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Functional dissection of *Escherichia coli* promoters: information in the transcribed region is involved in late steps of the overall process

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After binding to a promoter *Escherichia coli* RNA polymerase is in contact with a region of about 70 bp. Around 20 bp of this sequence are transcribed. Information encoded within this transcribed region is involved in late steps of the functional program of a promoter. By changing such 'downstream' sequences promoter strength *in vivo* can be varied more than 10-fold. By contrast, information for early steps of the promoter program such as recognition by the enzyme and formation of a stable complex resides in a central core region of about 35 bp. Our data show that the strength of a promoter can be limited at different levels of the overall process. Consequently promoters of identical strength can exhibit different structures due to an alternate optimization of their program.

Key words: E.coli promoters/signal elements/functional programmes

Introduction

Prokaryotic promoters encode a complex program whose ultimate goal is the release of a transcriptional elongation complex sometimes predisposed to interact differentially with signals such as terminators (Grayhack *et al.*, 1985) located distal to the promoter. In a simplified model the program of an unregulated *Escherichia coli* promoter can be subdivided into four major steps: (i) recognition of the sequence by RNA polymerase; (ii) isomerization of the initial complex into a conformation capable of initiation; (iii) initiation of RNA synthesis; and (iv) transition into an elongation complex and promoter clearance.

In principle each of these steps can be rate limiting for the overall function of a promoter. Thus, promoters of identical strength may differ in their structure due to alternate functional optimizations (Bujard, 1980; Deuschle *et al.*, 1986). Here we report the identification of structural elements which are responsible for partial functions of the overall process. Modification of such elements has allowed us to alter the properties of promoter sequences in a predictable manner by shifting the rate-limiting event to a different step of the overall process.

One promoter analyzed here (P_{N25}) is a typical representative of promoters found in the 'early' expression class of coliphage T5 (Gentz and Bujard, 1985). These promoters belong to the most efficient transcriptional initiation signals identified so far (Deuschle *et al.*, 1986). Their sequences show homologies not only within the region commonly considered to be essential for promoter function (between +1 and −36, +1 being the first nucleotide transcribed), but also around position −43 and between +1 and +20 (Figure 1). A second promoter included in this study is P_{con}, a sequence synthesized by Dobrynin *et al.* (1980) according to a consensus sequence proposed by Scherer *et al.* (1978). *In vitro* both promoters are readily recognized by RNA polymerase and form stable complexes with the enzyme. However, whereas P_{N25} initiates efficient RNA synthesis *in vivo* and *in vitro*, P_{con} is a rather poor promoter in both environments (Deuschle *et al.*, 1986). By exchanging defined sequence elements both promoters can be converted into signals closely resembling each other in their *in vivo* and *in vitro* properties.

Results

Experimental strategy

Based on the analysis of conserved sequences (Gentz and Bujard, 1985) and on footprint experiments (U.Peschke, unpublished results) of promoters from coliphage T5 we define a promoter as a sequence extending from position +20 to −50 and subdivide it rather arbitrarily into a 'core', an 'upstream' (USR) and a 'downstream' (DSR) region. Typical features for a DSR of some T5 promoters such as P_{N25} are the conserved pentamer around +7 and a stretch of purines between +9 and +18. The prominent motive of a USR is a block of As centered around position −43 (Figure 1). By contrast the design of P_{con} (Figure 1) was based exclusively on homologies found within the core region. Its DSR and USR are fortuitously dependent on the site of integration, or in the case of the DSR on sequences designed to function as translational start signals.

To probe a possible role of DSRs in promoter function the core/USR of both P_{N25} and P_{con} were fused with various DSRs and the resulting sequences were studied *in vitro* and *in vivo*. Two DSRs were synthesized, the '*anti*'- and the '*pex*'-DSR. Based on a consensus sequence of six 'early' T5 promoters (Gentz and Bujard, 1985), the *anti*-sequence was constructed by inserting C for A, T for G and vice versa but avoiding runs of more than three Gs or Cs. The *pex*-DSR is identical with the corresponding sequence of P_{N25} except that the pentameric sequence around position +7 is exchanged (GGGTC replaces TTTGA). The two synthetic sequences and several naturally occurring DSRs were used to construct the promoters depicted in Figure 1.

Promoter constructs

The synthetic DSRs *anti* and *pex* are 23-bp and 24-bp long oligonucleotides respectively flanked by a *Hin*fI and a *Bam*HI site. Using the *Hin*fI cleavage site centered around position −3 of P_{N25} fusions $P_{N25/anti}$ and $P_{N25/pex}$ were obtained. A third construct in which the *lac* operator was joined to the P_{N25} core region resulting in $P_{N25/lac}$ has been described previously (Stueber *et al.*, 1984). For fusing the various DSRs to the core sequence of P_{con} the *Rsa*I site of this promoter located around position −3 was converted into a *Hin*fI site. The resulting promoter sequences such as $P_{con/pex}$ are homologous to their P_{N25} counterparts (e.g. $P_{N25/pex}$) up to position −5. In addition the *Rsa*I site of P_{con} was directly used for fusions with the DSRs of P_{trp} (Yanofsky *et al.*, 1981), and $P_{D/E20}$ (Gentz and Bujard, 1985) since these promoters carry conveniently located *Rsa*I sites

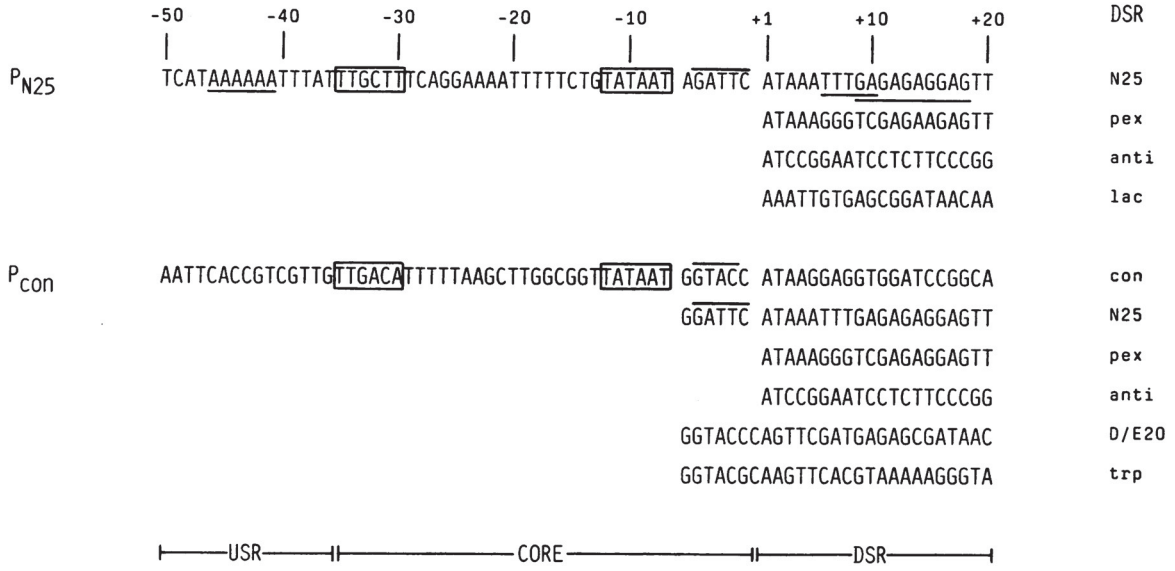

Fig. 1. Nucleotide sequences of the promoters studied. The sequence of coliphage T5 promoter P_{N25} comprising 70 bp from position +20 to −50 is shown in the upper part. The USR, core and DSR are delineated and highly conserved sequences are boxed. The conserved pentamer and the purine-rich sequence typical for a DSR of 'early' T5 promoters as well as the block of As around −43 are underlined. The starting nucleotide for RNA synthesis at +1 has been determined for P_{N25} (Stüber, 1980). The *Hin*fI cleavage site is overlined. The lower part shows the sequence of P_{con} (consensus sequence between +1 and −40; the *Rsa*I and *Hin*fI sites are overlined) and DSRs which were fused to the P_{con} core via the *Rsa*I site (*D/E20* and *trp*) or after changing the sequence of P_{con} via the *Hin*fI site (*N25, pex, anti*). In some of our constructs a spontaneous change from G to A at position +15 of P_{N25} has occurred. However, this base change did not affect the parameters examined in this study.

between their core and DSR's. The distance between the −10 region and the starting nucleotide (+1) of the latter two promoters ($P_{con/trp}$ and $P_{con/D/E20}$) is, however, increased by one nucleotide compared with the other constructs (Figure 1).

All the promoter sequences were flanked downstream by *Bam*HI and upstream by *Eco*RI or *Xho*I cleavage sites respectively. This allowed their oriented integration into the pDS1/$t_0$2 vector.

Recognition of promoters with altered DSR by E. coli RNA polymerase

Stable complexes between RNA polymerase and promoters are formed *in vitro* at distinct rates which can vary at least 50-fold (Brunner, 1986). Both promoters P_{N25} and P_{con} bind RNA polymerase with rates above $5 \times 10^7\ M^{-1}s^{-1}$ and form stable complexes with the enzyme (Brunner, 1986; Kammerer, 1986). To examine whether information within the DSR is contributing to the rate of complex formation, mixtures of fragments carrying the various promoter constructs were exposed to increasing but limiting amounts of RNA polymerase and the resulting complexes were monitored by adsorption onto nitrocellulose filters. As seen in Figure 2 replacement of the original DSRs by various sequences including the *anti*-DSR affects neither the rate of complex formation of RNA polymerase to P_{N25} nor to P_{con} derived sequences. These findings are supported by the experiment depicted in Figure 3. Here we have compared the interaction of RNA polymerase with the intact sequence of P_{con} and with a version of this promoter truncated at position −4. Again the enzyme binds with comparable rates to both sequences. We therefore conclude that the information required for promoter recognition and for the formation of stable complexes must reside upstream of position −4.

The effect of different downstream sequences on RNA synthesis in vitro

The efficiency of a promoter *in vitro* depends strongly on assay conditions. Therefore in experiments described here promoters were either directly compared with each other under competitive conditions, or their strength was determined in relation to an internal standard. In a first set of experiments stochiometric mixtures of fragments carrying the various promoters were used as templates to produce 'run off' transcripts of different sizes at RNA polymerase concentrations of about two enzyme molecules per promoter. When P_{N25} was compared with $P_{N25/anti}$ a most striking result was obtained. As seen in Figure 4 (lane 12) $P_{N25/anti}$ is a much less efficient promoter than P_{N25}. Replacing the *anti*-DSR by the *lac* operator results in $P_{N25/lac}$, a promoter of intermediate strength. By contrast, P_{con}, a poor promoter under competitive conditions, increases in strength if its core sequence is combined with the DSRs of P_{N25} or $P_{D/E20}$ (Figure 4, lanes 6−8). A minor increase in promoter strength is observed when the DSR of P_{trp} is fused to P_{con} ($P_{con/trp}$). The fusion of the *anti*-DSR to P_{con} ($P_{con/anti}$) diminishes even the low activity of this promoter.

Quantitative data for some of the promoter constructs were obtained by determining the *in vitro* RNA obtained from supercoiled templates following the procedure of Deuschle *et al.* (1986). The results (Table I) show that the replacement of the DSR of P_{N25} by the synthetic *anti*-DSR reduces the promoter strength 10-fold. Similarly, P_{con} loses efficiency upon transition to $P_{con/anti}$ (Figure 4). However, if $P_{con/anti}$ is compared with $P_{con/N25}$ a 4- to 5-fold increase in promoter efficiency is observed (Figure 4 and Table I). Thus, information relevant for promoter strength *in vitro* is encoded downstream of the transcriptional start site.

The influence of DSRs on the in vivo activity of promoters

Several promoter constructs (Table I) integrated in plasmid pDS1/$t_0$2 were transformed into *E. coli* C600 cells. RNA of logarithmically growing cultures (OD_{600} = 0.7) was pulse-labelled for 1 min with [^{3}H]uridine and quantified according to Deuschle *et al.* (1986). The results summarized in Table I show that also *in vivo* the replacement of the natural DSR by the *anti*-DSR reduces

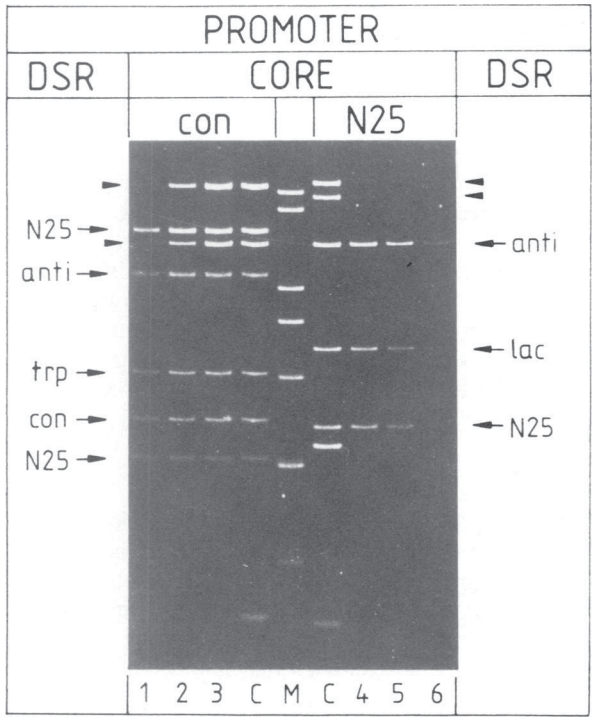

Fig. 2. The signal strength of P_{N25}, P_{con} and hybrid sequences. DNA mixtures containing stoichiometric amounts of promoter carrying fragments **(lanes C)** were exposed to increasing but limiting amounts of RNA polymerase under standard conditions. The enzyme/DNA complexes retained on nitrocellulose filters were analyzed by PAGE (6%, 8 V/cm, 4 h, stained with ethidium bromide). The left part of the gel shows the analysis of sequences composed of the P_{con} core region and DSRs as indicated. A corresponding experiment for P_{N25} sequences is shown at the right part of the gel. The actual RNAP/promoter ratios were 0.3, 1.0 and 3.0 in **lanes 1–3** and 1.0, 0.3 and 0.1 in **lanes 4–6** respectively. The position of fragments carrying the β-lactamase promoter are indicated (▶). In **lanes C** promoter-free fragments which are not bound by RNAP can be identified. M denotes a size marker (*Hin*fI digest of pBR322 with fragments between 153 and 517 bp in length).

the strength of P_{N25} by a factor of 10. The *lac* operator ($P_{N25/lac}$) though clearly better than $P_{N25/anti}$ still diminishes the efficiency of P_{N25} about 3-fold. P_{con}, a rather inefficient promoter *in vivo*, loses strength when fused to the *anti* sequence ($P_{con/anti}$). However, the combination $P_{con/N25}$ is a more than 10-fold better promoter than $P_{con/anti}$ and reaches the strength of P_{N25}.

After placing the *anti*-DSR distal to a promoter we have found no effect on transcription (data not shown) demonstrating that it does not cause termination of an elongating complex but instead acts in concert with other promoter functions.

To examine whether the conserved pentamer TTTGA within the DSRs of certain T5 promoters is an essential element the *pex*-DSR was fused to several core promoter sequences. As seen in Table I replacement of the natural pentameric sequence by GGGTC reduces the strength of both P_{N25} and $P_{con/N25}$ by less than 30%. Thus, this pentamer is not the sole contributor to the effect observed with the *N25* downstream sequence.

DSRs act in late stages of promoter function

The above data show that signal elements involved in promoter recognition and formation of a stable RNAP/promoter complex are located upstream of position −4. DSRs must therefore contribute to the later steps of the overall process. We have therefore examined the *in vitro* RNA synthesis initiated by promoters with identical core and USR but different DSR sequences and vice versa, with identical DSRs and differing core and USR sequences. Thus, a mixture of fragments containing P_{N25} and $P_{N25/anti}$ as well as P_{lacUV5} and P_{tacI} was transcribed *in vitro* and the run-

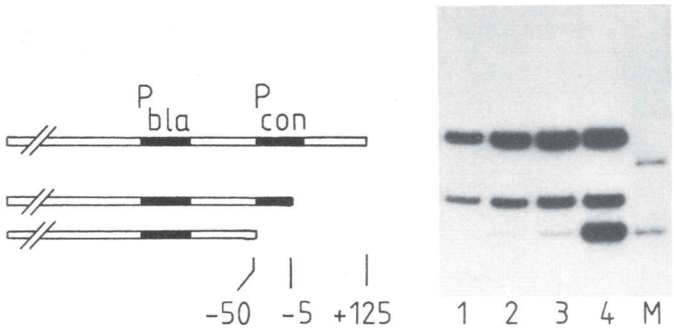

Fig. 3. Sequences essential for specific RNA polymerase binding. A ^{32}P end-labelled DNA fragment of 700 bp carrying both P_{bla} and P_{con} was cleaved at position −4 and −50 of P_{con} by *Rsa*I or *Eco*RI respectively (left part of the figure). Mixtures of these fragments **(lane 4)** were exposed to increasing amounts of RNAP. The enzyme DNA complexes were collected on nitrocellulose filters and analyzed by PAGE (6% polyacrylamide, 8 M urea, 1 mA/cm, 1.5 h) and autoradiography. The RNAP/promoter ratios were 0.25, 0.5 and 1.5, for **lanes 1–3** respectively. M contains size markers of 622 and 504 bp in length.

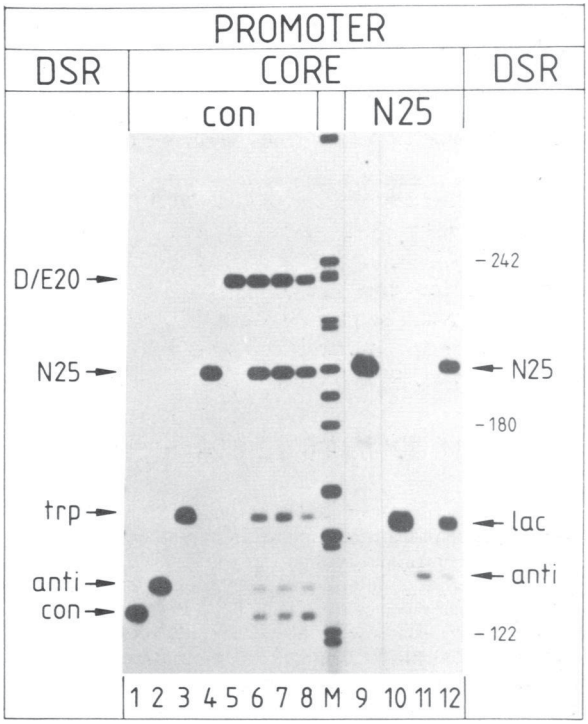

Fig. 4. The strength of hybrid promoters *in vitro*. P_{con} and P_{N25} as well as various core/DSR combinations were transcribed *in vitro* individually **(lanes 1–5** and **9–11)** and in assays containing stoichiometric amounts of fragments carrying the respective promoters **(lanes 6–8** and **12)**. The fragments were sized in such a way that run-off transcripts of different lengths are obtained. The [α-^{32}P]-UTP labelled RNAs were analyzed by PAGE (4% polyacrylamide, 8 M urea, 0.5 mA/cm, 2 h) and autoradiography. The left part of the figure shows transcripts from various $P_{con/DSR}$ constructs as indicated. **Lanes 6–8** contain mixtures of transcripts obtained with P_{con} derivatives at 200 **(lanes 6** and **7)** and 300 mM **(lane 8)** KCl respectively. The analysis of P_{N25} and two of its derivatives is shown in the right part of the figure. With the exception of the assay analyzed in **lane 8** all experiments were carried out in 200 mM KCl. M denotes a size marker (*Hpa*II digest of pBR322, the length in bp for some fragments is given on the right side).

Table I. Promoter strength *in vivo* and *in vitro* of P_{N25}, P_{con} and their derivatives

Promoter		Relative promoter strength	
Core	DSR	*In vitro*	*In vivo*
N25	*N25*	18	25
	anti	2	2.7
	pex	–	15
	lac	–	8
con	*con*	–	4
	anti	3	1.8
	N25	12	25
	pex	–	16
	D/E20	–	13
	trp	–	8

The relative promoter strengths were determined according to Deuschle *et al.* (1986). The leftmost column indicates the core and USR sequences as shown in Figure 1. The various sequence combinations are indicated by the 'DSR' column. Both *in vitro* and *in vivo* promoter strengths are related to the promoter of the β-lactamase (*bla*) and are given in 'P_{bla}-units' (Deuschle *et al.*, 1986). The maximal deviations are around ± 10%.

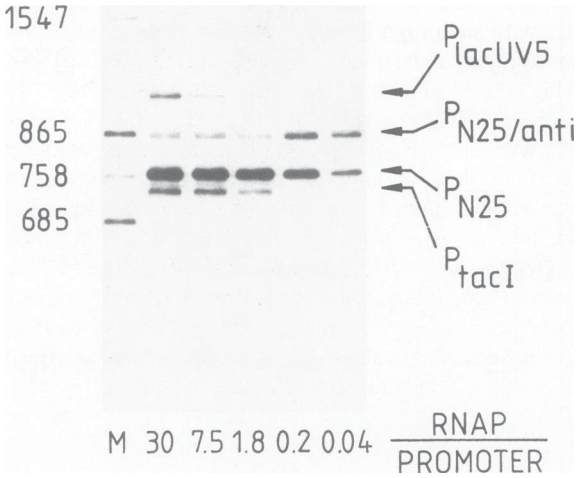

Fig. 5. Dependence of the relative promoter strength on the concentration of RNA polymerase. Fragments carrying various promoters were prepared to give run-off transcripts of different size. For transcription these fragments were mixed in molar ratios of 2:2:1:1 for P_{UV5}, $P_{N25/anti}$, P_{N25} and P_{tacI} respectively. These mixtures were transcribed in the presence of [α-^{32}P]-UTP and 200 mM KCl at the RNAP/promoter ratios indicated. Aliquots of the various assays were applied to the gel to give roughly constant amounts of $P_{N25/anti}$ transcripts after separation in PAGE (4% polyacrylamide, 8 M urea, 0.6 mA/cm, 2.5 h) and autoradiography. The positions of the various transcripts and the length of the size markers (M) are indicated.

off transcripts were analyzed. As seen in Figure 5 at very low enzyme concentrations (lane 5) P_{N25} and $P_{N25/anti}$ produce comparable amounts of RNA. Under these conditions the complex formation is limiting and since both promoters compete equally well for the enzyme (Figure 2) and also form stable complexes with the enzyme, differences at later steps of the process have little impact. At higher enzyme concentrations (lanes 1–4), however, the amount of RNA produced from $P_{N25/anti}$ is clearly limited by the function of the *anti*-DSR. Thus, the difference in promoter strength of these two promoters as seen at higher enzyme levels (lanes 1–4) is due to events controlled by the DSR. A complementary observation is made with P_{tacI} and P_{lacUV5}. Both promoters have identical DSRs (P_{tacI} is a hybrid sequence between P_{trp} and P_{lac}; Amann *et al.*, 1983); however, P_{tacI} binds RNA polymerase five times more efficiently than P_{lacUV5} (Kammerer, 1986). Since these two promoters should not differ in processes directed by their DSRs the difference seen in promoter strength should be comparable with the different rates of complex formation. This becomes obvious when the concentration of RNA polymerase is lowered (Figure 5, lanes 1–3): the amount of RNA synthesized from P_{lacUV5} decreases strongly followed by P_{tacI} specified transcripts. At very low enzyme concentrations both promoters are competed out by P_{N25} and $P_{N25/anti}$. At conditions of excess RNAP, P_{lacUV5} and P_{tacI} still differ somewhat in their *in vitro* strength — indicating that the rate of complex formation is not the only parameter which determines the functional difference of these two promoters. Thus, the relative strength of promoters *in vitro* also depends strongly upon the concentration of RNA polymerase.

Discussion

It has generally been accepted that the information essential for the function of unregulated *E. coli* promoters is stored within about 35 bp, spanning from position +1, the starting position for RNA synthesis, to about position −35. It is this region where the most striking sequence homologies among promoters are found and where the overwhelming number of promoter mutations were mapped. Several lines of evidence, however, suggest that sequence information flanking this region may be important for promoter function as well. (i) When bound to a promoter, *E. coli* RNA polymerase covers close to 70 bp (Schmitz and Galas, 1979; Siebenlist *et al.*, 1980). Promoter function might therefore not be independent of contacts between the enzyme and the sequences outside of the 35-bp region. (ii) The sigma subunit of the enzyme is only released after a sequence of 8–11 nucleotides has been transcribed (Hansen and McClure, 1980; Straney and Crothers, 1985). Information encoded in this region may participate in this process. (iii) Conserved sequences upstream and downstream of the 35-bp core region are found in some strong promoters (Bujard, 1980; Bujard *et al.*, 1983; Gentz and Bujard, 1985).

In this study we have defined a promoter as a 70-bp sequence containing a 'core' of 35 bp, a USR and a DSR of 15 and 20 bp respectively (Figure 1) and have examined the potential function of the region downstream of position +1.

DSRs can influence promoter strength

Based on a prototype DSR typical for the sequence between +1 and +20 of some phage T5 promoters such as P_{N25} (Gentz and Bujard, 1986) we have synthesized an *anti*-DSR. This sequence was fused to the core region of P_{N25} and P_{con} (Figure 1). In both cases the *in vitro* and the *in vivo* promoter strength was significantly reduced (Figure 4, Table I). By contrast, when the *anti*-DSR of $P_{con/anti}$ was replaced by the original DSR of P_{N25} yielding $P_{con/N25}$ the promoter activity *in vivo* was raised 14-fold (Table I). Similarly, when P_{con} was combined with the DSR of $P_{D/E20}$, another T5 promoter, the strength was again increased *in vivo* and *in vitro* (Figure 4, Table I). DSRs of other promoters like P_{lac} and P_{trp} caused intermediate effects: they decreased the strength of P_{N25} but increased the activity of P_{con} (Table I, Figure 4). Thus, by changing the sequence within the first 20 bp of the transcribed region the *in vivo* activity of promoters P_{con} and P_{N25} was varied up to 14-fold. We have attempted to identify a signal within the DSR of the T5 promoters and have therefore exchanged the conserved pentameric sequence TTTGA within the *N25*-DSR. The resulting promoter construct $P_{N25/pex}$ (Figure 1) showed a reduced activity (Table I) which, however,

demonstrates that this sequence is not the only information involved in the observed downstream effects. Nevertheless it is obvious that the sequence of the P_{N25} DSR must contain information which can increase decisively the strength of at least some promoters.

DSRs encode 'late' promoter functions

To examine whether DSRs are involved in the recognition process we have compared the rate of complex formation between RNA polymerase and various promoter constructs. In experiments as depicted in Figure 2 it was shown that information within the downstream region does not contribute to the recognition process. In fact, as demonstrated in Figures 2 and 3 the sequence elements essential for recognition must reside upstream of position −5. Similarly, no effect on promoter recognition was observed when sequences upstream of position −36 were removed (Kammerer, 1986).

From these results we conclude that the promoter region extending between +2 and −36, which we define as 'core' (Figure 1), contains the essential elements for the early steps of the interaction between a promoter and RNA polymerase; these include recognition of a sequence by the enzyme and isomerization of the initial complex into a state capable of starting RNA synthesis. DSRs must therefore contain signal functions required at a later stage of the overall process.

These conclusions are supported by experiments as depicted in Figure 5. At limiting RNA polymerase concentrations P_{N25} and $P_{N25/anti}$ which both compete equally well for the enzyme produce similar amounts of transcripts, i.e. P_{N25} equals $P_{N25/anti}$. At non-limiting enzyme concentrations, however, the overall efficiency of the two promoters is determined by the function of their DSRs, i.e. $P_{N25} > P_{N25/anti}$. The same experiment shows, furthermore, that the strength of P_{lacUV5} and P_{tacI} which both have identical DSRs correlates with the rate of complex formation between enzyme and promoter. Thus, the four promoter sequences differ in strength for different reasons. Their sequences are the result of diverse optimization processes and *in vitro* their functional hierarchy is influenced by the concentration of RNAP: at high enzyme to promoter ratios $P_{N25} > P_{tacI} > P_{lacUV5} > P_{N25/anti}$ whereas at low enzyme concentrations $P_{N25} \sim P_{N25/anti} > P_{tacI} > P_{lacUV5}$.

Conclusions and implications

Are these latter considerations relevant for the situation *in vivo*? Our data show that promoters which bind RNAP with high forward rate constants (e.g. P_{N25} or P_{con}) can lose efficiency when combined with DSRs which apparently slow down later steps of the overall process (Kammerer, 1986). This suggests that high forward rate constants and efficient DSRs may be prerequisites for an optimal promoter. On the other hand some of the strongest promoters identified so far (P_{A1} and $P_{D/E20}$ from phage T7 and T5 respectively; Deuschle *et al.*, 1986) bind RNAP five times less efficiently than P_{N25} (Kammerer, 1986), indicating that optimal function of a promoter *in vivo* does not necessarily depend on a highly effective recognition of the sequence. By contrast, for P_{tacI} and P_{lacUV5} the *in vivo* strength (5-fold difference; Deuschle *et al.*, 1986) correlates well with the rate of complex formation between RNAP and promoter found *in vitro* (Kammerer, 1986). This suggests strongly that it is indeed the recognition by the enzyme which causes the difference in strength between these two promoters.

How can these apparent contradictions be resolved? We propose that promoters with a rather low forward rate constant for RNAP binding ($< 5 \times 10^6\ M^{-1}s^{-1}$) are limited by processes such as recognition of the sequence and formation of a stable enzyme/ promoter complex. For such sequences RNA chain initiation and promoter clearance can be slow as long as the complex is stable enough to ensure the start of each enzyme bound. Mutations within the DSR of such promoters would in general not be detected. Above a certain rate of complex formation ($> 10^7\ M^{-1}s^{-1}$), however, later steps of the overall process such as initiation and promoter clearance can become rate limiting and consequently information contained within the DSR will be relevant. Both genetically well-studied promoter systems P_{trp} and P_{lac} are recognized by RNAP rather inefficiently (unpublished results). The discovery of promoter mutations within the DSR is therefore unlikely, although such mutations were reported for P_{lac} (Maquat *et al.*, 1980). However, by encoding the 5′ terminal 20 nucleotides of a mRNA, a DSR usually also contains information relevant for translation (Shine and Dalgarno, 1975) and for mRNA stability (Yamamoto and Immamoto, 1975). Rigorous proof of a promoter mutation in this region has therefore to be provided at the level of RNA synthesis.

As mentioned above some of the strongest promoters *in vivo* (P_{A1} and $P_{D/E20}$) are recognized five times less efficiently by RNAP than, for example, P_{H207} or P_{N25}. Why then did promoters with such high forward rate constants evolve? The promoter strengths determined *in vivo* were all obtained from fast growing cells in mid log phase (Deuschle *et al.*, 1986). The concentration of RNAP at this stage of growth may not favor promoters with optimal recognition properties, and promoters like some of coliphage T5 may be optimized for conditions where stringent competition for the enzyme is required. In this context it appears intriguing to us that promoters which are optimized according to different principles can form different functional hierarchies depending on the concentration of active polymerase. Thus, controlling for example the concentration of the σ-subunit at different growth conditions could profoundly change the pattern of mRNA abundance thereby affecting the physiological state of the cell.

Materials and methods

Plasmids and DNA sequences

The plasmids of the pDS system, their nomenclature and their preparation were described previously (Stüber and Bujard, 1982; Deuschle *et al.*, 1986). The cloning and characterization of the promoters P_{N25}, $P_{D/E20}$, P_{lacUV5}, P_{tacI} and P_{con} has been described in detail previously (Deuschle *et al.*, 1986). Promoter P_{trp} was isolated from plasmid ptrpH1 (Amann *et al.*, 1983). Oligonucleotides were synthesized using the triester method and purified by gel electrophoresis in 8 M urea. The sequences of all promoters and their derivatives were verified by dideoxy sequencing (Sanger *et al.*, 1977).

Preparation of in vitro RNA

The standard assay for 'run-off' transcripts contained in a volume of 50 μl was: 20 mM Tris/HCl pH 8; 10 mM $MgCl_2$; 5% glycerol, 1 mM DTT, 200 mM KCl; 300 μM each of ATP and GTP; 150 μM CTP; 50 μM UTP combined with 30−150 nM [α-^{32}P]UTP (3000 Ci/mmol).

After pre-incubation of 0.2 pmol template per promoter fragment in the reaction mixture at 37°C for 1 min transcription was started by the addition of 1 pmol RNAP. The concentration of UTP was raised to 1 mM after 2 min and the incubation was continued for another minute. Samples were prepared for electrophoresis (8 M urea, 4% polyacrylamide) by mixing 1/10 of the assay with 5 μl sample buffer (95% formamide, 1 × TBE containing bromophenol blue and xylenexyanol FF). Autoradiograms were quantitatively evaluated using a densitometer. Transcripts from supercoiled templates were obtained in an assay which was modified as follows: the volume and the amount of template were doubled and the ratio of RNAP:promoter was 50:1.

Quantitation of RNA

For determing promoter strengths *in vitro* or *in vivo* RNA was labelled, isolated and subjected to hybridization according to Deuschle *et al.* (1986). In this method the promoter under investigation transcribes the coding sequence of the dihydro-

folate reductase of the mouse (*dhfr*). The *dhfr*-specific RNA is compared with an internal standard, the β-lactamase (*bla*) specific RNA which is transcribed from the same plasmid but under the control of P_{bla}.

Analysis of promoter/RNAP complexes by adsorption to nitrocellulose

DNA fragment mixtures (0.25 pmol per fragment) in a volume of 200 μl containing 120 mM KCl; 20 mM Tris−HCl pH 8.0; 10 mM $MgCl_2$, 5% glycerol and 1 mM DTT as well as a small amount of a ^{32}P-labelled promoter containing fragment were incubated at 37°C for 2 min. One aliquot (50 μl) was removed and stored on ice as control, before the mixture was divided into 50-μl portions to which different dilutions of RNAP in 50 μl of assay buffer and pre-warmed to 37°C were added. After 5 min at 37°C competitor DNA was added (0.5−2 μg of single-stranded fd DNA per assay in binding buffer without KCl) and incubation was continued for another 5 min before the mixture was filtered through nitrocellulose (4.5 μm pore size, Schleicher and Schüll) pre-equilibrated with binding buffer without KCl. The filters were rinsed twice with 200 μl of binding buffer containing 60 mM KCl and the adsorbed fragments were eluted with three 50-μl portions of 10 mM Tris−HCl pH 8.0; 1 mM EDTA; 0.1% SDS. Complete removal of DNA from the filter was examined by monitoring the radioactivity of the labelled fragment. The DNA was precipitated by ethanol and the redissolved pellet was analyzed by PAGE and ethidium bromide staining, or by autoradiography of the dried gels with Kodak X-ray film.

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