other hand, binding of RNA polymerase to a DNA fragment containing promoter mutation *gal* (-35G) does not differ from that seen with the corresponding wild-type fragment (cf. lanes C with lanes B in Figure 6). Originally, this exchange was identified by Busby *et al.* (1982) and shown to prevent binding of the cAMP–CRP complex to the *gal* promoters. This mutation has been reconstructed in the same plasmid background as used for the other mutations (unpublished experiments by A.Krause in our laboratories).

Discussion

Differential effects of promoter-up mutations on RNA polymerase binding and on rate of transcription

We have presented evidence that rendering the 'Pribnow box' region of *gal* promoters P_1 or P_2 identical to the canonical sequence TATAAT greatly enhances their capacity to form a stable complex with RNA polymerase. The effect of the same mutations on the rates of transcription from P_1 and P_2, on the other hand, is strikingly different. Whereas mutation *gal* (-8A, -9A) has a strong P_1-up phenotype, the corresponding mutation *gal* (-13A, -14A) displays only moderately increased transcription from promoter P_2. Apparently, a later step than the formation of a stable complex with RNA polymerase is rate-limiting in the transcriptional activity of this mutant, as has previously been considered for other *E. coli* promoters (Stefano and Gralla, 1979; Straney and Crothers, 1985). Perhaps the combination of an ideal Pribnow box with other less well understood features of P_2 structure leads to RNA polymerase binding too strong for efficient initiation of transcription.

In summary, one is left with the notion that the same type of structural change introduced into different promoters can lead to strikingly different changes in phenotype. It has to be noted, however, that until a detailed kinetic analysis of the various promoters discussed here is carried out, our arguments strictly pertain only to overall rates of productive RNA synthesis. We have measured amounts of RNA accepting the oligonucleotide primer for the reverse transcriptase reaction. It should be kept in mind that these amounts do not necessarily reflect only the different rates of initiation at P_1 and P_2. Any initiation leading to prematurely terminated transcripts (i.e. before the region of primer hybridization – nucleotides +36 to +54 – is transcribed) would go unnoticed in our assay. Indeed, wild-type *gal* promoter P_2 is prone to some extent to initiate such prematurely terminated RNA synthesis (cf. DiLauro *et al.*, 1979). It remains to be tested whether the transition from an abortive cycling reaction of RNA polymerase (see Gralla *et al.*, 1980; Straney and Crothers, 1985) to productive transcription has been affected by any one of our promoter mutations.

Consequences of competition for RNA polymerase between gal promoters P_1 and P_2

Complex transcription units containing overlapping promoters as exemplified by the *E. coli* galactose operon have the special property of being sensitive to indirect effects attributable to changes in the relative strength of the two promoters (under the term 'strength' we subsume both binding affinity to RNA polymerase and rate of initiation from the final complex). Findings presented in this paper suggest that this effect could account for the *gal* P_1-down phenotype of promoter mutation *gal* (-13A, -14A). This mutation results in enhanced binding of RNA polymerase at promoter P_2. Its P_1-down phenotype would then be based on drainage of RNA polymerase away from P_1 into the strong binding site at P_2.

The same rationale can be applied to explain the unexpected distribution of mutational exchanges observed by Bingham *et al.* (1986) in a selection designed to yield *gal* P_2 promoter-down mutations. The starting plasmid used by these authors carried a transcriptionally inactive *gal* P_1 promoter. Despite a high selective pressure which strongly favoured reduced *gal* operon expression, only a small fraction of plasmid-linked Gal-negative mutants was found, and among them only a surprisingly low

number carrying different *gal* P_2 promoter mutations, most of which did not consist of an exchange within one of the conserved sequences.

The initial mutation *gal* (-14A) employed in this selection had been identified by Busby *et al.* (1984) as a phenotypically P_1-negative mutation. It contains one of the base changes present in our double mutation *gal* (-13A, -14A) described above. Our results suggest that the P_1-negative phenotype of the single mutation *gal* (-14A) may be an indirect effect of an increased affinity of P_2 for RNA polymerase, as this exchange also improves the homology between the P_2 promoter and the canonical Pribnow box sequence. This interpretation is supported by the original description of this mutant as exhibiting an elevated P_2 promoter activity (Busby *et al.*, 1984). Any additional mutation which would reduce the binding of RNA polymerase to promoter P_2 would then result in a concomitant restoration of P_1 activity and would thus be missed in the selection procedure, unless the same mutational change would simultaneously affect P_1 directly as was indeed observed by Bingham *et al.* (1986) with mutations at -12, in which the replaced nucleotide pair is shared by the overlapping Pribnow box sequences.

According to this interpretation, the difficulties of Bingham *et al.* (1986) in obtaining P_2 mutations with structural changes in the conserved sequences were not due to an intrinsic property of *gal* promoter P_2 but rather a consequence of the particular selection employed. Indeed, we have previously demonstrated that mutation *gal* (-16C), a Pribnow mutation of promoter P_2, exhibits a completely P_2-negative phenotype. Moreover, in the present study we show that the same mutation (obtained by oligonucleotide-directed mutation construction without phenotypic selection) has lost RNA polymerase binding affinity. Thus, there is no paradoxical response of P_2 function to the structure of its conserved regions. Special effects can be explained on the basis of competition between P_1 and P_2 for RNA polymerase binding.

Does binding of RNA polymerase to a promoter induce DNA bending?

Wrapping of DNA around the nucleosome core provides the crystallographically documented precedent of DNA bending upon contact with protein (Richmond *et al.*, 1984). DNA bending was also observed or postulated in the interaction between DNA binding proteins and their respective cognate DNA sequences (Frederick *et al.*, 1984; Weber and Steitz, 1984; Matthew and Ohlendorf, 1985). DNA bending brought about by proteins directly involved in gene expression and its implications for gene regulation are the subject of current debate and different experimental approaches (Wu and Crothers, 1984; Takahashi *et al.*, 1986; Hochschild and Ptashne, 1986; Ryder *et al.*, 1986; see recent review by Ptashne, 1986).

We suggest RNA polymerase induced DNA bending as the most straightforward explanation for the data laid out in Figure 4. We are, however, aware of alternative explanations such as different three-dimensional structures of RNA polymerase in the various DNA/protein complexes analysed — a non-trivial possibility in its own right. We think the following observations favour different DNA structures rather than different protein conformations as the more likely cause of the effects. Firstly, the shorter fragment F-5 shows a much more pronounced retardation upon RNA polymerase binding to the different *gal* promoters than the longer fragment F-4. This is in accord with existing concepts since in F-5 the *gal* promoters are located closer to the centre of the fragment (cf. Wu and Crothers, 1984). Incidentally, fragment F-4 is retarded to an even greater extent by RNA polymerase bound to the promoter of the pBR322 β-lactamase gene which is located still closer to the centre of the DNA fragment. Secondly, when switching from the F-5 to the F-4 series, the order of electrophoretic mobility of the different wild-type and mutant fragments is inverted. Within each series the DNA–protein complex displaying the largest effect is the one in which RNA polymerase occupies the promoter site localized more toward the interior of the DNA molecule: *gal* (-8A, -9A) in F-5 and *gal* (-13A, -14A) in F-4.

Clearly, the phenomenon observed requires and deserves further investigation. RNA polymerase-induced promoter bending as discussed above is mechanistically distinct from but may be functionally related to DNA curvature as an intrinsic DNA property (cf. Bossi and Smith, 1984; Ryder *et al.*, 1986). Different three-dimensional DNA structures (in the absence of proteins) as the consequence of *lac* promoter-up mutations were recently discussed by Spassky and Sigman (1985).

Assays developed for DNA/protein association and for the occurrence of bends in double-stranded DNA (Marini *et al.*, 1982; Wu and Crothers, 1984) are based on measuring decreased electrophoretic mobility of DNA fragments in polyacrylamide gels. The dilemma with using this parameter for the measurement of protein-induced DNA bending lies in the fact that it is difficult to separate the respective contributions to total electrophoretic retardation made by protein binding as such and by protein-induced DNA-bending.

The mutations we have described in this study may be favourable for further analysis by these methods, as one and the same protein, RNA polymerase, can be observed to interact with various derivatives of a given DNA fragment. These derivatives are characterized by having exactly the same physical length and almost exactly the same nucleotide sequence. The gross DNA/protein stoichiometry may then be expected to be constant for the different complexes studied and comparison may reveal more subtle differences such as exact positioning of the protein on the DNA and/or differences in protein-induced DNA bending. Moreover, the DNA fragments described here also contain binding sites for the *gal* repressor and for the cyclic AMP receptor protein. Binding studies with these proteins could serve as controls, as their recognition sequences *per se* have not been affected by the newly introduced promoter mutations.

Materials and methods

Materials

The materials used were from the following sources: avian myeloblastosis virus reverse transcriptase, *E. coli* exonuclease III and *E. coli* RNA polymerase from Boehringer (Mannheim, FRG); RNA polymerase from PL-Pharmacia (Freiburg, FRG) has also been used and was found to give the same results. Substrates for the cell-free transcription–translation reaction, other enzymes and chemicals were from sources indicated previously (Fritz *et al.*, 1983; Kuhnke *et al.*, 1986).

Bacteria and plasmids

Escherichia coli strains used for transformation and preparation of plasmid DNA and for the preparation of protein-synthesizing extracts as well as the parent plasmid pLF101 (see Figure 2a) have been described (Kuhnke *et al.*, 1986). This pBR322 derivative contains an *E. coli* galactose operon which differs from the wild type by elimination of the *Hin*dIII restriction site originally present within the *galE* structural gene and by the introduction of a new unique *Hin*dIII site into the leader sequence (Fritz *et al.*, 1983).

Construction of gal promoter mutations

The gapped-duplex approach for oligonucleotide-directed mutation construction elaborated by Kramer *et al.* (1984) has been applied to introduce structurally predetermined mutations into the *gal* promoter region located on the 83-bp *Eco*RI–*Hin*dIII fragment (cf. Kuhnke *et al.*, 1986). All plasmid derivatives used in this study differ from the parent plasmid pLF101 only in the positions indicated, as ascertained by recloning and sequence analysis. The designation defined

by Kuhnke *et al.* (1986) to indicate *gal* mutations and the corresponding pLF101 plasmid derivatives is also used here: the nucleotides differing in the mutant *gal* sequence from that of the parent plasmid ('upper strand') are indicated in relation to position +1, the transcriptional start site of cAMP-activated transcription.

Quantitation of gal mRNA by primer extension assay

Preparation of *gal* mRNA and its quantitation by a primer extension assay were as described by Kuhnke *et al.* (1986). Briefly, mRNA was prepared from cell-free transcription–translation reaction mixtures programmed by the different plasmid DNAs. cAMP and D-fucose, if added as indicated, were present at 0.5 mM and 20 mM final concentration. Complete elongation of the primer by reverse transcriptase on *gal* mRNA yields cDNAs of 54 and 59 nucleotides length for the *gal* transcripts initiated at the transcriptional start sites of promoters P_1 and P_2. Their position in the gel is marked as S_1 and S_2, respectively. The synthetic oligonucleotide primer is complementary to the mRNA sequence from position +36 to +54, position 1 being the first nucleotide of the cAMP stimulated *gal* mRNA. It has to be stressed that only transcripts elongated at least into this interval are detected by the primer extension assay used in this study.

RNA polymerase binding studies

Standard methods were used for the preparation of restriction fragments F-1 to F-5 from pLF101 DNA. They were end-labeled with T4 polynucleotide kinase and [^{32}P]ATP (Maniatis *et al.*, 1982). Incubation of DNA fragments (~50 fmole per test) with RNA polymerase at 37°C was in the 'standard buffer' given by Spassky *et al.* (1984). Details are indicated for each figure. When complex formation was to be analysed electrophoretically, free RNA polymerase was inactivated after 10 min at 37°C by the addition of heparin to 125 μg/ml final concentration. Electrophoresis through 4% nondenaturing polyacrylamide gels (ratio of acrylamide:bisacrylamide 29:1) was used to separate free DNA fragments from the corresponding fragments complexed with RNA polymerase. Electrophoresis was at 37°C under conditions given by Shanblatt and Revzin (1984).

For nuclease protection studies conditions for RNA polymerase binding were as indicated above. Details for digestion with either exonuclease III or with DNase I are stated in the figure legends. The reaction was terminated by the addition of a mixture of EDTA, sodium acetate and sonicated calf thymus DNA to 20 mM, 150 mM and 50 μg/ml final concentrations. The DNAs were precipitated with ethanol and separated on a 10% denaturing polyacrylamide gel (Maxam and Gilbert, 1980).

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