The EMBO Journal vol.10 no.9 pp.2589 – 2594, 1991

Flexibility of the DNA enhances promoter affinity of *Escherichia coli* RNA polymerase

Wladyslaw Werel[2], Peter Schickor[1] and Hermann Heumann[1]

[1]Max Planck Institute of Biochemistry, D-8033 Martinsried, FRG and [2]University of Gdansk, Department of Molecular Biology, 80-822 Gdansk, ul. Kladki 24, Poland

Communicated by W.Zillig

Two types of mechanisms are discussed for the formation of active protein–DNA complexes: contacts with specific bases and interaction via specific DNA structures within the cognate DNA. We have studied the effect of a single nucleoside deletion on the interaction of *Escherichia coli* RNA polymerase with a strong promoter. This study reveals three patterns of interaction which can be attributed to different sites of the promoter, (i) direct base contact with the template strand in the '−35 region' (the 'recognition domain'), (ii) a DNA structure dependent interaction in the '−10 region' (the 'melting domain'), and (iii) an interaction which is based on a defined spatial relationship between the two domains of a promoter, namely the 'recognition domain' and the 'melting domain'.

Key words: DNA dependent RNA polymerase/DNA flexibility/hydroxyl radicals

Introduction

DNA sequences mediating specific functions are distinguished by consensus sequences. The existence of consensus sequences suggests that a specific interaction between bases of this sequence and the protein is necessary for the formation of an active complex. To what extent the interaction of sequence specific binding proteins with DNA is mediated by direct contacts of specific bases within the consensus sequence or by special structural features of the DNA such as flexibility, is the subject of our present investigation. Using hydroxyl radical pretreated DNA as a template, we show that different regions within an *Escherichia coli* promoter, such as the '−35 region' and the '−10 region', display different modes of interaction with *E.coli* RNA polymerase.

Hydroxyl radicals were previously used to determine the 'footprint' of sequence specific binding proteins at its cognate DNA (Tullius and Dombroski, 1986; Metzger *et al.*, 1989; Schickor *et al.*, 1990). The inverse approach uses hydroxyl radical pretreated DNA as a template and measures the effect of the treatment on the affinity of sequence specific binding proteins (Chalepakis and Beato, 1989; Hayes and Tullius, 1989; Schreck *et al.*, 1990). The interpretation of the obtained interference binding pattern requires knowledge of the chemical and the structural modification introduced by the hydroxyl radicals. Hydroxyl radicals eliminate a single base, leaving phosphate groups, both at the 5′- and the 3′-positions next to the gap (Shafer *et al.*, 1989). This technique of eliminating a single nucleoside from a recognition sequence was used previously to investigate the influence of missing contacts between the bases of the cognate DNA sequence and the homologous binding protein in the lambda repressor system, the lambda cro system and the GCN4 system (Hayes and Tullius, 1989; Brunelle and Schleif, 1987; Gartenberg *et al.*, 1990). In these systems a decrease in the binding affinity was found, if a specific base within the cognate DNA was deleted. We show that the elimination of single bases in the *E.coli* promoter A1 of the phage T7 can lead to a lower or a higher affinity of RNA polymerase to the promoter. This effect depends upon the position of the deletion, implying that the single bases of the promoter play different mechanistic roles in the RNA polymerase–promoter interaction.

Results and discussion

Deletion of a single base increases the DNA flexibility

Little is known about the structural changes introduced to the DNA by elimination of a single base. Crystal structure analysis of nicked DNA shows no drastic deviation from the canonical B-DNA structure (Aymami *et al.*, 1990). Whether this result obtained with nicked DNA can be applied to gapped DNA is questionable. It is rather obvious that a deletion enhances the flexibility of the DNA. In order to answer the question of whether eliminating a single nucleoside within a DNA fragment generates a bending center, gapped duplex DNA was subjected to an electrophoretic analysis analog to the permutation assay (Wu and Crothers, 1984). The rationale of this approach is the previous finding that a DNA fragment having a bend in the middle is more strongly retarded than those having the bend at the end of the fragment.

A 112 bp DNA fragment containing the phage T7 A1 promoter was treated with iron/EDTA-generated hydroxyl radicals in order to produce a fractional population of gapped duplexes. This mixture of partially modified DNA fragments containing random deletions of single nucleosides was subjected to two subsequent electrophoretic runs. In the first run the DNA fragments were separated under non-denaturing conditions. The 'smear' in Figure 1a indicates that hydroxyl radical treatment generates DNA fragments having a lower electrophoretic mobility. Charge effects cannot account for the observed change in the mobility, since the charge difference due to the deletion of a single base in each DNA fragment is too small. In order to show that a correlation exists between the electrophoretic mobility and the position of the gap, the gel was cut into slices, the DNA fragments in the slices were collected and applied to a sequencing gel for size determination (Figure 1a). For the sake of clarity, we show in Figure 1b a schematic representation of the electrophoretic pattern which would be obtained if the observed decrease of the electrophoretic mobility of gapped DNA in the first, non-denaturing gel is a consequence of

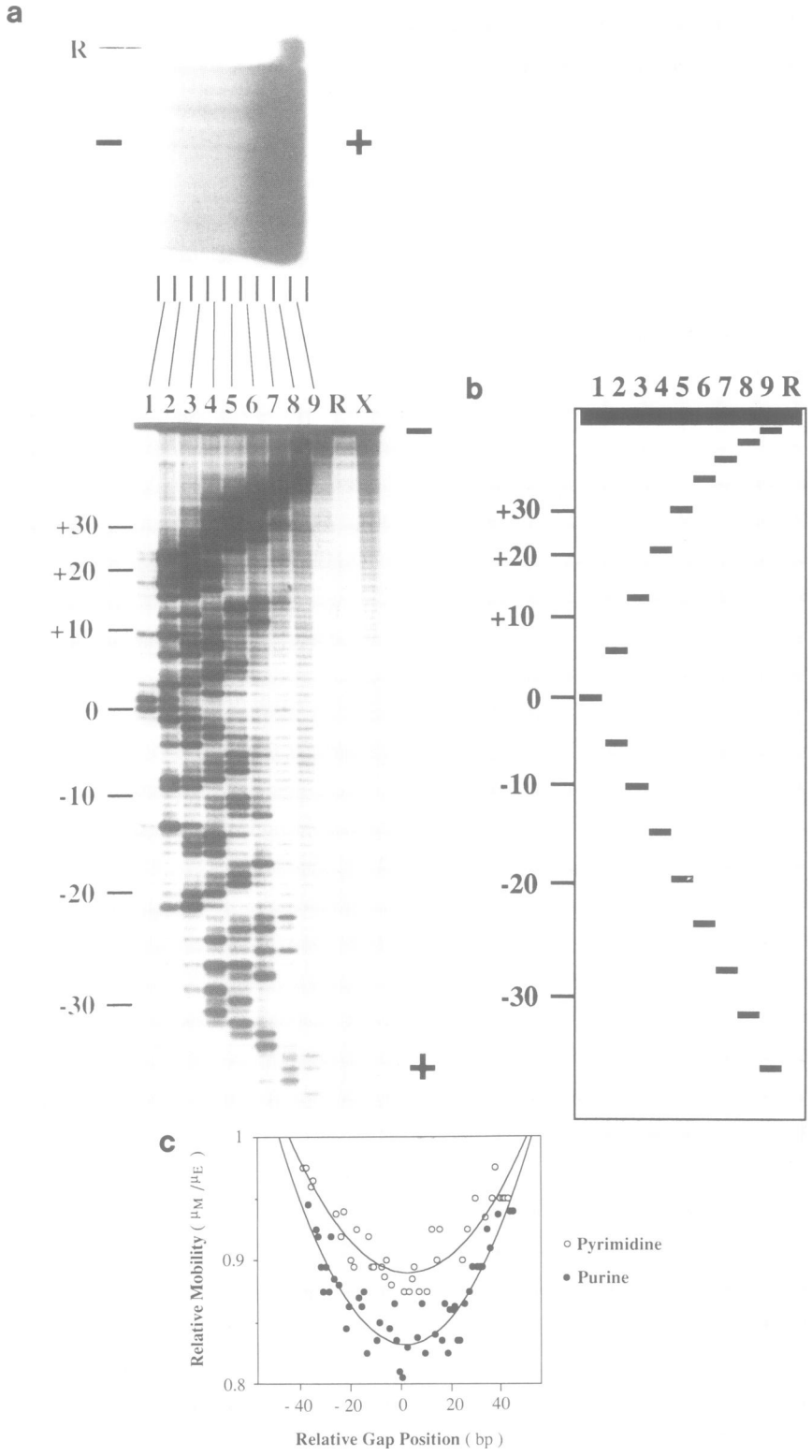

Fig. 1. Influence of the elimination of a single nucleoside on electrophoretic mobility. A DNA fragment of 112 bp carrying the A1 promoter of the phage T7 labeled at the 5′ end upstream of the promoter was subjected to hydroxyl radical treatment, yielding a small fraction (~10%) of singly gapped DNA. The elimination of a single nucleoside generated topologically different isomers which differ in their electrophoretic mobility. The different isomers were analyzed by two subsequent gel electrophoreses (a). (**a**) Electrophoresis under non-denaturing conditions for the separation of topologically different isomers according to their electrophoretic mobility. Fragments exhibiting different mobilities were cut from the gel, collected in nine fractions and separated according to size on a sequencing gel. The amounts of DNA applied on each lane were adjusted to have approximately the same radioactivity in each lane. Lane R represents the non-gapped DNA, which shows no retardation, lane X represents the gapped DNA without previous separation on the non-denaturing gel. (**b**) A schematic representation of the electrophoresis pattern predicted for flexible or bent DNA fragments. The numbers represent the position of the gap. The 0 position refers to the midpoint of the fragment. (**c**) A graphic representation of the electrophoretic mobility of DNA fragments having a deletion at the position as indicated on the abscissa. The numbers of the gap positions refer to the middle of the fragment (0 position). The ordinate shows the electrophoretic mobility of the gapped DNA (μ_M) normalized to the mobility of non-gapped DNA (μ_E). The data were taken from (a). Open circles represent the DNA fragments having at the gap position a pyrimidine and closed circles those with a purine.

bending of the DNA at the gap assuming the same bending angle at each gap (Lerman and Frisch, 1982). The solid line in Figure 1c represents the calculated electrophoretic mobility. The experimentally obtained data points with gapped DNA in Figure 1c, which were derived from the electrophoretic pattern in Figure 1a, display essentially the characteristic, position-dependent electrophoretic mobility of bent DNA (Wu and Crothers, 1984). DNA fragments having a gap in the middle are the most retarded, consistent with the view that a gap is a center of bending or enhanced flexibility. Moreover, the extent of retardation depends upon the type of the eliminated base. A deleted purine promotes a larger gel shift than a pyrimidine, as indicated by the segregation into two groups of data points highlighted in Figure 1c. From this follows that elimination of a purine creates a stronger flexibility or bending in the DNA than a pyrimidine.

However, the position-dependent mobility assay does not permit a decision on whether the gap creates a center of enhanced flexibility or a rigid bend. This is obvious, since both kinds of conformational changes lead to a decrease in the mean end-to-end distance of the DNA fragment, the parameter which is assumed to be responsible for the observed decrease of the electrophoretic mobility (Lerman and Frisch, 1982).

From these studies we conclude that elimination of a single nucleoside leads to a change of the DNA structure which can be explained by an enhancement of the flexibility of the DNA at the position of the deleted nucleoside. The degree of flexibility depends on the eliminated base type.

Elimination of a single nucleoside within the promoter modulates the RNA polymerase binding in a position-dependent manner

The promoter-containing DNA fragment used in the previous experiment was treated with hydroxyl radicals to produce a small population (~ 10%) of randomly gapped duplexes. This partially modified DNA was incubated with RNA polymerase at different temperatures as described in Materials and methods. Free RNA polymerase, free DNA and the complex were separated by electrophoresis on a non-denaturing acrylamide gel and the DNA-containing fractions were analyzed on a sequencing gel (Figure 2). The difference in the intensity of the bands in the complex containing fractions (lanes 3 and 5 of Figure 2) and the reference DNA containing fractions (lane 1 in Figure 2) reflects the modulation of the affinity of RNA polymerase for the DNA due to elimination of the corresponding base. [It is consistent that the fraction containing free DNA (lanes 2 and 4 in Figure 2) shows an intensity pattern complementary to that of the complex-containing fraction.] The relative binding free energy due to the elimination of a single nucleoside was calculated, as shown in Materials and methods, from the concentration of the complex, which was estimated from the relative change of the intensity of the bands. Figure 3 shows the change in relative binding free energy at 4°C and 22°C for both strands (panels A and B in Figure 2).

The most striking effect on the binding affinity of RNA polymerase and promoter DNA is observed if a base in the region between base positions +2 and −14, the 'melting domain' of the promoter (Metzger *et al.*, 1989; Schickor *et al.*, 1990), is eliminated. Surprisingly, a deletion in this region enhances the affinity. Missing contacts due to the deletion of a single base cannot account for this effect, because missing contacts would decrease the affinity, as shown for other specific protein−DNA complexes (Hayes and Tullius, 1989). Obviously, another mechanism must account for the observed effect. The increase in affinity is more distinct at a low temperature (4°C) than at a high temperature (37°C) (see Figure 2). The reduced affinity of RNA polymerase for intact promoter DNA at low temperature is fully compensated for by eliminating a single nucleoside in the melting region (Figure 4).

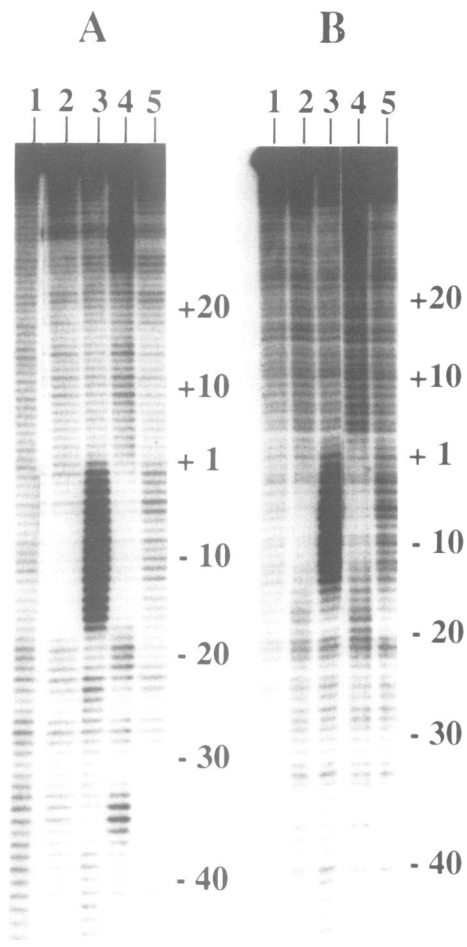

Fig. 2. Electrophoretic pattern of gapped promoter DNA free and in the complex with RNA polymerase. A DNA fragment of 112 bp carrying the T7 A1 promoter with single nucleoside deletions was incubated with RNA polymerase at 4°C (lanes 2 and 3) and 22°C (lanes 4 and 5). DNA free and in the complex was separated on a non-denaturing gel, collected and applied to a sequencing gel. Lane 2 shows the free DNA, lane 3 the DNA in the complex, both at 4°C; lanes 4 and 5 show the same DNA fraction obtained at the incubation temperature of 22°C. Lane 1 shows the DNA without RNA polymerase. Panel **A** shows the template strand, panel **B** the non-template strand. The numbers refer to the starting point of transcription.

Based on the gel shift studies on gapped duplex DNA shown above, we suggest that this increase of the affinity at low temperature is a consequence of enhanced flexibility of the DNA at the gap, which facilitates the adaptation of the proper conformation for optimal RNA polymerase binding. At high temperature the effect is small, probably due to the already high affinity of the RNA polymerase to intact A1 promoter at elevated temperature.

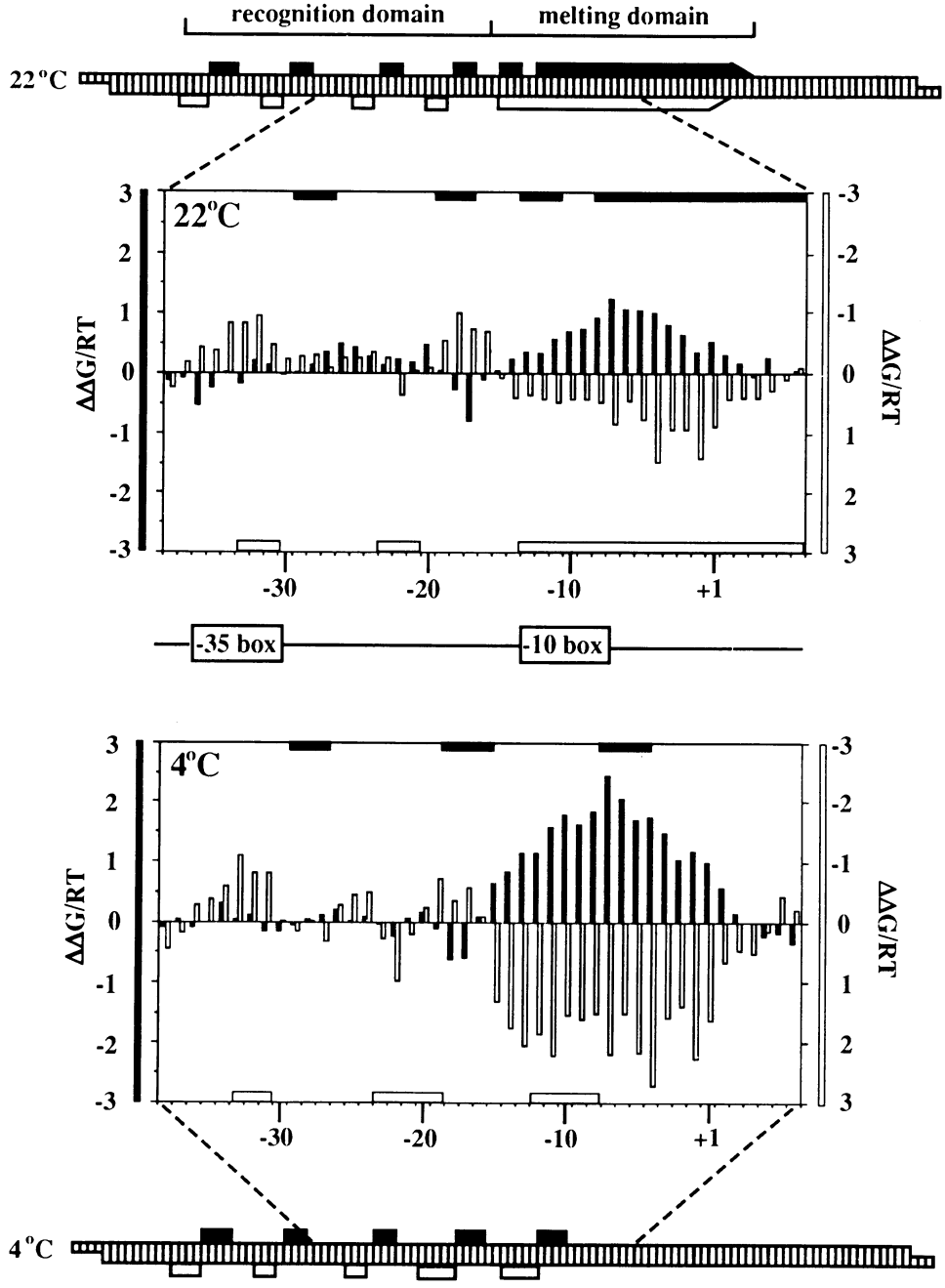

Fig. 3. The influence of missing nucleosides on RNA polymerase binding. The change of the relative binding Gibbs free energy in kcal/mol at 4°C and 22°C are depicted by the vertical bars as a function of the gap position (filled bars, non-template strand; open bars, template strand). The analogous protection patterns of RNA polymerase obtained by hydroxyl radical footprinting (Schickor *et al.*, 1990) are indicated by horizontal bars (filled bars, non-template strand; open bars, template strand).

The increase in affinity caused by a single base deletion is a specific effect. It is restricted to that region of the promoter which contains the transcription bubble (von Hippel, 1982), namely the region between base positions +2 and −8 (Kirkegaard *et al.*, 1983; Schickor *et al.*, 1990). NMR studies have shown an enhanced structural flexibility of this DNA region depending on the temperature (Chou *et al.*, 1984), which might facilitate the formation of an open complex by RNA polymerase (Amouyal and Buc, 1987). Genetic data suggest that the contacts between RNA polymerase and the DNA within the melting domain are provided by the phosphate groups of the DNA (Siegele *et al.*, 1989). All these findings support the view that the interaction of RNA polymerase with the melting domain is mediated by the DNA structure, rather than by direct contacts between a specific base of the melting domain and the RNA polymerase.

We know that a promoter is used by RNA polymerase for initiating RNA synthesis, if the operating temperature is high enough to allow open complex formation. Our data suggest that this temperature requirement can be overcome by elimination of a single nucleoside within the melting domain. It is in line with our suggestion that at low temperature the effect due to a gap in the DNA extends downstream of the region protected by RNA polymerase (Figure 3). This could be a consequence of a long-range

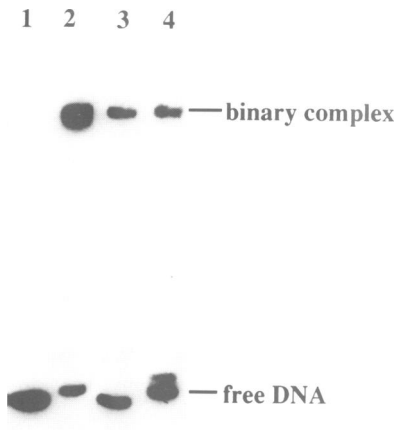

Fig. 4. Gel-shift assay with a DNA fragment having a single base deletion within the promoter. The 112 bp promoter carrying DNA fragment (radioactively labeled at the 5'-strand) was incubated with RNA polymerase at different temperatures under conditions described in Materials and methods. The incubation temperatures were 4°C (lanes 1 and 2) and 22°C (lanes 3 and 4). Lanes 2 and 4 show the complexes formed with a DNA template having a single gap at the base position −8. Lanes 1 and 3 show the complexes formed with intact DNA.

effect of the gaps allowing the DNA to adopt the proper conformation.

The use of hydroxyl radical treated promoter DNA raises the question of whether the modification changes the ability of the DNA to act as a promoter. Therefore, we compared the RNA synthesis activity and the protection pattern of RNA polymerase bound to gapped and intact promoter DNA. For these studies a homogeneous population of singly gapped DNA was required, instead of the partially and randomly gapped duplex DNA used in the previous experiment. Therefore, we prepared a DNA fragment by chemical synthesis having a gap at a single position in the template strand. We have chosen base position −8, since the effect due to the deletion is pronounced here. As expected, the affinity of RNA polymerase for this specifically gapped DNA is greatly enhanced at low temperature and only slightly at high temperature compared with unmodified template DNA (Figure 4). Hydroxyl radical footprinting studies reveal (Figure 3) no discernible differences in the protection pattern using modified (data not shown) or unmodified template (Schickor *et al.*, 1990). Furthermore, the elimination of this single nucleoside in the DNA does not interfere with RNA synthesis activity. The amount and size of products obtained under standard assay conditions at 37°C using either the DNA with a single gap at base position −8 or intact DNA do not differ as time dependent gel electrophoretic analysis of the RNA products revealed (data not shown).

Gaps in the 'recognition domain' upstream of the base position −20 have a minor effect on RNA polymerase affinity, with the exception of a stretch of the template strand between base positions −31 and −34. This region is identical with the '−35' or 'recognition' sequence, one of the consensus sequences of a promoter. Gaps in this region decrease the affinity significantly, indicating that effects due to missing contacts are predominant. It is striking that the effect is confined to that strand which has contact with RNA polymerase, as revealed by protection studies [(Schickor *et al.*, 1980) and Figure 3], suggesting that these contacts between the template strand and the RNA polymerase are responsible for the recognition process.

A gap in a window of 2−4 bp centered at base position −18 positioned in either the template or the non-template strand decreases the affinity. This window is positioned between two functional domains of the DNA, the melting domain and the recognition domain. This region is affected despite a lack of direct contacts between the RNA polymerase and the DNA in this region, as revealed by protection studies [Figure 3 and Schickor *et al.* (1990)]. We speculate that the spatially correct juxtaposition of the '−35 box' and the '−10 box' with respect to the corresponding interaction sites on the RNA polymerase is lost by the enhanced flexibility of the DNA, if a single nucleoside in the region around base position −18 is deleted.

Our finding that a gap in the spacer region around base position −18 decreases the binding affinity of RNA polymerase seems to be in conflict with the notion of Ayers *et al.* (1989) who claim that a gap in the spacer region between the '−10 box' and the '−35 box' increases the affinity. They studied the effect of a gap, asking the question of whether torsional stress applied on the spacer region during RNA polymerase promoter complex formation could be relieved by a single base deletion. However, our data in Figure 3 show that such a problem can only be tackled if the influence of a single gap is known in a larger sequence context. Figure 3 shows that even within a short base sequence such as the spacer region, a single gap can have rather different effects. Ayers *et al.* (1989) deleted a single base at position −22 on the template strand. Figure 3 shows that a deletion at this position has a minor effect on the RNA polymerase−promoter interaction. Even if they used a different promoter as we did, we would propose that a template having a deletion at base position −18 would have been much more sensible for studying the torsional stress relief by single gap formation.

Conclusion

The single base deletion assay provides information about the significance of a single base for the interaction of a sequence specific binding protein with its cognate DNA. This assay probes two effects: (i) missing contacts between protein and DNA and (ii) a possible structural change of the DNA due to the elimination of a base. This change can be an enhanced flexibility and/or a bend of the DNA.

Using the gel shift assay, changes in affinity due to the deletion of a single base within the cognate DNA can be determined quantitatively. Such changes were shown for the RNA polymerase−promoter complex. Different modes of interaction were deduced from the affinity pattern. In the recognition domain (the −35 region) specific contacts between bases of the codogenic strand and the RNA polymerase are important, whereas in the melting domain (the −10 region) the adoption of a specific DNA conformation is essential for specific complex formation. A third feature of the RNA polymerase promoter interaction is the correct steric arrangement of the two sites on the DNA with respect to RNA polymerase.

The success of this study concerning the significance of a single base for the mode of RNA polymerase−promoter interaction is based on the information obtained from two sources, the 'single base deletion assay' and the

'hydroxyl radical footprinting assay'. Both assays provide complementary information. The ease of their implementation makes this approach particularly useful for the investigation of protein–DNA complexes displaying different modes of interaction.

Materials and methods

Preparation of RNA polymerase

RNA polymerase was prepared according to Zillig *et al.* (1970). σ factor was isolated from the overproducing strain M 5219/pMRG 8 using the method of Gribskow and Burgess (1983). RNA polymerase holoenzyme was reconstituted from core enzyme and σ factor as previously described (Heumann *et al.*, 1986).

Preparation of DNA fragments

A 112 bp DNA fragment carrying the T7 A1 promoter was prepared and labeled as described by Schickor *et al.* (1990). The same DNA fragment having a single gap at position −8 (with respect to the starting point of transcription) in the template strand was obtained by chemical synthesis of the single strands and subsequent reannealing of the single-stranded fragments.

Hydroxyl radical treatment of promoter DNA

Hydroxyl radical treatment was performed as described by Hayes and Tullius (1989).

Electrophoresis

Non-denaturing conditions. Different DNA conformers were separated by electrophoresis on 12% acrylamide gels (20 × 55 × 0.05 cm^3) at 4°C for 20 h at 10 V/cm. DNA fractions exhibiting different mobilities were cut from the gel and eluted as described by Metzger *et al.* (1989).

Binary complexes of RNA polymerase and hydroxyl radical treated promoter fragment were separated from unbound DNA by gel electrophoresis under non-denaturing conditions as described by Fried and Crothers (1981) and Heumann *et al.* (1986). DNA containing bands were cut from the gel and eluted.

Denaturing conditions. Determination of the size of the DNA fragments was performed by electrophoresis on a 10% acrylamide gel (0.2–0.4 mm) as described for DNA sequence analysis (Maxam and Gilbert, 1977) (7 M urea, 100 mM Tris–Cl, pH 8.6, 84 mM borate, 1 mM EDTA). The electrophoresis was performed at 50 W at a temperature of 60°C.

Preparation of binary complexes

For binary complex formation, either synthesized promoter fragments (having a discrete gap at position −8) or hydroxyl radical pretreated promoter fragments (randomly gapped) were incubated with RNA polymerase in a buffer containing 8 mM Tris–Cl, pH 7.9, 50 mM NaCl, 1 mM β-mercaptoethanol for 15 min at temperatures as indicated. The concentration of RNA polymerase was 0.25 mg/ml and that of the DNA 0.025 mg/ml. The volume of the assay was 20 μl. In order to stabilize the RNA polymerase–promoter complex, the sample was dialyzed against 8 mM Tris–Cl, pH 7.9. A 10-fold molar excess of heparin was added in order to destroy nonspecific complexes. The complexes were kept at the indicated temperatures throughout the experiment including non-denaturing gel electrophoresis.

Densitometric analysis of the electrophoretic pattern

X-ray films of the electrophoretic pattern of bound and unbound DNA were scanned on a Hirschmann photodensitometer. Different scans were normalized by using the intensities of bands within regions not affected by binding of RNA polymerase.

Determination of the relative binding free energy of RNA polymerase – promoter interaction

The relative change of the binding free energy ($\Delta\Delta F$) due to a deletion at base position i was calculated according to Gartenberg *et al.* (1990): $\Delta\Delta f_i = -RT \ln f_i$, with $f_i = K_{i,Gap+}/K_{i,Gap-}$, the ratio of the binding constants (K_i) for RNA polymerase–DNA complex formation. Since $K_{i,Gap+}/K_{i,Gap-} = c_{i,Gap+}/c_{i,Gap-}$, $\Delta\Delta F_i$ is obtained from the ratio of the intensities of the bands with and without a gap, assuming proportionality between the complex concentrations (c_i) and the intensity of the corresponding band: $I_{i,Gap\pm} \propto c_{i,Gap\pm}$.

Acknowledgements

We thank Drs F.Pfeifer, M.Horne and A.Whelan for a critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft.

References

Amouyal,M. and Buc,H. (1987) *J. Mol. Biol.*, **195**, 795–808.
Ayers,D.G., Auble,D.T. and Dehaseth,P.L. (1989) *J. Mol. Biol.*, **207**, 749–756.
Aymami,J., Coll,M., van der Marel,G.A., van Boom,J.H., Wang,A.H.-J. and Rich,A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2526–2530.
Brunelle,A. and Schleif,R.F. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6673–6676.
Chalepakis,G. and Beato,M. (1989) *Nucleic Acids Res.*, **17**, 1783.
Chou,S.-H., Wemmer,D.E., Hare,D.R. and Reid,B.R. (1984) *Biochemistry*, **23**, 2257–2262.
Fried,M.G. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
Gartenberg,M.R., Ampe,C., Steitz,T.A. and Crothers,D.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6034–6038.
Gribskow,M. and Burgess,R.R. (1983) *Gene*, **26**, 109–118.
Hayes,J.J. and Tullius,Th.D. (1989) *Biochemistry*, **28**, 9521–9527.
Heumann,H., Metzger,W. and Niehörster,M. (1986) *Eur. J. Biochem.*, **158**, 575–579.
Kirkegaard,K., Buc,H., Spassky,A. and Wang,J.C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2544–2548.
Lerman,L.S. and Frisch,H.L. (1982) *Biopolymers*, **21**, 995–997.
Maxam,A.M. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560–564.
Metzger,W., Schickor,P. and Heumann,H. (1989) *EMBO J.*, **8**, 2745–2754.
Schickor,P., Metzger.W., Werel,W., Lederer,H. and Heumann,H. (1990) *EMBO J.*, **9**, 2215–2220.
Schreck,R., Zorbs,H., Winnacker,E.L. and Bauerle,P.A. (1990) *Nucleic Acids Res.*, **18**, 6497–6502.
Shafer,G.E., Price,M.A. and Tullius,Th.D. (1989) *Electrophoresis*, **10**, 397–404.
Siegele,D.A., Hu,J.C., Walter,W.A. and Gross,C.A. (1989) *J. Mol. Biol.*, **206**, 591–603.
Tullius,T.D. and Dombroski,B.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5469–5473.
von Hippel,P.H., Bear,D.G., Winter,R.B. and Berg,O.G. (1982) In Rodriguez,R.L. and Chamberlin,M.J. (eds), *Promoters Structure and Function*. Praeger, New York, pp. 3–31.
Wu,H.M. and Crothers,D.M. (1984) *Nature*, **308**, 509–513.
Zillig,W., Zechel,K. and Halbwachs,H.J. (1970) *Hoppe Seyler's Z. Physiol. Chem.*, **351**, 221–224.

Received on April 10, 1991; revised on June 4, 1991