

Promoter search and strength of a promoter: two important means for regulation of gene expression in *Escherichia coli**

RAKESH K MISHRA and DIPANKAR CHATTERJI

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

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Abstract. Search for a promoter element by RNA polymerase from the extremely large DNA base sequence is thought to be the slowest and rate-determining for the regulation of transcription process. Few direct experiments we described here which have tried to follow the mechanistic implications of this promoter search. However, once the promoter is located, transcription complex, constituting mainly the RNA polymerase molecule and few transcription factors has to unidirectionally clear the promoter and elongate the RNA chain through a series of steps which altogether define the initiation of transcription process. Thus, it appears that the promoter sequence acts as a trap for RNA polymerase associated with a large binding constant, although to clear the promoter and to elongate the transcript such energy barrier has to be overcome. Topological state of the DNA, particularly in the neighbourhood of the promoter plays an important role in the energetics of the whole process.

Keywords. *Escherichia coli* promoters; promoter strength; abortive cycling; sigma cycle.

1. Introduction

In prokaryotes, the DNA-dependent RNA polymerase is the enzyme responsible for transcription. The enzyme from *Escherichia coli* is a complex multimeric protein with subunit composition α_2 , β , β' , σ (figure 1). We have recently discussed in detail the subunit function and molecular anatomy of the transcription complex (Chatterji and Kumar 1992). The *E. coli* genome consists of different classes of promoters recognized by the holoenzyme containing different σ -factors. Promoters recognized by σ -70 (vegetative sigma) holoenzyme comprise the major class and the present paper pertains mainly to this class. When it binds to DNA the holoenzyme covers about 70 bp (– 50 to + 20) of DNA and the DNA sequence within this region and perhaps in the immediate neighbourhood provide identity to a promoter in terms of its efficiency of productive transcription.

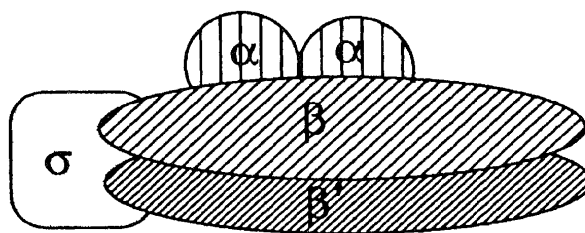


Figure 1. *E. coli* RNA polymerase subunits.

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2. Promoters

Detailed studies on more than 100 *E. coli* promoter have led to an understanding of the role of primary DNA sequence and the modular nature of such promoters (figure 2) (Hawley and McClure 1983; Travers 1987; Gralla 1991). In addition to the conserved hexamer boxes (– 10 and –35 boxes) and the distance between them (17 ± 2 bp), the overall flexibility of the DNA double helix in promoter region seems to be a conserved feature (Nussinov 1984; Margalit *et al* 1988; Werel *et al* 1991). Variation in promoter strength has been observed due to variation in the DNA sequence in the promoter region. Although, very often increase in homology to the conserved sequence leads to increase in promoter strength, there are exceptions to

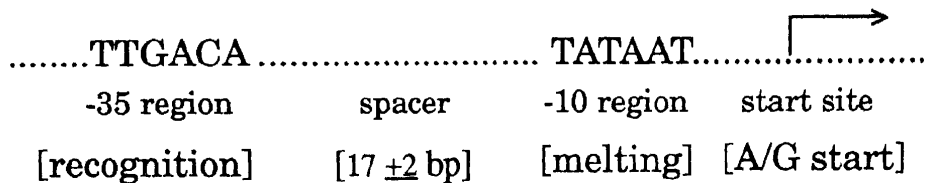


Figure 2. Modular nature of *E. coli* promoter.

this trend (Hansen and McClure 1980; McClure 1985; Leirimo and Record 1990). Sometimes a change in the primary sequence outside the conserved region also influences promoter strength. It is therefore not clear as to what in a DNA sequence in the promoter region determines the promoter strength. However, it appears from these studies that it is the overall DNA sequence in the context of a particular promoter that influences the exact characteristics of the promoter. Further studies directed at primary sequences and the secondary and tertiary structures along with promoter properties should reveal the rules of structure-strength relations for promoters. Our studies on the mechanism of initiation of transcription indicate that whatever the rules may be, the effect is brought about by influencing the various steps in the process of initiation of transcription differentially (Mishra and Chatterji 1991). We discuss this aspect in detail in subsequent sections.

Most *E. coli* promoters direct transcription to start with a purine residue; the significance of this observation is not yet known. However, there are a few promoters for which the 5' terminus of the transcript is a pyrimidine. We have recently discovered that RNA polymerase has different domains for promoter recognition with purine *vs* pyrimidine starts and they behave differently towards rifampicin (Kumar and Chatterji 1992; Reddy P S and Chatterji D, unpublished). The two observations are likely to be interrelated, *i.e.* the latter may be a consequence of the former. Upstream of the start site, around position –10, is a hexanucleotide (TATAAT) sequence that is referred to as a melting module, because during the initiation process, the melting of the DNA double helix starts from this box. Another conserved hexanucleotide stretch, called the – 35 box (TTGACA), is found 17 ± 2 bp upstream of the – 10 box. The –35 box is called the 'recognition module'. The 17 bp distance between the two conserved hexanucleotides is also functionally important. The spacer perhaps plays a role in the phasing of the two conserved boxes and contributes to the RNA polymerase-promoter interaction. Upstream of

the -35 box, many promoters have a polyA track which has been implicated to provide a bent conformation of the DNA and help in promoter search, recognition and binding (Travers 1987). In addition to these modules there may be other *cis* acting regions which by themselves or with the help of proteins might regulate transcription.

The effect of DNA topology on promoter activity has been studied extensively. There are three kinds of promoters which show different responses with respect to the supercoiling of the DNA: (i) those that show increase of activity upon increase in negative supercoiling, *e.g.* *topA*, *proU*, *nifA*, T7A2 (Dimri and Das 1988; Mishra *et al* 1990; Palecek 1991); (ii) those that show decrease of promoter activity upon increase in negative supercoiling, *e.g.* *gyrA*, *gyrB* (Palecek 1991) and (iii) those that show no change in activity upon change in supercoiling status *e.g.* T7A1 (Mishra *et al* 1990), *lac UV5* (Borowiec and Gralla 1987). Supercoiling influences the energetics of the template DNA and is governed by the DNA structure or the primary sequence. By altering the sequence between the conserved hexamer boxes we noticed that strength of a promoter is not related to GC content. Rather, the establishment of sequence specific contacts between the template and enzyme to less or more favourable extents by conformational modulations, supercoiling of DNA may be responsible for variable influence on promoter activity (Jyothirmal and Mishra 1992). We discuss the contribution of supercoiling energy to the mechanism of initiation of transcription in a subsequent section.

3. Initiation of transcription

Initiation of transcription in *E. coli* involves search for promoter sites by the RNA polymerase, recognition and binding of the enzyme to the promoter and transition of the initiating complex to the elongation mode (McClure 1985; Straney and Crothers 1987; Leirimo and Record 1990). In the following sections these steps and their possible roles in the regulation of gene expression are presented.

3.1 Promoter search

It is now clear that an important factor in determining the transcriptional activity of any given gene is the rate at which RNA polymerase approaches specific sites (promoters) on the DNA template. However, the first step in gene transcription is the formation of a binary complex between DNA and RNA polymerase. This complex subsequently can bind substrates to initiate RNA synthesis. Two types of binary complexes can be formed: (i) specific complexes formed with promoter sites and (ii) non-specific complexes with all other regions of DNA. The search for and recognition of promoter sequences and discrimination between promoters and other DNA sequences is one of the most fascinating aspect of RNA polymerase function and is probably the primary basis for specific gene expression. Furthermore, because non-specific sites are present in much higher numbers than are specific sites on the DNA, it is generally believed that non-specific site binding must also play a significant role in promoter binding (Park *et al* 1982a).

There are only a limited number of specific binding sites on DNA for RNA polymerase. Bacteriophage T7 DNA has only eight such sites (three major and five

minor promoters), while the number of non-specific sites is much higher (McGhee and von Hippel 1974). It is now known that RNA chain initiation at a specific promoter site on T7 DNA takes only 1-2 min (Shimamoto *et al* 1981). However, this time range is much shorter than that allowed by three-dimensional diffusion involving a protein as large as RNA polymerase and a few promoter sequences (Wu and Tweedy 1982). As the bimolecular association rate constant between bacteriophage T7 or T3 promoters and RNA polymerase is more than $10^{10} \text{ M}^{-1}\text{s}^{-1}$ (von Hippel *et al* 1984), a value apparently higher than the diffusion-controlled limit for molecules of their size (Wu 1990), it is of interest to find out the mechanism through which such high rates can be achieved. One should keep in mind that no simple bimolecular association can occur faster than diffusion.

In an attempt to explain the above observations, certain important postulations were made (von Hippel *et al* 1984). It was thought that DNA in solution is not homogeneous in nature and thus may have local structures which are different from each other. These structural domains act as traps for RNA polymerase with varying affinity and varying dissociation rate. The protein will eventually locate the promoter site *via* a series of interdomain transfer events and thereupon bind tightly to it. Various mechanisms have been proposed for this interdomain transfer. These include linear diffusion of protein along the DNA (sliding), on-off mechanism (hopping) or direct transfer between segments. It appears that any of these mechanism can facilitate promoter search without violating the diffusion-controlled limit because of a reduction in dimensionality or a diminution in the volume to be searched (Wu 1990).

Thus far, two direct approaches have been used to follow the movement of the RNA polymerase molecule along a DNA template starting from promoter search to elongation of RNA chains. Although a simple measurement of the lag period during incorporation of radioactive UTP into acid-insoluble RNA had been taken to represent the time for promoter search (Nierman and Chamberlin 1979), more direct methods to determine this time constant were warranted. We describe below two such methods.

4. Rapid mixing/photocross-linking technique

It is of importance to demonstrate kinetically how a protein molecule such as RNA polymerase can transfer itself among a series of non-specific binding sites on a DNA molecule during the process of searching for its promoter target. In order to provide such experimental evidence for linear diffusion, Wu and his colleagues developed an interesting technique described below (Park *et al* 1982a,b; Wu *et al* 1983; Singer and Wu 1987, 1988).

In this technique, RNA polymerase and DNA are rapidly mixed in a quartz tube of a stopped-flow apparatus. At specific time points following the mixing, a high-intensity xenon lamp is used to generate a very brief ($10 \mu\text{s}$) high intensity flash of UV light, which cross links the proteins to the DNA (figure 3). Due to the short lifetime of the excited state, this results in the covalent attachment of RNA polymerase to locations along the DNA strand according to the distribution of the protein at the time of the flash. Following the cleavage of the DNA into resolvable fragments by an appropriate restriction enzyme, covalent DNA-protein complexes

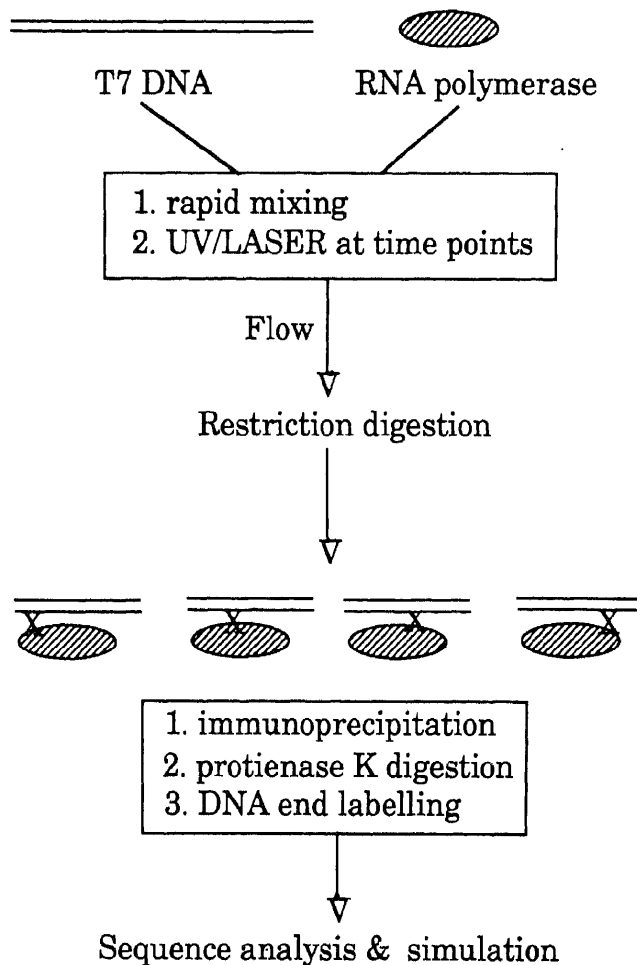


Figure 3. Rapid mixing and photo-crosslinking.

are isolated using anti-RNA polymerase antibodies. The covalently attached protein is then removed using proteinase K. At this point, the collection of DNA fragments in each sample is representative of the distribution of RNA polymerase at the time the sample was flashed. The DNA fragments can be end-labelled and used for quantitative analysis of the results.

5. Promoter search on linear template

Following the above method in an experimental system consisting of an equimolar mixture of *E. coli* RNA polymerase and ΔD_{III} T7 DNA (with one promoter, T7A1) some very interesting conclusions were drawn. In fact the kinetics of the distribution variation of RNA polymerase over the DNA template were first plotted with experimental data points which were later fitted to simulated models. It was observed that (i) initial binding is rapid and takes place mostly at non-specific sites

on T7 DNA, (ii) the rate of loss of polymerase from any site is proportional to the distance from the promoter, (iii) the movement of polymerase towards the promoter is bidirectional in nature and (iv) promoters are the final destination of the polymerase.

However, one of the major difficulties that arose in developing a one-dimensional model for promoter search over a linear DNA was that the ends of the DNA were also found to have strong affinity for RNA polymerase. This ambiguity restricted the quantitative interpretation of the diffusion mechanism. To eliminate this problem Singer and Wu (1987, 1988) have recently used a circular DNA as template and they have observed that the rate of loss of polymerase from a non-specific site is only dependent on its distance from the promoter, They have developed a kinetic model and the effects of salt, temperature, etc., have been studied based on this model. Moreover, the approach described above is suitable to understand the effect of "road-block" on the movement of RNA polymerase over a DNA template.

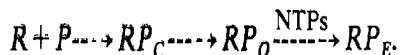
6. Promoter recognition and binding

The σ -factor of RNA polymerase is called the eye of the enzyme because it is the factor that actually provides specificity to the interaction between DNA and RNA polymerase, σ -factor directly interacts with promoter sequences by stabilizing specific contacts with both of the conserved hexamers through helix-turn-helix motifs (Gardella *et al* 1989; Siegel *et al* 1989). Only the σ -bound holoenzyme can initiate transcription from promoter sites whereas the core enzyme can initiate transcription (without the sigma) or carry out elongation from nicks or non-specific sites. This highlights the importance of the σ -factor in the process of initiation of transcription. It is however not understood if σ -mediated interaction of RNA polymerase with the promoter also determines promoter strength. This is an interesting possibility, since mutations in the conserved hexamers, where σ -factor has direct sequence specific contacts, alter promoter efficiency most dramatically (Gardella *et al* 1989; Siegel *et al* 1989). Studies on the structure of the σ -factor and its interaction with promoter DNA sequences should be significant in this regard. Although core enzyme does not bind DNA in a sequence specific manner, it does have a single strand specific binding site, which favours the melting of the DNA double helix from the middle of the -10 box to about position + 8. Such a process might also help to bring the appropriate strand and nucleotide near the initiation and catalytic sites of the enzyme.

7. Intermediates in the initiation of transcription

Initiation of transcription is a multi-step process involving various intermediates (McClure 1985; Straney and Crothers 1987; Leirimo and Record 1990). In a simplistic model, RNA polymerase (R) binds to promoter DNA (P) to form a 'closed complex' (RP_C). Subsequently, the RP_C isomerizes to an 'open complex' (RP_O) where one and a half turns of the DNA double helix melt near the melting module which include the start site. Upon addition of the appropriate NTPs this open complex initiates synthesis of an RNA chain, releases σ -factor, and goes to the

elongation mode (RP_E) (Krummel and Chamberlin 1989).



It becomes evident that the interaction of RNA polymerase with promoter DNA is a rather dynamic process and involves promoter binding and promoter clearance, two antagonistic steps. This is where the complexity of the process lies. The recognition is sigma mediated and promoter clearance is accompanied by release of sigma, leaving the RNA polymerase in the elongation mode. This process is also associated with stabilization of the nascent RNA chain leading to the formation of a ternary complex. In the initiation mode such a transcript may get released giving abortive transcripts. The transition from initiation to elongation mode is a crucial step for productive transcription and this is probably the step, as described below, that determines the strength of the promoter. Transition from initiation to elongation mode is accompanied by: (i) ternary-complex formation, (ii) rifampicin resistance, (iii) release of σ -factor, (iv) loss of promoter specific contacts, (v) salt resistance, (vi) stopping of abortive cycling, (vii) reduction in the stretch of protected DNA and (viii) other major configurational alterations in the constituents. The process starts after the formation of RP_o and is accompanied by nascent RNA synthesis. We have focussed our attention on understanding the trigger for the transition from initiation to elongation mode of the transcription complex which eventually leads to the establishment of the order of events during the initiation of transcription (Mishra and Chatterji 1993). With the help of these findings and using existing information we present here a model for initiation of transcription, (figure.4) which includes the role of abortive synthesis in the process and the mechanistic determinants of promoter strength (see later). We carried out our studies on a supercoiled template since it represents a topological state close to the physiological one. Our strategy was to arrest the transcription initiation process at various steps by limiting the number of NTPs in the reaction mixture. We have isolated and analysed the molecular details of such intermediates which help to generalize the findings in terms of the mechanism of initiation of transcription (Mishra and Chatterji 1993).

8. Abortive cycling and promoter strength

Our findings can be fitted into a model for initiation of transcription shown in figure 4. After the formation of RP_o , NTPs are taken up and small transcripts are made on an initiating binary complex (RP_{IBC}). These transcripts have two fates: (i) to get released in the form of abortive products reverting the RP_{IBC} to RP_o ; or (ii) to be lengthened by further uptake of NTPs forming initiating ternary complex RP_{ITC} . The RP_{ITC} can at a very low rate abort, the transcript (behave like RP_{IBC}) and go back to RP_o . We call this ternary complex the initiating ternary complex, RP_{ITC} , for this reason. This complex is also susceptible to rifampicin. RP_{ITC} contains σ -factor. Upon subsequent uptake of NTP and increase in RNA length, major configurational changes take place in the complex leading to the release of σ -factor and formation of elongating ternary complex RP_{ETC} .

Our results (Mishra and Chatterji 1993) suggest that the trigger for the transition

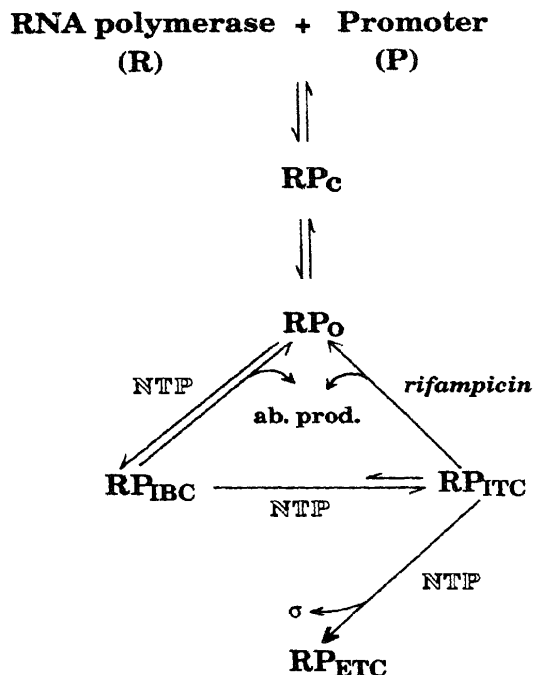


Figure 4. Initiation of transcription.

from initiation to elongation mode is the length of the growing RNA chain. We have also observed that it is not the sequence of the RNA chain that stabilizes the ternary complex, rather, the overall context in which the RNA chain is being synthesized is important. In other-words, the length of the RNA chain at which the ternary complex will be stable is a promoter specific property. We found that, on a supercoiled template, formation of the ternary complex takes place at a very short transcript length (as short as 2-3 nucleotides) where the promoters are known to be strong, whereas weaker promoters may require the synthesis of transcripts more than eight nucleotides long to stabilize the ternary complex. We can thus correlate the length of the abortive transcript to the strength of the promoter. Strong promoters lead to such an interaction with RNA polymerase that the RNA is stabilized on the initiating complex at a very early stage and, therefore, there is less time and scope for the nascent RNA to be released as abortive product. On the other hand, a weak promoter would not be able to undergo promoter clearance followed by ternary complex formation until a long transcript is synthesized. During this process the nascent RNA could be aborted and thereby net productive transcription might be reduced. A requirement for longer transcripts to convert the RP_{IBC} into RP_{ETC} indicates greater energy requirement for promoter clearance and thus greater chance for abortive release from a strained complex (Straney and Crothers 1987). These aspects are illustrated in a energy diagram (figure 5). The figure shows that increase of RNA length from an RP_o causes accumulation of energy within the complex (Gill *et al* 1990). This energy can be released by forming

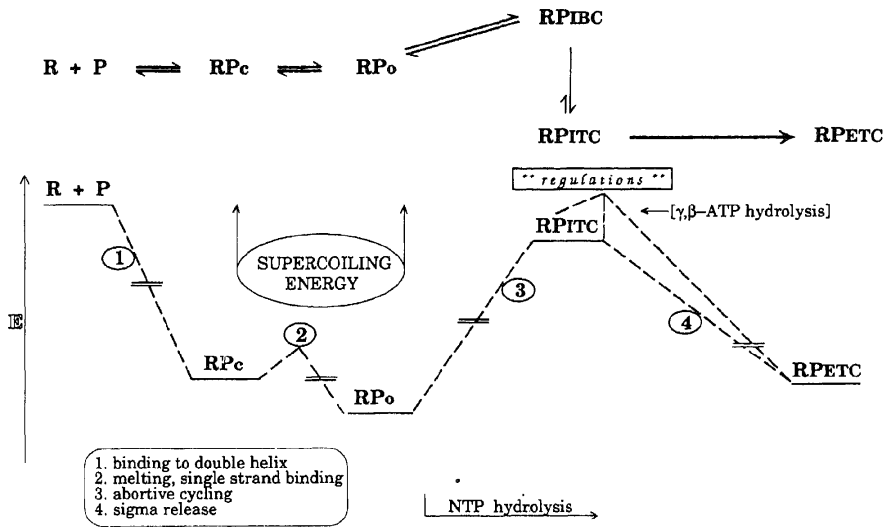


Figure 5. A relative energy profile representation of transcription.

RP_{ETC} . The energy barrier between RP_o/RP_{IBC} and RP_{ETC} peaks around RP_{ITC} , whereupon addition of a few more NTPs or hydrolysis of ATP at a site away from the RNA synthesis start site cause promoter clearance concomitant with the release of σ -factor. Therefore it becomes obvious that stronger promoters will have a smaller energy hill to climb to attain RP_{ETC} . Also, perhaps, it is the gap between RP_o/RP_{IBC} and RP_{ETC} that might provide a regulatory handle since any factor lowering the energy peak of RP_{ITC} would facilitate the transcription, and *vice versa*. The negatively supercoiled form is a higher energy state of the double helical DNA as compared to the relaxed or linear forms. This topological distinction and energy store makes supercoiled DNA a better template for transcription. Also, a change in the topological state of the template may, therefore, regulate expression of genes. By providing a suitable looping in the form of writhe it may facilitate 'step 1' of figure 5, *i. e.* formation of RP_c . Further, the link deficient nature of supercoiled DNA may facilitate melting of the double helix and lead to the isomerization of RP_c to RP_o , 'step 2' of figure 5. Finally, the higher energy content of template may raise the energy levels of RP_c and RP_o , thus bringing down the energy barrier in 'step 3' of figure 5. This would facilitate the formation of RP_{ITC} and block abortive turnover, resulting in higher yield of RP_{ETC} .

The proposed model for the mechanism of initiation of transcription in *E. coli* appears to hold in principle for other prokaryotes (Whipple and Sonenshein 1992) and also for eukaryotes. A recent report shows that mutations in eukaryotic promoters that lead to decrease in promoter strength increase abortive product compared to the wild type promoter *in vitro* (Jacob *et al* 1991). Transition from initiation to elongation mode can be influenced by factors affecting the kinetics or thermodynamics or both of the process by altering binary-complex formation, rate of isomerization (open to closed complex), rate of phosphodiester bond formation

(at each step) and rate of release of transcript (at each step). If the rate of phosphodiester bond formation is decreased or the rate of release of nascent RNA is increased by any means (e.g. mutations in the promoter), the resulting promoter will be a weaker promoter. When a series of phosphodiester bonds are being formed, a particular one would lead to the formation of the elongation complex. The stage at which this would occur is, in our opinion, a promoter-specific property. Higher rate of release of abortive product will lead to reduced productive transcript. So increased release of abortive product on a promoter will make it weaker and therefore provide a kinetic control. A different promoter may be weaker because it aborts transcripts of greater lengths, thus delaying promoter clearance; this exemplifies thermodynamic control.

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