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Kinetic studies of the modulation of *ada* promoter activity by upstream elements

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We have determined the kinetics of initiation of transcription of the wild-type *ada* promoter by abortive initiation assays. In the absence of activation, it is a weak promoter, with an association constant K_B and an isomerization rate constant k_2 comparable to those obtained under the same conditions for other positively regulated promoters (0.36×10^7 M^{-1} and 1.7×10^{-2} s^{-1}, respectively, at 37°C and 50 mM NaCl, on a supercoiled template). As already observed for other promoters, these constants are modulated by varying the supercoiling of the plasmid. However, the strength of the promoter (given by the $K_B \cdot k_2$ product) remains almost constant, because the maximum value of K_B and k_2 are obtained for different values of the superhelical density. The *ada* promoter has a stretch of seven adenosine residues (A_7) in its upstream region. We have analysed the effect of this upstream sequence on the efficiency of initiation of the *ada* promoter by comparing the wild-type sequence with an up-mutant promoter characterized by the inversion of the central base pair in the sequence (A_7) leading to the sequence (A_3TA_3). Although the mutation, which is located outside the promoter consensus regions, has no effect on the isomerization step, it affects the equilibrium constant K_B that characterizes the association step. In the mutant promoters, the supercoiling of the plasmid modulates the isomerization and association constants in such a way that both K_B and k_2 are maximum for the same superhelical density (−0.05), leading to a 12-fold increase of the strength of the promoter, on a supercoiled template. By two-dimensional polyacrylamide gel electrophoresis of the ligation products of two 31 bp oligonculeotides harboring the wild-type and the mutant sequences, we have shown that the mutation induces an increased bending or flexibility of the 31mer. We suggest that this increased flexibility facilitates the wrapping of the DNA around the RNA polymerase in the initiation complex, thus leading to an increased efficiency of the initiation of transcription observed for the mutant promoter.

Key words: adaptative system/DNA flexibility/promoter strength/upstream elements

Introduction

The initiation of transcription of procaryotic genes by the *Escherichia coli* RNA polymerase is regulated through a fine balance between the effects of several different control elements affecting the efficiency of the interaction of a given promoter with the RNA polymerase. If the strength of a promoter can be related to its agreement with the consensus sequences (the −10 and the −35 regions McClure, 1985), the intrinsic promoter strength can also be modulated by perturbations of the medium, such as temperature, ionic strength or pH (Lohman *et al.*, 1980). The local three-dimensional structure of the promoter region increasingly appears to play a prominent regulatory role. In particular, variation in superhelicity of the DNA harbouring the promoter can modify the efficiency of initiation (Gellert, 1981; Bertrand-Burggraf *et al.*, 1984a,b; Malan *et al.*, 1984; Brahms *et al.*, 1985; Borowiec and Gralla, 1987; Pruss and Drlica, 1989). Additional signals, upstream from the promoter have also been identified as regulatory elements (Jo *et al.*, 1986). Such prokaryotic elements affect initiation in a way similar to that of eukaryotic enhancer regions. Moreover, these 'activator regions' often contain tracks of adenine residues, that are known to be involved in DNA bending (Marini *et al.*, 1982). Such upstream sequences may contribute to the formation of 'nucleosome-like' structures, in which the promoter DNA wraps in a left handed supercoil around the RNA polymerase, during the initiation process (Amouyal and Buc, 1987; Travers, 1989). Finally, DNA binding proteins that enhance or reduce the promoter efficiency regulate the initiation of transcription by direct protein−protein contacts with the RNA polymerase (Ptashne, 1986).

The Ada protein is the central regulatory element of the adaptative system induced in response to alkylation damage of DNA (for review, see Lindahl *et al.*, 1988). When *E.coli* is exposed to low levels of alkylating agents, the activaton of the genes of this system (*alkA, aklB, ada, aidA, aidB*, etc.) by Ada protein results in an increased resistance of the cells to the mutagenic and lethal effects of such agents.

It has been shown that the operator site for Ada protein (*ada* box) on its own gene is located near the '−35 region' of the promoter with an (AT) rich region flanking the '*ada* box' on the 5′ side (Figure 1). In this paper we analyse the influence of this (AT) rich region on the efficency of initiaton of the *ada* promoter. The kinetics of initiation of transcription of the wild-type *ada* promoter is compared with that of a constitutive mutant, characterized by an AT→TA transversion at the central base pair of the (AT) stretch. The results are discussed in terms of promoter structure and flexibility.

Results

Production of different plasmid topoisomers

In order to study the effect of supercoiling on the activity of both wild-type and mutant promoters (on plasmids pTN232 and pTN301, respectively), we have used plasmids with different topological constraints. The plasmids were first

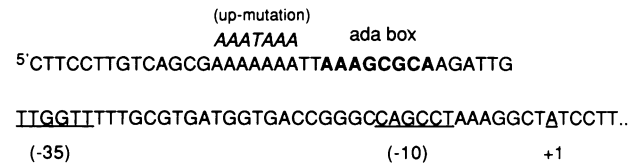

Fig. 1. Organization and sequence of the *ada* promoter region. By convention, position +1 refers to the initiation site of the *ada* mRNA. Underlines show the position of the '−10' and '−35' promoter regions. The *ada* box is also indicated by bold characters.

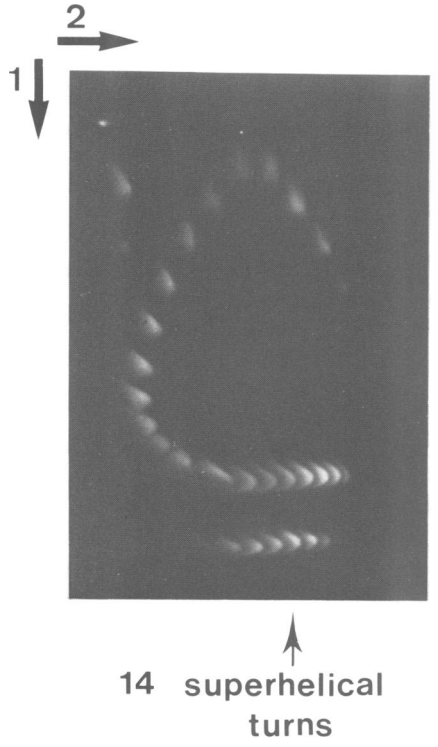

Fig. 2. Two-dimensional agarose gel electrophoresis of topoisomers of plasmid pTN232. The directions of migration are indicated by arrows. The upper distribution shows the different topological species generated by topoisomerase I in the presence of various concentrations of ethidium bromide, and the lower distribution represents the topoisomers as they were isolated from the culture (the 'natural' superhelical density).

studied at 'natural' superhelical density after extraction from wild-type *E.coli*. With a measured average linking number of −14 (Figure 2), the mean superhelical density σ of the distribution of topoisomers is equal to −0.05. New topoisomers distributions with less and also with more supercoiling were generated (see Materials and methods). In the less supercoiled collections, the topoisomer distribution (σ = −0.036) is characterized by the loss of an average of four superturns with respect to the 'natural' topoisomers distribution (Figure 3). More supercoiled distributions were produced with an average of three (σ = 0.06) and four (σ = −0.064) additive superhelical turns for the plasmids harbouring the wild-type and the *ada* mutant promoter, respectively (Figure 3). Plasmids linearized with restriction enzyme *Hin*dIII were used as a fully relaxed control.

Comparative study of the wild-type and mutated ada promoters

The kinetics of abortive initiation was monitored using the increase in fluorescence of 1-naphthylamine-5-sulphonic acid

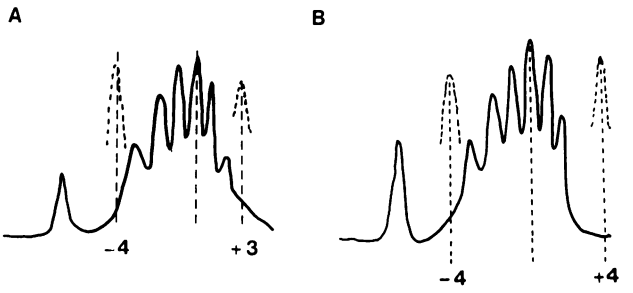

Fig. 3. Representative scans from 1.5% one-dimensional agarose gels of the different topoisomer species generated for plasmid pTN232 (**A**) and plasmid pTN301 (**B**). The whole distributions (____) are given for the two plasmids with the 'natural' superhelical density (σ = −0.05). Only the central position of the distributions are given for the topoisomers with less or more negative superhelical turns. The middle of all the gaussian distributions are indicated by dashed lines. Gels were run in presence of ethidium bromide (0.025 μg/ml).

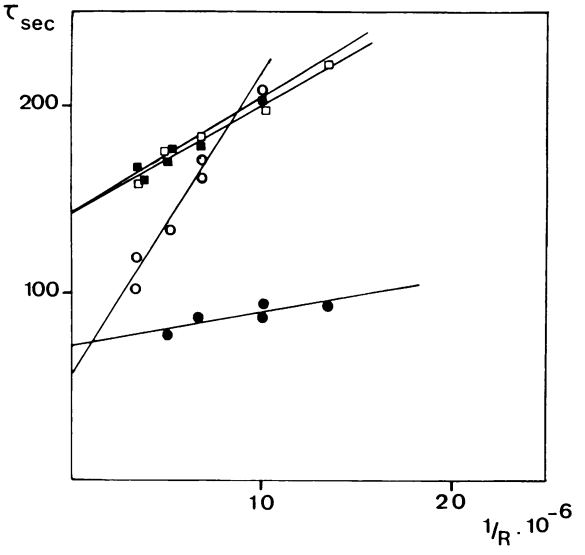

Fig. 4. Tau plots for the two *ada* promoters on linear and supercoiled templates. The lag times τ observed during UpApU synthesis are plotted versus the reciprocal of the RNA polymerase concentration: wild-type (o) and mutant (●) supercoiled promoters; wild-type (□) and mutant (■) linear promoter.

(ANS)-labelled UTP (UTPγANS) when it is cleaved by RNA polymerase prior to its incorporation in the nascent oligonucleotide (Bertrand-Burggraf *et al.*, 1984a,b). The assays were performed with both plasmids containing the wild-type and the mutant *ada* promoter. A dinucleotide was used in order to ensure that the specificity of the starting site (in our case UpA, see Materials and methods). The kinetics of the abortive initiation of transcription can be described by the following scheme (McClure, 1980):

$$R + P \underset{}{\overset{K_B}{\rightleftharpoons}} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o$$

where K_B is the association constant for the closed complex formation, k_2 and k_{-2} are the on and off isomerization rate constants, R is the RNA polymerase, P is the promoter, and RP_c is the closed complex, which isomerizes in the open complex RP_o. Transcription can be initiated from the open complex only. The observed kinetics are characterized by a lag time τ, which corresponds to the time necessary for

open complex formation. According to McClure (1980), the lag time τ is related to the kinetic parameters by:

$$\tau = 1/k_2 + 1/k_2 \cdot K_B[R] \quad (1)$$

where [R] corresponds to the RNA polymerase concentration. When τ is plotted versus $[R]^{-1}$ (tau plot), a linear relationship is obtained,where the y-axis intercept equals $1/k_2$ and the ratio of intercept to slope equals K_B.

It was necessary to check whether there were potential sites for initiation other than the *ada* promoter that would be able to use the UpA dinucleotide and UTPγANS. To do so, we performed the abortive initiation assay with the parental supercoiled plasmid lacking the *ada* promoter. The fluorescence increase observed (data not shown) was negligible, indicating that no initiation of transcription occurs outside the *ada* promoter on these plasmids under the conditions of our assays. We did not investigate the activity of other potential promoters starting with the UpApU sequence on the parental plasmid as a function of the superhelical density. The blank test was only performed at a superhelical density of −0.05, which corresponds to the optimal value for the activation of the mtuant *ada* promoter (see below). Any other promoters which would have been activated at other superhelical densities would have led to higher values of measured k_2 and K_B. On the contrary, at these other superhelical densities, we have observed a rather low promoter activity detected by fluorescence (see below).

The tau plots for both promoters inserted in linear and 'naturally' supercoiled plasmids are all linear indicating that scheme (1) is valid (Figure 4). The values of the isomerization rate constant k_2 and association constant K_B are reported in Table I, together with the product $K_B \cdot k_2$, which is a measure of the strength of the promoter.

The wild-type *ada* promoter is relatively weak, as the values of the product $K_B \cdot k_2$, measured at several superhelical densities, range in the lower part of the known promoters (Hawley *et al.*, 1982). There is a slight effect of the superhelical density on the efficiency of transcription initiation, producing a 2- to 3-fold decrease of $K_B \cdot k_2$ when going from linear to supercoiled plasmids.

The strength of the mutant promoter depends more strongly on the supercoiling of the plasmid. In absence of topological constraints ($\sigma = 0$), it behaves like the wild-type *ada* promoter, with a low $K_B \cdot k_2$ product (1.75×10^5 M^{-1} s^{-1}). However, the $K_B \cdot k_2$ product exhibits a net maximum for a superturn density of −0.05, corresponding to a 10-fold increase of the promoter strength (Figure 5).

For both promoters, the variations of K_B and k_2 with the superhelical density are not monotonous. The isomerization rate constants vary similarly in wild-type and mutant promoters with superhelical density. However, major differences between the wild-type and mutant promoters are seen in the modulations of the association constants K_B by the superhelical density: the variation in the mutant promoter appears to be shifted relatively to the variation observed in the wild-type promoter (Table I). For a density of superturns of −0.05, the association constant of the wild-type promoter is at its minimum while that of the mutant is at its maximum. When the supercoiling of the plasmid increases further, the K_B increases for the wild-type promoter and decreases for the mutant promoter (Table I). The conjunction of maximum values of K_B and k_2 occurring at the same superhelical density ($\sigma = -0.05$) for the mutant promoter leads to the large value of the overall kinetic constant $K_B \cdot k_2$, exceeding by a factor of 10 the value measured for the wild-type promoter (Figure 5).

Table I. Kinetic parameters for the initiation of transcription at wild-type (*ada* WT) and mutant (*ada* m) promoters at various superhelical densities: L = linear, S = supercoiled (the experimental conditions are indicated in Materials and methods).

	σ (density of superturns)	k_2 10^2 (s^{-1})	K_B 10^{-7} (M^{-1})	$k_2 \cdot K_B$ 10^{-5} ($M^{-1}s^{-1}$)
ada WTL	0.00	0.70	2.28	1.60
ada WTS$^-$	−0.04	0.60	0.97	0.58
ada WTS	−0.05	1.70	0.36	0.61
ada WTS$^+$	−0.06	0.35	3.00	1.05
ada mL	0.00	0.70	2.50	1.75
ada mS$^-$	−0.04	0.56	1.10	0.60
ada mS	−0.05	1.38	4.20	5.80
ada mS$^+$	−0.07	0.76	0.78	0.60

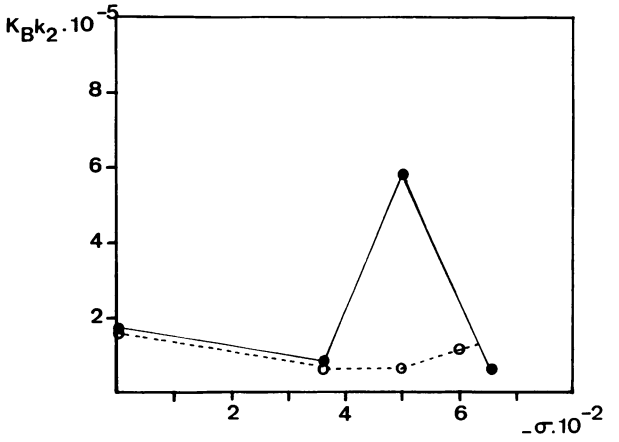

Fig. 5. Variation of the strength ($K_B \cdot k_2$) of the wild-type (o) and the mutant promoter (●) with the of superhelical density (σ) of the plasmid.

Comparative analysis of the conformation of both promoter upstream regions by two-dimensional gel electrophoresis

In order to measure the flexibility of the two promoter upstream regions, we ligated 31 bp long duplex DNA, corresponding to the natural sequences that contain in their centre either the A_7 stretch (found in the wild-type promoter sequence) or the mutant sequence (A_3TA_3). Flanked by 4 bp long sticky ends, these duplexes can be ligated unidirectionally into multimers in which the A_7 or A_3TA_3 sequences are separated from one another by approximately three helical turns (see Materials and methods). We first compared the two ligation products on a 10% polyacrylamide gel; no signficiant difference in migration was observed between the two 31 bp long oligonucleotides. However, the migration of the mutant multimers was slightly slower than that of the wild-type multimers (data not shown).

To quantify the difference in DNA curvature responsible for this effect, we analysed the ligation products by two-dimensional gel electrophoresis (Ulanovsky *et al.*, 1986; Hussain *et al.*, 1988). The ligation mixture was first run on a 5% polyacrylamide gel in order to separate the molecules according to their length, and then in the second dimension,

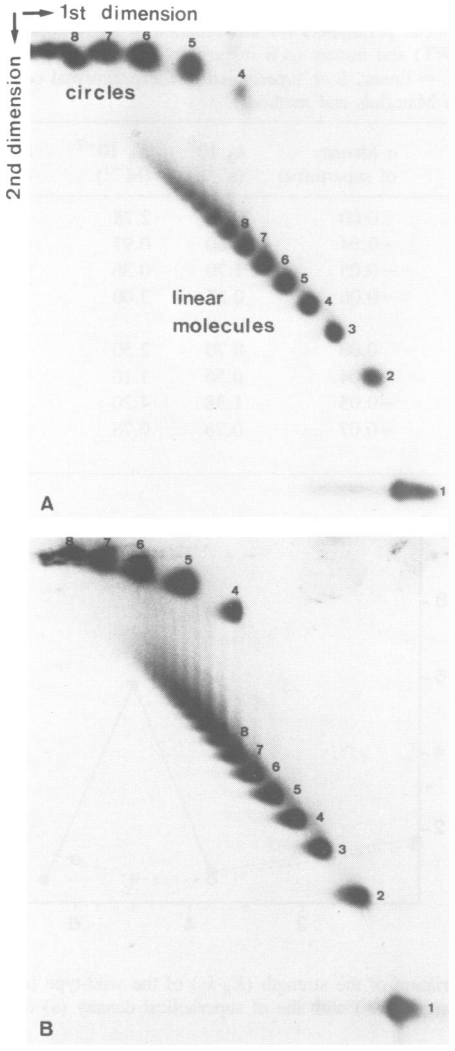

Fig. 6. Two-dimensional analysis of the two families of ligation products obtained with the wild-type oligonucleotide (**A**) and the mutant oligonucleotide (**B**). The upper distributions of spots correspond to the circular species, and the lower distributions to noncircular species. The ligation products were first electrophoresed (left to right) in a 5% polyacrylamide gel and then in the second dimension (top to bottom) in a 10% polyacrylamide gel containing 0.025 μg/ml ethidium bromide. Spots were recovered and identified on a 5% denaturing polyacrylamide gel in the presence of 7 M urea. The numbers 1–8 indicate the length of DNA as multimers of the repeating 31mers present in the spots.

in presence of the intercalating agent (ethidium bromide) in order to separate linear and covalently closed circular molecules.

For the two 31mers used, two distributions of spots were obtained (Figure 6), corresponding to the two different families of multimers generated, the linear and the circular molecules, respectively. The multimers obtained in both families were analysed further by running the DNA extracted from the spots on denaturing polyacrylamide gels in the presence of 7 M urea (data not shown) in order to determine their size.

The intensities of the spots on the two-dimensional gel (Figure 6) were quantified by Cerenkov counting. The fraction of circular DNA formed was then determined by taking the ratio of spot intensities for the circular ligation

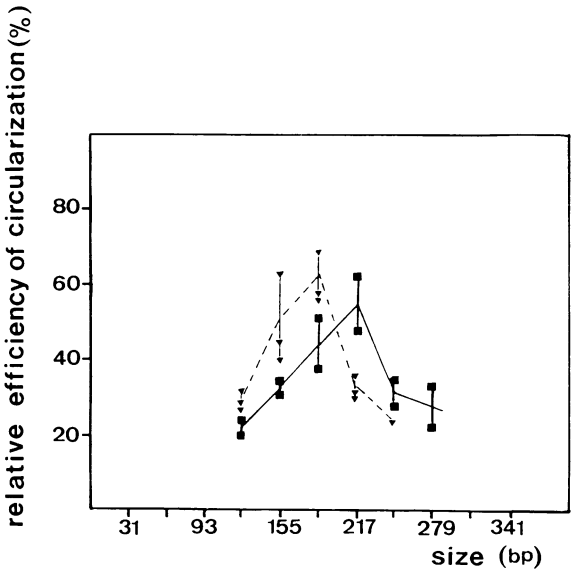

Fig. 7. Fraction of circular DNA formed in our experimental conditions. This quantity is calculated by taking the ratio of spot intensities for the circular ligation products to that of total ligation products of a given size for the wild-type (■) and mutant (▽) sequences. The spots were extracted and the radioactivity in each species was quantitated by Cerenkov counting. The results plotted are the average values from three independent experiments.

products to that of the total ligation products of a given size (Figure 7). This quantification indicates that the mutant duplex (containing A_3TA_3) forms smaller circles than the duplex containing the wild-type sequence (A_7). Indeed, a length of 186 bp (6 × 31mer) appears to be optimum for circularizing multimers of the mutant duplex, compared to 217 bp (7 × 31mer) for the wild-type sequence.

Discussion

The *ada* promoter is a weak promoter which can be activated by the methylated Ada protein. The so called *ada* box is located upstream of the −35 consensus sequence of the promoter. This upstream region also bears a stretch of seven adenine residues (A_7; between −56 and −62), which has been identified, in other promoters, as a *cis*-acting regulatory signal for transcription initiation (Zahn and Blattner, 1985; Jo *et al.*, 1986; Bracco *et al.*, 1989). A mutation of the central base pair in the A_7 sequence (an AT→TA transversion leading to the sequence A_3TA_3) gives rise to a new promoter, which can no longer be activated by the Ada protein, but which is constitutively stronger than the wild-type-promoter. It is worthwhile to notice that this mutation does not affect either the −10 or the −35 sequences and lies near the binding site of the Ada activating protein (Nakamura *et al.*, 1988).

As the interaction between the RNA polymerase and a promoter is influenced by the structure of the nucleic acid and its ability to be deformed (intrinsic bending and bendability; for reviews, see Travers, 1987, 1989), we will first discuss the effect of the A_7 to A_3TA_3 transversion on the conformation and flexibility of the *ada* promoter. Then we will analyse the modulation induced by this mutation on the thermodynamic and kinetic parameters of the transcription initiation from the *ada* promoter, and try to interpret it on a molecular conformation and flexibility point of view.

The bending of the DNA appears to be greater in the A_3TA_3 mutant promoter than in the wild-type sequence

Sequences harbouring A tracks are known to have unusual electrophoretic mobilities (Marini *et al.*, 1982) and the structure of A tracks has been studied by NMR (Lane *et al.*, 1986; Nadeau and Crothers, 1989) and X-ray crystallography (Nelson *et al.*, 1987; DiGabriele *et al.*, 1989). The base pairs in these sequences exhibit large propeller twists that allow a better base stacking. The crystallographic structures are bent, probably due to the crystal packing forces, as pointed out by DiGabriele *et al.* (1989).

The migration (on a 10% polyacrylamide gel) of the ligation products of the 31 bp oligonucleotides containing the sequences (A_7) and (A_3TA_3) respectively, were only slightly different, allowing no conclusion (data not shown). The analysis made by two-dimensional gel electrophoresis (Figure 6) reflects the competition between circularization and elongation by an additional unit sequence of a fragment of given length. The latter event depends on the oligonucleotides concentrations, which are identical in both experiments. The different species formed (circles and linear fragments) result from the ligation of non-covalent prehybridized fragments. These fragments are partitioned according to the Boltzmann law so that the population of each species depends on the logarithm of their free energy of formation. In the conditions used,the optimal number of units necessary for circularization are clearly different between the two sequences. The (A_7) sequence circularizes the best with seven units while the maximum fraction of circles are obtained with six units only with the (A_3TA_3) sequence. The bending of the oligonucleotides when included in these optimal circles can be approximately calculated: 360/7 = 51° per 31mer and 360/6 = 60° per 31mer for the wild-type and mutant sequences, respectively.

The circularization efficiency depends either on a permanent curvature or on the flexibility of the oligonucleotide. Our results show that the A_3TA_3 sequence is either more curved or more flexible than the A_7 track.

The first hypothesis agrees with the observation in the crystal structure of the AT base pair track (Nelson *et al.*, 1987; DiGabriele *et al.*, 1989) that bends are introduced in the molecule at the interruption points of the track. Also, studies of AT-rich sequences, using chemical probes (Hagerman, 1985; Burkoff and Tullius, 1988) suggest that AT steps are associated with local and stable distortion of the helix. The resulting effects of disposing these AT dinucleotides in phase along the sequence can be a macroscopic curvature of the molecule.

However, the latter hypothesis is supported by the fact that the A_7 to A_3AT_3 transversion introduces a phasing in the sequence which better fits the consensus bendability pattern. In this pattern, AT-rich sequences are found where the minor groove points inside the curvature (the AT step being the best bender) while GC-rich sequences are positioned where the minor groove points out (Gartenberg and Crothers, 1988; Travers, 1989).

Finally, the circularization efficiency can be influenced by the planarity of the molecule or the anisotropy which characterizes the flexibility in DNA molecules (Travers, 1989). In conclusion, our results can be interpreted in either way: compared to the wild-type sequence (A_7), the mutant sequence (A_3TA_3) can introduce either a greater curvature of the molecule, or an additional curvature which could compensate for the non-planarity of the (A_7) oligonucleotide, or a particular bendability which lowers the energy of formation of smaller circles.

As will be shown below, it is necessary to invoke the higher bendability of the mutant sequence in order to explain the activation of the transcription initiation observed with the A_3TA_3 mutant promoter. However, it should be noticed that the curvature and the flexibility hypothesis are not exclusive from one another.

The ada promoter and its (A_3TA_3) mutant are both weak promoters

Most of the *E.coli* promoters are relatively weak promoters (McClure, 1985). They are limited, either for the association step ($K_B < 10^8\ M^{-1}$) or for the isomerization step ($k_2 < 10^{-2} s^{-1}$), leading to a $K_B \cdot k_2$ product which ranges between 10^5 and $10^7\ M^{-1}\ s^{-1}$. In that respect, the values of the association constant K_B and the isomerization rate constants k_2 measured in this study show that the wild-type *ada* promoter is weak, as expected for a positively regulated promoter (Raibaud and Schwartz, 1984). Values are comparable to those obtained under similar conditions for the lac promoter in the absence of activation by the cAMP–CRP complex (Malan and McClure, 1984). This result is not surprising in view of the significant deviation from consensus sequences of both −10 and −35 boxes. In the *ada* promoter these sequences are CAGCCT and TTGGTT, at −10 and −35, respectively. Statistical analysis of known promoters have shown the consensus sequences to be TATAAT and TTGACa (Hawley and McClure, 1983; Harley and Reynolds, 1987). Moreover the −10 and −35 boxes are separated by 20 bp, while a 17 bp spacing is generally thought to be optimal (Hawley and McClure, 1983; Harley and Reynolds, 1987).

The A_3TA_3 mutant of the *ada* promoter is also a weak promoter. It remains limited for the association even at a superhelical density of −0.05 for which the $K_B \cdot k_2$ product exceeds 10 times the value obtained for the wild-type promoter.

The A7 to A_3TA_3 mutation affects the binding of the RNA polymerase to the promoter and not the isomerization step

It has already been observed that the supercoiling state of the DNA modulates the activity of some promoters (Malan *et al.*, 1984; Brahms *et al.*, 1985; Borowiec and Gralla, 1987; Pruss and Drlica, 1989). In some cases, the effect of the supercoiling on the constants k_2 and K_B was analysed separately (Bertrand-Burggraf *et al.*, 1984b). No definite rules can be extracted from these observations, since the supercoiling of the DNA either enhances or inhibits the transcription initiation from different types of promoters. A typical illustration of this is the transcription of the *gyr* (A and B) and *top* genes which responds to supercoiling in opposite direction (Menzel and Gellert, 1987; Tse-Dinh and Beran, 1988).

The AT→TA mutation in the A_7 track has no effect on the isomerization step, as variations of k_2 are similar for both the wild-type and mutant promoters. Rather, it affects the formation of the closed complex, which is characterized by the association constant K_B.

Our results offer a phenomenological explanation for the

activation of the *ada* promoter by the transversion in the upstream region. This mutation produces a shift of the superhelical density at which the association constant is maximum. Hence, the activation of the promoter, induced by this up-mutation, is due to the simultaneous enhancement of both the association and isomerization rate constants.

Molecular basis for the activation of the ada promoter by the A_7 to A_3TA_3 mutation

A first explantion for the activation of the transcription observed in the A_3TA_3 mutant sequence could be that the mutation produces a second promoter-like sequence, which would allow the interaction with a second RNA polymerase. Such double interaction sites have been recognized to produce an activation of the transcription initiation (Travers *et al.*, 1983; Duval-Valentin and Ehrlich, 1988). However, examination of the upstream sequence does not reveal strong homology with promoter consensus sequences. Moreover, it is difficult to fit this explanation with the need of supercoiling the DNA in order to obtain the mentioned activation.

The fact that the A_7 to A_3TA_3 transversion affects the modulation by the supercoiling by the association constant alone (and not that of the isomerization constant) indicates that upstream regions are involved in the primary contact between RNA polymerase and the *ada* promoter. In fact, Sakumi and Sekiguchi (1989) have shown by DNase I footprinting that this upstream region (also harbouring the Ada binding site) is essential for the interaction of the RNA polymerase with the *ada* promoter. This conclusion also agrees with the 'nucleosome-like' model proposed by Amouyal and Buc (1987), in which the promoter wraps around the RNA polymerase in order to form the first closed complex prior to isomerization. In this model, the upstream region comes into contact with the enzyme.

The question is now to understand what particular feature in the upstream DNA sequence leads to the difference in the mutant and wild-type promoter behaviour.

If the effect of the mutation was to induce a permanent curvature of the DNA differently from that of the wild-type sequence, non-similar association constants K_B would have been observed for each promoter when included in linear plasmids. On the contrary, the effect of the mutation on the transcription intiation is only revealed by the supercoiling of the plasmid. This suggests that the toplogical stress induces different structures in the wild-type and mutant sequences, which are recognized differently by the RNA polymerase. It is more a difference of bendability between the A_7 and A_3TA_3 sequences than a difference of curvature which can explain our observations.

It is not clear whether the A track itself or its interrupted version is recognized by the polymerase. It is also possible that the interaction of the RNA polymerase with these regions might require a particular spatial arrangement. The optimal topology (which leads to the stronger association constant) is obtained in the A_3TA_3 mutant for a smaller superhelical density than that needed by the wild-type sequence. In other words, the energy needed to fold the DNA in an optimal topology for its interaction with the RNA polymerase is smaller when the A_7 track is replaced by the A_3TA_3 sequence. This can be related to a higher bendability of the DNA introduced by the latter sequence.

Comparison between in vivo and in vitro activation of the promoters

Using β-galactosidase assays, Nakamura *et al.* (1988) showed that the transcription with the A_3TA_3 up-mutant promoter is $\sim$40 times stronger than with the wild-type *ada* promoter in absence of activation. The methylated Ada protein increases transcription of the wild-type *ada* promoter by a factor of 190, while it has no effect on the mutant promoter. Taking into account the difference in conditions between the two assays and the experimental errors, one can consider that our observations are compatible with the results obtained *in vivo* by Nakamura *et al.* (1988).

The activation of transcription in the mutant promoter is due to in-phase modulation of the k_2 and K_B constants which happen to be maximum for the same superhelical density. This optimal superhelical density is equal to -0.05, which is in fact the superhelical density of the plasmid as obtained after extraction from the *E.coli* cells.

This raises the question of the real supercoiling state of the plasmid in the cell. Indeed, the supercoiling of the plasmid *in vivo* has been measured to be equal to half this optimum value: -0.025 (Greaves *et al.*, 1985; Peck and Wang, 1985). However, by comparison between the *in vivo* and *in vitro* activity of *lac* mutant promoters (UV5 and p^s), Borowiec and Gralla (1987) have shown that the chromosomic superhelical state is equal to -0.048 ± 0.005. In fact, the local supercoiling need not be the same all along the plasmid and may depend on the sequence (more precisely the bendability; Travers, 1989). Moreover, it is clear now that transcription affects locally the supercoiling of the DNA (Wu *et al.*, 1988; Lodge *et al.*, 1989. Rahmouni and Wells, 1989; Tsao *et al.*, 1989). When transcribing, the RNA polymerase generates positive supercoiling ahead and negative supercoiling behind. As the stimulation of the A_3TA_3 mutant promoter occurs at a superhelical density of -0.05, we can conclude that this is also the value of the superhelical density around the *ada* promoter *in vivo*. This particularly high negative supercoiling could be due to the activity of the RNA polymerases engaged in the transcription complex on the gene following the promoter.

Materials and methods

Materials

Plasmids pTN232 and pTN301, containing the wild-type sequence and the (−59) A→T up-mutant, respectively, were generous gifts from Dr Nakamura (Nakamura *et al.*, 1988).

The RNA polymerase was extracted from an *E.coli* K12 strain, according to Burgess and Jendrisak (1975). The specific activity on calf thymus DNA was 899 U/mg, where 1 U is equivalent to 1 nmol UTP incorporated in 10 min.

Unlabelled nucleoside triphosphate and dinucleotide monophosphates were purchased from Sigma. ANS was from Fluka. UTPγANS was prepared as described by Yarbrough *et al.*, (1979).

DNA topoiosomers

The different topoisomers of the two plasmids were generated by the action of calf thymus topoisomerase I in presence of different amounts of ethidium bromide.

The superhelical densities of the plasmids were determined by two-dimensional agarose gel electrophoresis (Peck and Wang, 1983).

Both one- and two-dimensional electrophoresis of the topoisomers were carried out at room temperature in a horizontal gel apparatus, using 1.5% agarose gels equilibrated in the electrophoresis buffer (40 mM Tris−HCl, pH 8, 20 mM sodium acetate, 5 mM EDTA) and in presence or absence of ethidium bromide. A field of 3 V/cm was applied to the gel overnight.

Each gel was stained with 10 μg/ml ethidium bromide and photographed with polaroid type 665 film. Negatives were traced by a scanning densitometer. For two-dimensional gels, the first dimension was run without eithidum bromide, whereas the second dimension was run in presence of 0.02 μg/ml ethidium bromide.

Abortive initiation assays

This technique relies on the production of short oligonucleotides (McClure, 1980). In our case, using the dinucleotide UpA as the starting dinucleotide and UTPγANS as the elongating nucleotide, we obtained a trinucleotide UpApU for the *ada* promoters. Standard 'transcription buffer' was 50 mM HEPES (pH 7.9), 0.4 mM potassium phosphate buffer, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 100 μg/ml bovine serum albumin (BSA), 0.5 mM UpA and 0.1 mM UTPγANS. The DNA template in standard buffer was 5 nM plasmid DNA; RNA polymerase concentrations varied from 50 to 400 nM in the same buffer.

Fluorescence measurements were performed on a Jobin−Yvon JY 3D spectrofluorimeter. The temperature inside the cell was kept constant. After 10 min for temperature equilibrium at 37°C, the RNA polymerase was directly mixed with DNA. In order to record the first seconds of the initiation kinetics, we adapted a rapid mixing system to the fluorescence cell (Bertrand-Burggraf *et al.*, 1984a). The total volume resulting from the mixing of the two solutions was 600 μl. The excitation wavelength was selected at 360 nm, and the fluorescence intensity was recorded at 430 nm. The variation of the fluroescence intensity with time was recorded discontinuously, with the interval between each measurement varying from 1 to 2 s.

The fluroescence time-course curve was analysed according to the method described previously (Bertrand-Burggraf *et al.*, 1984a).

DNA curvature analysis

Constructions. The two 31mers used for the ligation product analysis have the following sequences:

```
5'                          T                  3'
AGCTTCCTTGTCAGCGAAAAAAATTAAAGCG
     AGGAACAGTCGCTTTTTTTAATTTCGCTCGA
3'                          A                  5'
```

One can notice that none of the four strands would form a hairpin, that the sticky ends are cohesive over four bases in the duplexes, and that the sequences $(A)_7$ and (A_3TA_3) are in phase with the DNA helical repeat.

The two upper DNA strands were extracted from plasmids pTN232 and pTN301, respectively, digested with the restriction enzymes *Hin*dIII and *Hal* (Biolabs), end-labelled at their 5′ ends with ^{32}P and isolated on denaturing polyacrylamide gels in presence of 7 M urea.

The two lower stands were synthetic oligonculeotides, generously provided by E.Seeberg.

Ligation and gel analysis. After hybridization, the two 31mer duplexes were isolated on a non-denaturing 12% polaycrylamide gel. Ligations were performed at 16°C for 2 h, at a concentration of 0.2 μM DNA in the presence of 400 U T4 DNA ligase (Biolabs), and in presence of excess *Hin*dIII (Biolabs) and *Sac*I (Biolabs) restriction enzymes so as to affect directional ligation. The ligation products were analysed by two-dimensional gel electrophoresis under conditions similar to those described by Husain *et al.* (1988). Briefly, after separating the DNA fragments on a 5% polyacrylamide gel in the first dimension, we separated the linear and circular molecules by running the second dimension on a 10% polyacrylamide gel containing ethidium bromide (0.025 μg/ml).

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References

Amouyal,M. and Buc,H. (1987) *J. Mol. Biol.*, **195**, 795−808.
Betrand-Burggraf,E., Lefèvre,J.F. and Daune,M. (1984a) *Nucleic Acids Res.*, **12**, 1697−1706.
Bertrand-Burggraf,E., Schnarr,M., Lefèvre,J.F. and Daune,M. (1984b) *Nucleic Acids Res.*, **12**, 7741−7752.
Borowiec,J.A. and Gralla,J.D. (1987) *J. Mol. Biol.*, **184**, 587−598.
Brahms,J.G., Dargouge,O., Brahms,S., Ohara,Y. and Vagner,V. (1985) *J. Mol. Biol.*, **181**, 455−465.
Braco,L., Kotlartz,D., Kolb,A., Diekmann,S. and Buc,H. (1989) *EMBO J.*, **8**, 4289−4296.
Burgess,R.R. and Jendrisak,J.J. (1975) *Bichemistry*, **14**, 4634−4638.
Burkhoff,A.M. and Tullius,T.D. (1988) *Nature*, **331**, 455−457.
DiGabriele,A.D., Sanderson,M.R. and Steitz,T.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1816−1820.
Duval-Valentin,G. and Ehrlich,R. (1988) *Nucleic Acids Res.*,**16**, 2031−2044.
Gartenberg,M.C. and Crothers,D.M. (1988) *Nature*, **333**, 824−829.
Greaves,D., Patient,R. and Lilley,D. (1985) *J. Mol. Biol.*, **185**, 461−478.
Hagerman,P.J. (1985) *Biochemistry*, **24**, 7033−7037.
Harley,C.B. and Reynolds,R.P. (1987) *Nucleic Acids Res.*, **15**, 2343−2361.
Hawley,C.B. and McClure,W.R. (1983) *Nucleic Acids Res.*, **11**, 2237−2255.
Hawley,D.K., Malan,T.P., Mulligan,M.E. and McClure,W.R. (1982) In Chamberlin,M.J. and Rodriguez,R.L. (eds), *Promoters*. Praeger, New York, 54−68.
Husain,I., Griffin,J. and Sancar,A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2558−2562.
Jo,Y.L., Nara,F., Ichihara,S., Mizuno,T. and Mizushima,S. (1986) *J. Biol. Chem.*, **261**, 15252−15256.
Lane,A.N., Lefèvre,J.F. and Jardetzky,O. (1986) *Bochim. Biophys. Acta*, **867**, 45−56.
Lindahl,T., Sedgwick,B., Sekigushi,M. and Nakabeppu,Y. (1988) *Annu. Rev. Biochem.*, **57**, 133−157.
Lohman,T.M., de Haseth,P.L. and Record,M.T.,Jr (1980) *Biochemistry*, **19**, 3522−3530.
Lodge,J.K., Kazic,T. and Berg,D. (1989) *J. Bacteriol.*, **171**, 2181−2187.
Malan,T.P. and McClure,W.R. (1984) *Cell*, **39**, 173−180.
Malan,T.P., Kolb,A., Buc,H. and McClure,W.R. (1984) *J. Mol. Biol.*, **180**, 881−909.
Marini,J.C., Levene,S.D., Crothers,D.M. and Englund,P.T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7664−7668.
McClure,W.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5634−5638.
McClure,W.R. (1985) *Annu. Rev. Biochem.*, **54**, 171−204.
Menzel,R. and Gellert,M. (1987) *J. Bacteriol.*, **169**, 1272−1278.
Nadeau,J.G. and Crothers,D.M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2622−2626.
Nakamura,T., Tokumoto,Y., Sakumi,K., Koike,G., Nakabeppu,Y. and Sekiguchi,M. (1988) *J. Mol. Biol.*, **202**, 483−494.
Nelson,H.C.M., Finch,J.T., Luisi,B.F. and Klug,A. (1987) *Nature*, **330**, 221−226.
Peck,L.J. and Wang,J.C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6206−6211.
Peck,L.J. and Wang,J. (1985) *Cell*, **40**, 129−137.
Pruss,G.J. and Drlica,K. (1989) *Cell*, **56**, 521−523.
Ptashne,M. (1986) *Nature*, **322**, 697−701.
Raibaud,O. and Schwartz,M. (1984) *Annu. Rev. Genet.*, **18**, 173−206.
Rahmouni,A.R. and Wells,R.D. (1989) *Science*, **246**, 358−363.
Sakumi,K. and Sekiguchi,M. (1989) *J. Mol. Biol.*, **205**, 373−385.
Travers,A.A. (1987) *CRC Crit. Rev. Biochem.*, **22**, 181−219.
Travers,T.A. (1989) *Annu. Rev. Biochem.*, **58**. 427−452.
Travers,A.A., Lamond,A.I., Mace,H.A.F. and Berman,M.L. (1983) *Cell*, **35**, 265−273.
Tsao,Y.-P., Wu,H.-Y. and Liu,L.F. (1989) *Cell*, **56**, 111−118.
Tse-Dinh,Y.C. and Beran,R.K. (1988) *J. Mol. Biol.*, **202**, 735−742.
Ulanovsky,L., Bodner,M., Trifonov,E.N. and Choder,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 862−866.
Wu,H.-Y., Shyy,S., Wang,J. and Liu,L. (1988) *Cell*, **53**, 433−440.
Yarbrough,L.R., Schlageck,J.G. and Baughman,M. (1979) *J. Biol. Chem.*, **254**, 12069−12073.
Zahn,K. and Blattner,F.R. (1985) *Nature*, **317**, 451−453.

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