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Rationally designed insulator-like elements can block enhancer action *in vitro*

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Insulators are DNA sequences that are likely to be involved in formation of chromatin domains, functional units of gene expression in eukaryotes. Insulators can form domain boundaries and block inappropriate action of regulatory elements (such as transcriptional enhancers) in eukaryotic nuclei. Using an *in vitro* system supporting enhancer action over a large distance, the enhancer-blocking insulator activity has been recapitulated in a highly purified system. The insulator-like element was constructed using a sequence-specific DNA-binding protein making stable DNA loops (*lac* repressor). The insulation was entirely dependent on formation of a DNA loop that topologically isolates the enhancer from the promoter. This rationally designed, inducible insulator-like element recapitulates many key properties of eukaryotic insulators observed *in vivo*. The data suggest novel mechanisms of enhancer and insulator action.

Keywords: RNA polymerase/enhancers/initiation/insulators/transcription

Introduction

Insulators are eukaryotic DNA sequences that are defined based on two functional activities (for recent reviews see Gerasimova and Corces, 2001; West *et al*., 2002). The first is position-dependent enhancer-blocking activity: insulators block enhancer action only when placed between an enhancer and promoter but not upstream or downstream of an enhancer–promoter pair (Chung *et al*., 1993; Geyer and Corces, 1992; Kellum and Schedl, 1992). The second insulator activity is an ability to form chromatin boundaries (Ishii *et al*., 2002; Mutskov *et al*., 2002) and therefore confer position-independent transcription to transgenes stably integrated in the genome (Bonifer *et al*., 1990; Kellum and Schedl, 1991). Insulator activity has not been described in prokaryotes.

The mechanism of insulator action is unknown; two models were proposed to explain the enhancer-blocking activity of insulators. The 'promoter decoy' model suggests that insulators neutralize enhancer action by interacting with it and capturing a functionally active component of the enhancer (Geyer, 1997). The second, 'chromatin boundary' model suggests that insulators organize higher order chromatin structure, perhaps by formation of large chromatin loops (Schedl and Grosveld, 1995). An enhancer positioned within one chromatin loop cannot communicate with a promoter positioned within another chromatin domain. While a large body of data was obtained in support of both models (Geyer and Clark, 2002), the mechanism of insulator action remains hypothetical, in part because of the lack of an *in vitro* system supporting insulator action.

The mechanism of action of eukaryotic enhancers over a large distance is not well understood (see West *et al*., 2002 for review); this makes development of an *in vitro* enhancer-blocking assay extremely difficult. In contrast, enhancer-dependent promoters of *Escherichia coli* are well studied and highly active *in vitro*. Moreover, numerous data suggests that NtrC-dependent enhancer–promoter system has many properties characteristic of eukaryotic enhancer-dependent transcription, such as activation over a large distance, both upstream and downstream of the regulated promoters in an orientation-independent way (see Buck *et al*., 2000 for a review).

The *glnAp2* promoter activated by an NtrC-dependent enhancer is one of the best-studied promoters in *E.coli*. In this system, NtrC activator protein binds to the enhancer and, when phosphorylated by NtrB protein kinase, forms homo-oligomers and activates transcription of the *glnAp2* promoter (Porter *et al*., 1993; Wedel and Kustu, 1995; Wyman *et al*., 1997). Active enhancer-bound NtrC interacts with the RNA polymerase bound as a closed initiation complex at the promoter and stimulates transition to the open complex (Popham *et al*., 1989; Sasse-Dwight and Gralla, 1988). During the enhancer–promoter interaction intervening DNA is transiently looped out (Rippe *et al*., 1997; Su *et al*., 1990). More recently, it has been shown that DNA supercoiling dramatically facilitates enhancer–promoter communication over a large distance (Bondarenko *et al*., 2002; Liu *et al*., 2001).

In this work, rational design and construction of an insulator-like element having an enhancer-blocking activity have been attempted based on the detailed knowledge of the mechanism of enhancer action on the *glnAp2* promoter. Topologically isolated domains (DNA loops) were formed using *lac* repressor tetramer that can simultaneously bind two *lac* operator (*lacO*) sites on DNA. Placing the enhancer and promoter within different topologically isolated domains on the same supercoiled plasmid DNA molecule greatly inhibits enhancer–promoter communication. Moreover, a pair of *lac* operators recapitulates many key properties of eukaryotic insulators observed *in vivo*.

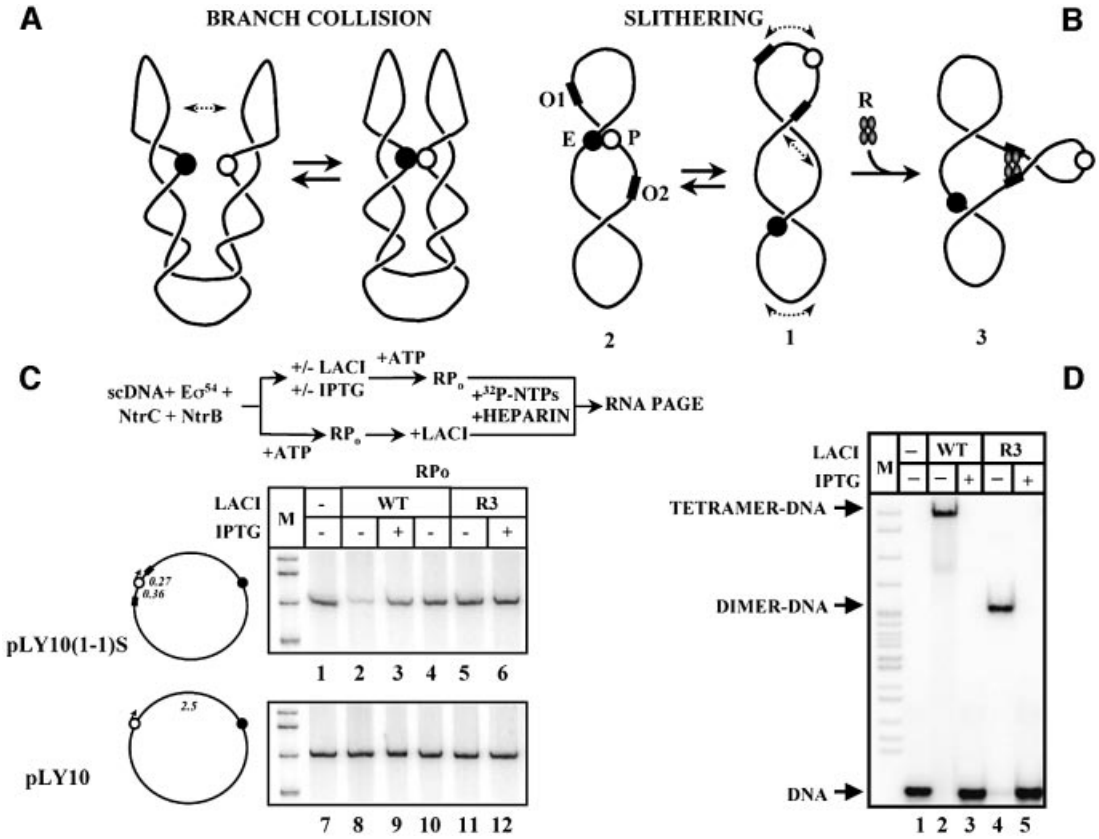

Fig. 1. The enhancer cannot *cis*-activate the *glnAp2* promoter localized within a topologically isolated DNA loop. (**A**) The branch collision mechanism. This mechanism was proposed to explain the effect of DNA supercoiling on the rate of communication between DNA regions on supercoiled DNA. Frequent collisions between branches formed on supercoiled DNA could facilitate communication between the enhancer and promoter localized on different branches of the same DNA molecule. The enhancer and promoter are indicated by black and white circles, respectively. (**B**) LacI-induced DNA loop formation topologically isolates the enhancer from the promoter. When spaced by 2.5 kb, the enhancer (E) and promoter (P) probably communicate by slithering on supercoiled DNA (structures 1 and 2). The slithering model suggests that intertwined DNA helixes can slide relative to each other on supercoiled DNA at a high rate; sliding greatly increases the probability of enhancer–promoter collision. LacI tetramer (R) can simultaneously bind two *lac* operators positioned upstream and downstream of the promoter (O1 and O2), form a DNA loop (structure 3), and thus could topologically isolate the enhancer from the promoter. (**C**) LacI-induced DNA loop formation greatly inhibits enhancer-dependent transcription. The experimental strategy is outlined at the top. Plasmid containing two *lac* operators localized 0.36 kb upstream and 0.27 kb downstream of the promoter [pLY10(1-1)S, upper panel] and control plasmid not containing *lac* operators (pLY10, lower panel) were transcribed in the presence or in the absence of wt LacI or the R3 mutant. R3 mutant binds DNA well but cannot form DNA loops. In some cases, LacI was added after formation of the open complexes (lanes 4 and 10). The distances between key regulatory elements are shown in italics. RP_o, open initiation complex. Other designations are as in (B). M, labeled pBR322-MspI markers. Note that, although LacI can present a weak road-block to transcript elongation (Oehler *et al*., 1990), the block was not detected on the templates used in this work. (**D**) LacI and the R3 mutant LacI are quantitatively bound to their binding sites. Analysis by native PAGE. Aliquots of transcription reactions described in (C), lanes 2, 3, 5 and 6, containing LacI or R3, respectively, but not containing plasmid DNA were incubated in the presence of labeled double-stranded oligonucleotide containing an 'ideal' *lac* operator. Mobilities of corresponding complexes in the gel are indicated.

Results

Designing an insulator-like element

Design of a regulatory element having enhancer-blocking activity was based on the knowledge of the mechanism of action of the NtrC-dependent enhancer over a large distance. DNA supercoiling greatly (~50-fold) facilitates enhancer–promoter communication; as a result, the enhancer works over short (0.11 kb) and large (2.5 kb) distances with similar efficiencies (Liu *et al*., 2001). Two models were proposed to explain the role of DNA supercoiling in facilitating communication between two DNA regions: 'slithering' (sliding of intertwined DNA double helixes) and 'branch collision' (Figure 1A and B; Chirico and Langowski, 1996; Vologodskii and Cozzarelli, 1996). Based on computer simulations of Brownian dynamics of supercoiled DNA it has been proposed that slithering is the predominant mechanism facilitating enhancer–promoter communication over distances up to 10 kb (Huang *et al*., 2001). Since plasmids used in this work have 2.5 or 3.3 kb enhancer–promoter spacing, an insulator-like element was designed based on the slithering model of enhancer action. This model suggests that placing the enhancer and promoter on topologically isolated DNA loops within the same supercoiled plasmid would prevent enhancer–promoter communication because sliding of DNA double helixes will only be possible within each domain, but not between the domains (Figures 1B and 6B).

To form stable DNA loops, a well-studied DNA looping protein (*lac* repressor, LacI) was used. LacI is a homotetramer that has two DNA-binding surfaces (Barkley *et al*., 1975; O'Gorman *et al*., 1980; Whitson *et al*., 1987) and is capable of binding two *lac* operators

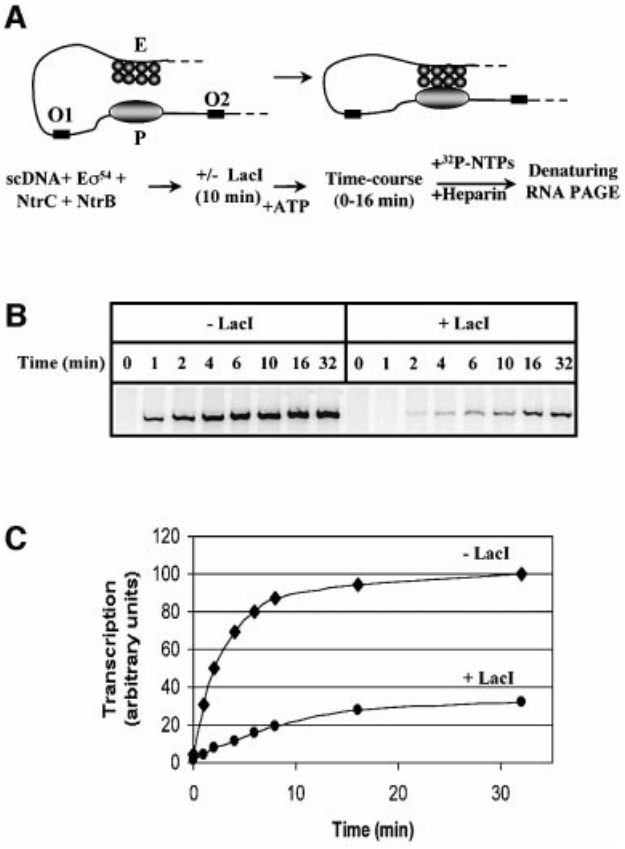

Fig. 2. Topological isolation of the enhancer from the promoter reduces the rate of enhancer–promoter communication. (**A**) Transcription assay for analysis of the effect of LacI on enhancer–promoter communication. The pLY10(1-1)S supercoiled template was pre-incubated with all components of the transcription machinery, and then incubated with or without LacI. Then ATP was added to allow enhancer–promoter communication. The rate of open complex formation was measured in a single-round transcription assay. NtrC activator octamer bound to the enhancer (E) and RNA polymerase bound to the promoter (P) are shown. Other designations are as in Figure 1C. (**B**) Analysis of labeled specific transcripts by denaturing PAGE. (**C**) The rate of enhancer–promoter communication is strongly decreased in the presence of LacI. DNA bands containing specific transcripts (B) were quantified and plotted as a function of time.

in vivo and *in vitro*, both on linear and supercoiled DNA (Hsieh *et al*., 1987; Kramer *et al*., 1987, 1988). The promoter was surrounded by two 'ideal' *lac* operators (Simons *et al*., 1984; Figure 1B). *Lac* operators were positioned far upstream and downstream the promoter (0.36 and 0.27 kb from the promoter, respectively) to avoid inhibition of transcription due to binding of LacI to promoter DNA (Borowiec *et al*., 1987; Choy and Adhya, 1992; Flashner and Gralla, 1988; Oehler *et al*., 1990). It was expected that at optimal concentration, LacI tetramer would bind both operators simultaneously, forming a stable DNA loop that would topologically isolate the promoter from the enhancer and thus prevent enhancer–promoter communication.

DNA loop formation topologically isolates the enhancer from the promoter

All plasmid constructs used in this work contain the *glnAp2* promoter and NtrC-dependent enhancer positioned 2.5 or 3.3 kb downstream of the promoter. Activity of the promoter entirely depends on the presence of the enhancer *in cis* (Liu *et al*., 2001). Transcription of the pLY10(1-1)S plasmid containing two LacI-binding sites positioned 0.36 kb upstream and 0.27 kb downstream of the *glnAp2* promoter, respectively, is strongly (5- to 10-fold) inhibited by LacI in a single-round transcription assay (Figure 1C, lanes 1 and 2). Addition of IPTG (an inducer of LacI which strongly inhibits its binding to the operator) almost completely reverses the inhibition (lane 3). There is no inhibition when the repressor is added after formation of the open initiation complex (RP_o) suggesting that the repressor acts before this step (lane 4). Moreover, R3, a mutant LacI that binds DNA equally well but cannot form the tetramer [it can only form dimers (Chen *et al*., 1994)] and therefore cannot form the DNA loop, does not have any effect on transcription (lanes 5 and 6). Neither repressor affects transcription of the control plasmid (not containing *lac* operators, lanes 7–12).

In a control experiment, aliquots of transcription reactions containing LacI and R3 (but not containing plasmid templates) were incubated with labeled double-stranded oligonucleotide containing 'perfect' *lacO* and analyzed in a native gel (Figure 1D). The oligonucleotide was present at the same molar amount as the plasmids in the experiment described in Figure 1C. As expected, only wt repressor forms the tetramer (R3 forms dimers, compare lanes 2 and 4), and addition of IPTG completely reverses binding of LacI and R3 to DNA (lanes 3 and 5). The data suggest that in the absence of IPTG, both LacI and R3 quantitatively occupy *lac* operators, but only wt repressor can form the DNA loop and inhibit transcription. Thus, quantitative DNA binding by R3 is not sufficient to inhibit transcription.

Taken together, the data suggest that LacI can strongly and specifically inhibit transcription initiation when its binding sites are positioned over more than 250 bp from the promoter. The novel type of inhibition over a distance requires the ability of the repressor to form the DNA loop; DNA binding *per se* is not sufficient for inhibition. These features of the repression make *lac* operators formally similar to eukaryotic 'insulators' blocking enhancer action over a large distance (see Introduction).

According to the slithering model of enhancer action (Figure 1B), LacI-mediated formation of the DNA loop should decrease the rate of enhancer–promoter communication. The rates of enhancer–promoter communication in the presence and in the absence of LacI were compared (Figure 2). First, all components of the transcription reaction were pre-incubated with the templates in the absence of ATP. Under these conditions, both RNA polymerase and NtrC bind to the promoter and the enhancer, respectively, but cannot functionally communicate with each other, and the polymerase cannot form the open complex (Buck and Cannon, 1992; Popham *et al*., 1989; Sasse-Dwight and Gralla, 1988). Enhancer–promoter communication was induced by adding ATP to the reaction to induce NtrC phosphorylation, and the rate of formation of the open complex [that reflects the rate of communication (Liu *et al*., 2001)] was measured in a single-round transcription assay. As expected, the rate of enhancer–promoter communication ($t_{1/2}$ = 1.5 min) is strongly (about 5-fold) decreased in the presence of LacI ($t_{1/2}$ = 8 min). In fact,

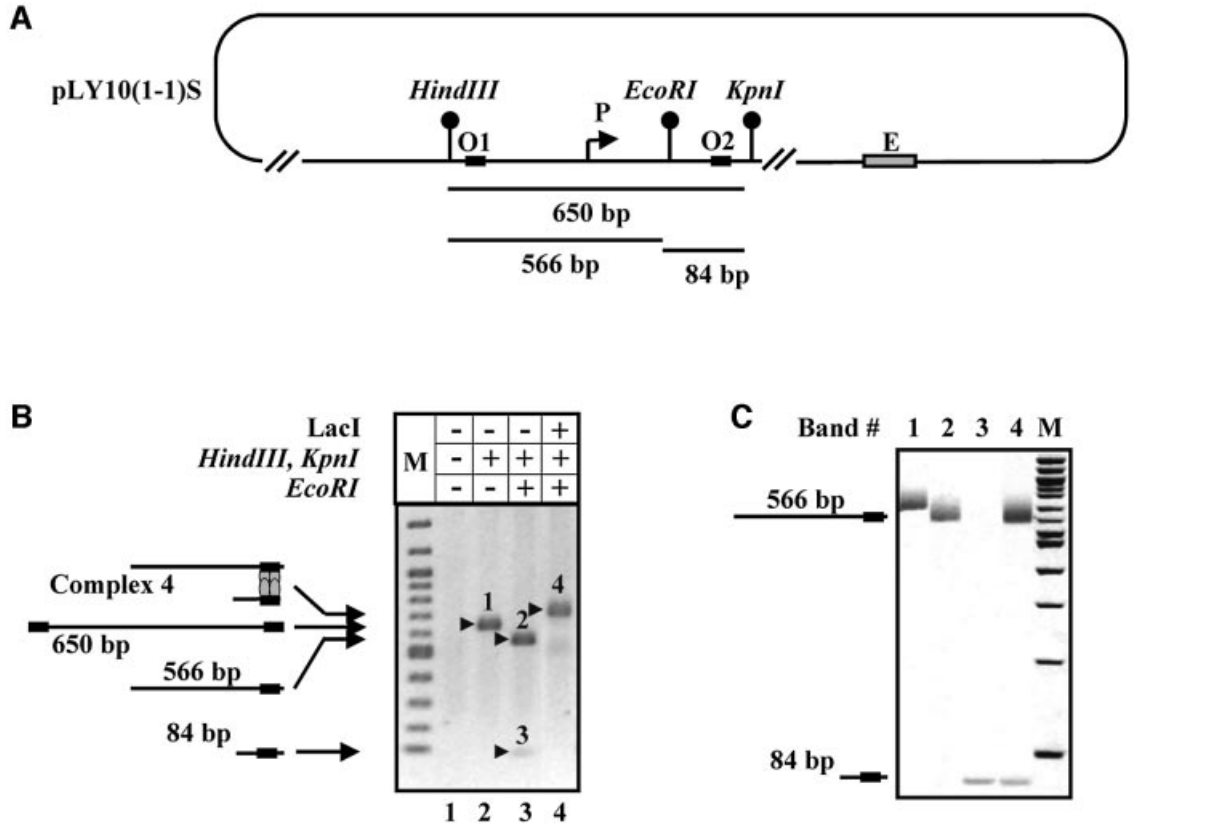

Fig. 3. LacI interacts with two *lac* operators and forms a stable loop on supercoiled DNA. (**A**) Partial restriction map of the pLY10(1-1)S plasmid. If LacI interacts with both *lac* operators (O1 and O2) on supercoiled DNA, two DNA fragments (84 and 566 bp) generated after digestion with restriction enzymes *Hin*dIII, *Eco*RI and *Kpn*I are expected to be bound to LacI and migrate as a single DNA–protein complex in a native gel. (**B**) LacI-induced DNA loop formation on supercoiled DNA: analysis of DNA–protein complexes. Supercoiled pLY10(1-1)S template was pre-incubated with or without LacI, then digested by different combinations of restriction enzymes and analyzed in a native agarose gel. The expected products of digestion are shown on the left. Different bands in the gel are arbitrarily numbered. M, 100 bp DNA ladder (NEB). (**C**) LacI-induced DNA loop formation on supercoiled DNA: analysis of DNA composition of different DNA–protein complexes. DNA was purified from different bands [1–4 in (B)] and analyzed by PAGE. M, 100 bp DNA ladder (NEB).

the data suggest that even this slow enhancer–promoter communication occurs only on a fraction of the templates; the majority of templates (70%) do not support transcription initiation in the presence of LacI (Figure 2C). In summary, LacI-induced formation of the DNA loop (see below) results in a strong decrease in the rate of enhancer–promoter communication.

LacI interacts with two lac operators and forms a stable loop on supercoiled DNA

The data described above are consistent with LacI-induced formation of a DNA loop on supercoiled DNA. The ability of LacI to form loops on linear DNA is well-documented (Hsieh *et al*., 1987; Kramer *et al*., 1987, 1988; Oehler *et al*., 1990). It has been suggested that LacI can also form loops on supercoiled DNA (Eismann and Muller-Hill, 1990; Whitson *et al*., 1987) but it has not been analyzed directly.

To analyze DNA loop formation, supercoiled pLY10(1-1)S template having two *lac* operators surrounding the P1 promoter (Figure 3A) was incubated in the presence of LacI and then digested with restriction enzymes producing 566 and 84 bp DNA fragments, each containing one *lacO* (Figure 3A). If the LacI tetramer interacts with both operators at the same time and forms a DNA loop, both fragments are expected to be bound to LacI and to migrate as one complex in a native gel. This was the case: neither 566 nor 84 bp free DNA fragments were detected after pre-incubation of supercoiled DNA with LacI and subsequent digestion (Figure 3B, compare lanes 3 and 4). Instead, a single complex having slower mobility in a native gel (complex 4) was detected. As expected, analysis of DNA extracted from this complex indicates that it contains both 566 and 84 bp DNA fragments present at a molar ratio of ~1:1 (Figure 3C).

The data suggest that the loop forms on supercoiled DNA and not after digestion with the restriction enzymes. Indeed, if LacI interacted with the DNA fragments after digestion, various combinations of the 566 and 84 bp DNA fragments (566–566, 566–84 and 84–84) were expected to be found in different complexes with LacI (data not shown). In contrast, only one discrete complex containing both fragments was observed (complex 4, Figure 3B) suggesting that the loop was formed on supercoiled DNA. The efficiency of loop formation was >80%. In summary, LacI binding to the *lac* operators flanking the promoter results in almost quantitative loop formation on supercoiled DNA.

Lac operators have many properties characteristic for eukaryotic insulators

To further analyze insulator properties of *lac* operators, a set of plasmids having 2.5 kb enhancer–promoter spacing

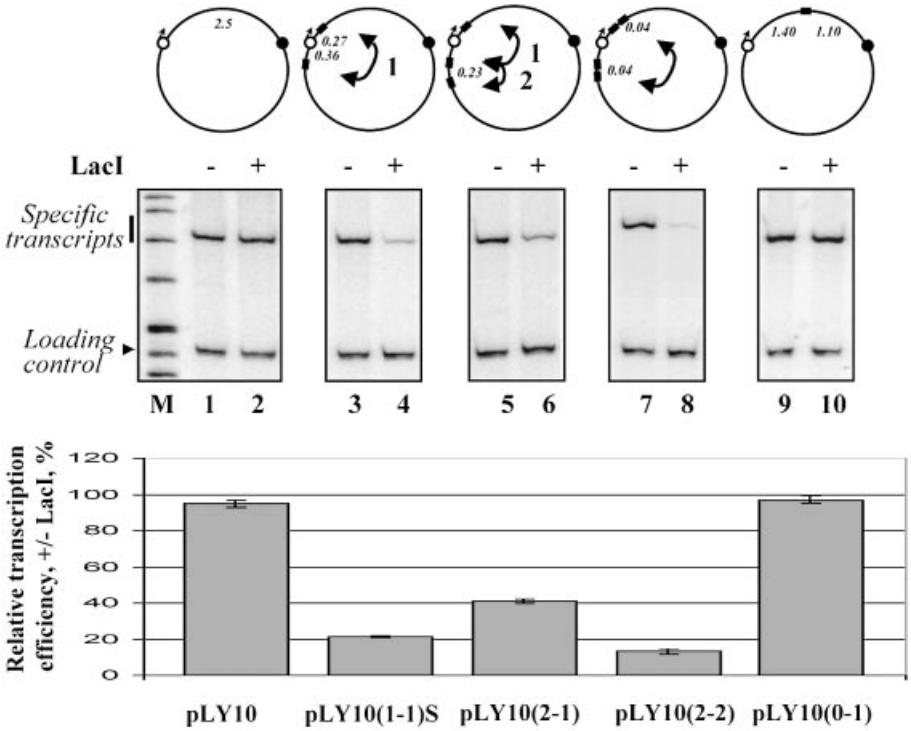

Fig. 4. Topological isolation of the enhancer from the promoter is required for insulation. Various constructs (upper panel), all containing the enhancer and promoter spaced by 2.5 kb but different numbers of 'ideal' *lac* operators positioned differently were transcribed in the presence or in the absence of LacI. The arrows between *lac* operators indicate the expected LacI-induced DNA loops. Two alternative loops formed on the pLY10(2-1) plasmid are indicated (arrows 1 and 2); other designations are as in Figure 1C. All constructs were transcribed in the same experiment; transcripts were analyzed by denaturing PAGE (middle panel). Equal amounts of a labeled DNA fragment were added to all samples immediately after termination of transcription (loading control). The histogram (lower panel) shows the ratio of the amounts of specific transcripts accumulated in the presence and in the absence of LacI. The data are averages of at least three experiments (standard deviations are indicated).

but containing a different number of 'ideal' *lac* operators positioned differently was constructed (Figure 4). Many of these plasmids were designed similarly to the constructs used for analysis of the mechanism of insulator action *in vivo*. This allowed comparison of the properties of *lac* operators *in vitro* with the properties of natural insulators *in vivo*. These constructs were transcribed in the presence and in the absence of LacI (Figure 4) and the levels of transcription inhibition by LacI were compared.

It has been shown that duplication of insulators can improve their activity (Chung *et al*., 1993). Duplication of *lac* operators also increases their activity ~2-fold (Figure 4, compare lanes 4 and 8), presumably because LacI forms a DNA loop that topologically isolates the enhancer from the promoter more efficiently. In this construct, short spacing (40 bp) between *lac* operators within each pair is likely to prevent interaction of the same LacI tetramer with two operators positioned next to each other. LacI bends DNA away from the repressor by 40° (Lewis *et al*., 1996), and once the tetramer binds to one DNA site the second DNA-binding surface is unlikely to reach the second site. This results in binding of two tetramers to each pair of operators and in more efficient formation of the DNA loop.

Duplication of some insulators placed between an enhancer and promoter can also considerably compromise activities of both insulators *in vivo* (Cai and Shen, 2001; Muravyova *et al*., 2001, but see also Melnikova *et al*., 2002; Kuhn *et al*., 2003). These findings can be recapitulated with *lac* operators: duplication of one of the two insulator-like elements flanking the promoter results in ~2-fold decrease in the repression (Figure 4, compare lanes 4 and 6). In this case, two *lac* operators positioned between the enhancer and promoter were spaced by 228 bp. This spacing is optimal for DNA loop formation by LacI (Law *et al*., 1993). Therefore, on this construct the DNA loop can be formed in either of two ways: the loop can include the promoter or be formed between the enhancer and promoter. In the former case, the loop (loop 1, Figure 4) inhibits enhancer–promoter communication (lane 4). If the loop is formed between the enhancer and promoter (loop 2), it is not expected to topologically isolate them from each other and would be neutral for promoter activity. In fact, this is the case (data not shown). However, since formation of loops 1 and 2 are competing processes, the overall effect of formation of loop 2 is a decrease in the efficiency of formation of loop 1 and promoter repression by LacI.

It has been proposed that natural insulators can work in pairs (Cai and Shen, 2001; Muravyova *et al*., 2001) or large clusters (Gerasimova *et al*., 2000; Gerasimova and Corces, 1998; Ishii *et al*., 2002). As expected, a single *lacO* placed between the enhancer and promoter did not inhibit enhancer-dependent transcription (Figure 4, compare lanes 4 and 10).

Finally, it has been shown that many insulators are 'neutral' DNA elements that do not inactivate promoters but presumably prevent enhancer–promoter communication *in vivo* (Cai and Levine, 1995; Scott and Geyer, 1995,

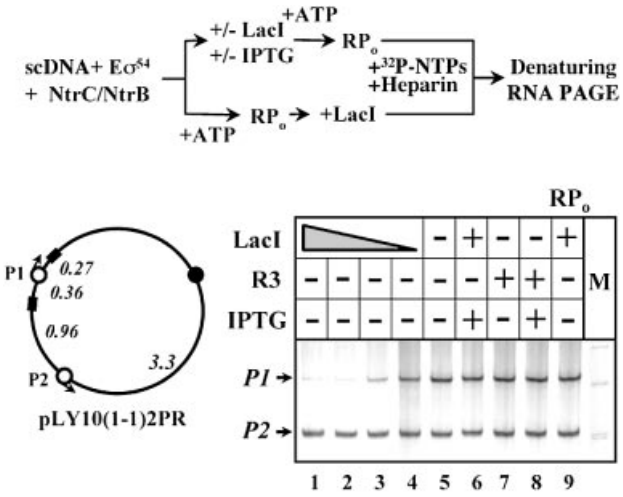

Fig. 5. LacI-induced DNA loop topologically isolates the enhancer from the promoter but does not inactivate them. The pLY10(1-1)2PR plasmid (upper panel) containing two identical *glnAp2* promoters (P1 and P2) under the control of one enhancer and two *lac* operators flanking promoter P1 was transcribed in the presence or in the absence of LacI and IPTG (see the diagram at the top). The regions transcribed from the P1 and P2 promoters are 401 and 309 nt long, respectively. The transcripts were analyzed by denaturing PAGE (lower panel). The template concentration was 3 nM and *lac* repressor was added to the following concentrations: lane 4, 2 nM; lane 3, 4 nM; lane 2, 6 nM and lanes 1 and 6–9, 8 nM. Other designations are as in Figure 1C.

but see also Wei and Brennan, 2000, 2001). To find out whether this property of insulators can be recapitulated *in vitro*, the pLY10(1-1)2PR plasmid was constructed (Figure 5). It contains two identical *glnAp2* promoters (P1 and P2), both activated by the same enhancer over distances of 2.5 and 3.3 kb, respectively. Only the P1 promoter is flanked by the *lac* operators. As expected, in the absence of LacI both enhancer-dependent promoters are active (lane 5) suggesting that the enhancer can work efficiently over a distance up to at least 3.3 kb. Adding increasing concentrations of LacI (lanes 4 to 1) results in progressive, selective and strong (up to 10-fold) inhibition of P1; P2 remains fully active. The data suggest that LacI-induced DNA loop formation does not inactivate either promoter P2 or enhancer. At the same time, activity of the promoter P1 localized within the DNA loop that topologically isolates the promoter from the enhancer is strongly inhibited. As expected, addition of IPTG reverses the inhibition (lane 6), inhibition was not observed after formation of the open complex (lane 9), and the R3 mutant repressor was unable to inhibit transcription (lanes 7 and 8).

In summary, many features of eukaryotic insulators observed *in vivo* can be recapitulated using *lac* operators in a highly purified enhancer–promoter system *in vitro*. *Lac* operators only work in pairs; placement of the enhancer and promoter in different topologically isolated domains is essential for insulator activity. Neither promoter nor enhancer is inactivated by the formation of the loop *per se*, but enhancer–promoter communication occurs with much lower efficiency.

Lac operators can insulate over a large distance

In all of the experiments described above, *lac* operators were inhibiting transcription over a relatively short distance: 0.27–0.36 kb from the promoter. Can *lac* operators, like eukaryotic insulators, work over a much larger distance? In order to address this question and to discriminate between the slithering and branch collision models of enhancer action (Figure 1A and B), pLY10(1-1)L template was constructed (Figure 6). On this plasmid, one *lacO* is positioned between the enhancer and promoter (1.4 kb downstream of the promoter); the second operator is placed at the maximal distance (3.7 kb) from the first one. The slithering model suggests that placement of the enhancer and promoter in topologically isolated domains is sufficient to inhibit communication between them: the exact positions of *lac* operators and spacing between them are not essential. At the same time, the pY10(1-1)L template is optimized for branch collision: the enhancer and promoter are localized almost symmetrically relative to the *lac* operators and over a large distance from the base of the loop. The large distance from the base of the loop maximizes flexibility of the 'branches' formed on supercoiled DNA. If the enhancer and promoter communicate by the branch collision mechanism, transcription of the pY10(1-1)L template would be inhibited by LacI less than transcription of the pY10(1-1)S plasmid where the enhancer and promoter are localized asymmetrically relative to the operators, the promoter is positioned much closer to the base of the loop and the DNA loop is much smaller.

Transcription of the pLY10(1-1)L plasmid is strongly (5- to 10-fold) inhibited by LacI (Figure 6A, compare lanes 7 and 8). Addition of IPTG almost completely reverses the inhibition (lane 9). There is no inhibition when the repressor is added after formation of the open complex (lanes 6 and 12). As expected, mutant R3 repressor does not have any effect on transcription (lanes 4, 5, 10 and 11). Neither repressor affects transcription of the control plasmid not containing *lac* operators (lanes 1–6). The data suggest that LacI can strongly and specifically inhibit transcription initiation when its binding sites are positioned 1.4–2.3 kb from the promoter and 3.7 kb from each other. The inhibition requires the ability of the repressor to form the DNA loop. The data are entirely consistent with the slithering model of enhancer action (Figures 1B and 6B).

Discussion

In summary, a pair of *lac* operators can work as a strong insulator-like element blocking enhancer action over a large distance in a highly purified *in vitro* system (Figure 1). Insulator activity requires formation of a stable LacI-induced DNA loop (Figure 3) that topologically isolates the enhancer from the promoter (Figure 4), but does not inactivate either enhancer or promoter (Figure 5). As a result, the rate of enhancer–promoter communication is greatly inhibited (Figure 2). The exact positioning of *lac* operators is flexible: they can work over distances of 0.3–2.3 kb from the promoter and 0.63–3.7 kb from each other (Figures 1 and 6).

The data have implications for the mechanism of enhancer action over a large distance. Two models were proposed to explain how enhancer–promoter communication over a distance could be facilitated on supercoiled DNA: the slithering and branch collision models

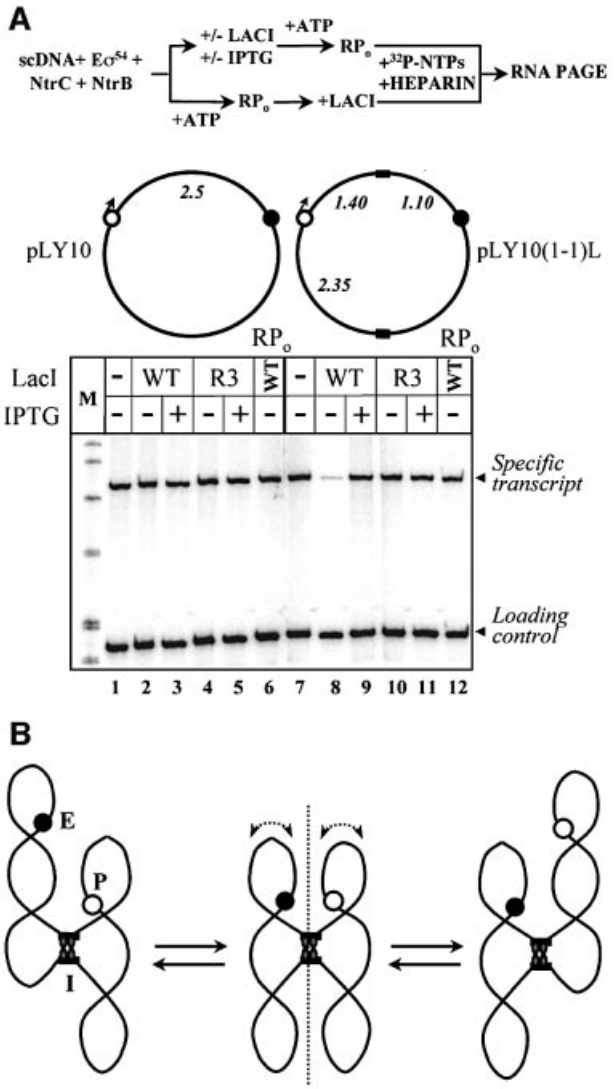

Fig. 6. Two *lac* operators can inhibit enhancer-dependent transcription over a large distance. (**A**) Two *lac* operators positioned 3.5 kb from each other can inhibit enhancer-dependent transcription. Plasmid containing two *lac* operators localized 3.7 kb from each other and at least 1.4 kb from the promoter [pLY10(1-1)L] and control plasmid not containing *lac* operators (pLY10) were transcribed in the presence or in the absence of LacI or the R3 mutant (see diagram at the top). A single-round assay was used. In some cases, LacI was added after formation of the open complexes (lanes 6 and 12). Labeled transcripts were analyzed in a denaturing gel. Labeled DNA fragment was added to all samples immediately after termination of transcription (loading control). Other designations are as in Figure 1C. (**B**) Slithering between two topologically isolated domains is impossible. Designations are as in Figure 1B. Binding of LacI tetramer to two *lac* operators results in separation of plasmid DNA into two topological domains. Slithering is only possible within each domain but not between them. Placing promoter and enhancer in topologically isolated domains prevents communication between them by slithering on supercoiled DNA.

(Figure 1A and B). The data are consistent with the slithering model of enhancer action. In particular, the ability of *lac* operators to prevent enhancer–promoter communication over a large distance and the data on partial inhibition of insulator activity by duplication of *lac* operators [Figure 4, construct pLY10(2-1)] are difficult to explain by the branch collision model. In contrast, the slithering model provides an immediate explanation for all of the experimental data. After LacI brings two *lac* operators together and forms a DNA loop, two topologically isolated domains are formed. Slithering is possible within each domain but not between them (Figure 6B). Therefore, the enhancer and promoter efficiently communicate only when they are located within the same domain on supercoiled DNA; communication from one domain to another is almost impossible, even when the domains are large (up to at least 3.7 kb). Communication between the domains by the branch collision mechanism (Figure 1A) could become possible when the size of the loops is >10 kb (Huang *et al*., 2001).

DNA supercoiling is essential for enhancer–promoter communication over a distance *in vitro* (Liu *et al*., 2001). Can DNA supercoiling facilitate enhancer–promoter interaction *in vivo*? The bulk of the eukaryotic genome does not contain unconstrained DNA supercoiling (Sinden *et al*., 1980) and there is no enzyme (gyrase) to generate it. However, localized negative DNA supercoiling could be generated by nucleosome displacement or remodeling (Camerini-Otero and Felsenfeld, 1977; Havas *et al*., 2000), and by transcript elongation (Liu and Wang, 1987; Wu *et al*., 1988). Indeed, transcriptionally active genome regions contain considerable levels of unconstrained DNA supercoiling (Kramer *et al*., 1999). Moreover, action of eukaryotic transcriptional and recombination enhancers over a large distance *in vitro* requires DNA supercoiling (Bagga and Emerson, 1997; Barton *et al*., 1997). It may seem unlikely that bulky chromatin structure would allow enhancer–promoter communication by slithering. However efficient enhancer–promoter communication occurs on supercoiled DNA containing bound R3 repressor (Figures 1 and 6) indicating that at least some bulky DNA–protein complexes are transparent for slithering. Many more studies are clearly required to establish to what extent the observations described here can be applied to a much more complicated intranuclear environment. In particular, it would be important to analyze whether DNA supercoiling is required for enhancer action on DNA organized in chromatin.

When the enhancer and promoter are positioned within different DNA loops on supercoiled DNA, changes in the structure of the complex that destabilize or stabilize the loops facilitate or inhibit enhancer–promoter communication, respectively. The loop can be destabilized using mutant R3 repressor or by disruption of LacI–DNA interactions by IPTG. Alternatively, the efficiency of formation of the 'inhibitory' loop can be decreased by formation of a competitive loop placing the enhancer and promoter in the same topological domain and thus allowing efficient communication between them [Figure 4, construct pLY10(2-1)]. In all cases a decrease in the efficiency of the 'inhibitory' loop formation results in transcription activation. In contrast, an increase in the stability of the loop results in stronger inhibition of transcription [Figure 4, construct pLY10(2-2)]. Thus, the data are entirely consistent with and support the slithering model of enhancer action (Chirico and Langowski, 1996; Vologodskii and Cozzarelli, 1996). The data suggest that two *lac* operators form a 'slithering barrier' at the border of the LacI-induced DNA loop.

LacI-dependent insulation by *lac* operators is clearly distinct from repression of the *lac* promoter that also

involves two *lac* operators and DNA loop formation by LacI (Borowiec *et al*., 1987; Choy and Adhya, 1992; Flashner and Gralla, 1988; Oehler *et al*., 1990). In the case of repression, one *lacO* overlaps the promoter and the function of the second operator is solely to increase promoter occupancy by LacI; the second *lacO* is only required at low concentration of LacI (Choy and Adhya, 1992; Oehler *et al*., 1990). Once bound at the promoter, LacI interferes with the escape of the polymerase from the promoter (Schmitz and Galas, 1979; Straney and Crothers, 1987). The presence of LacI at the promoter is essential for the repression. In contrast, insulation by *lac* operators does not require LacI binding at the promoter; in fact, *lac* operators can be placed 1.4 kb away from the promoter and still strongly inhibit transcription. At the same time, the presence of both *lac* operators and formation of the loop are essential for the insulator function.

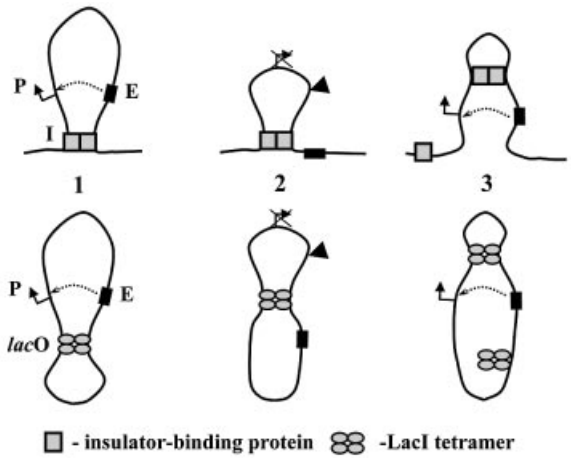

Fig. 7. Similarities between action of natural insulators *in vivo* and *lac* operators *in vitro*. An enhancer (E) and promoter (P) can efficiently communicate when flanked by insulators (I) or *lac* operators (*lac*O, structure 1). Insertion of an insulator or *lacO* between an enhancer and promoter may result in formation of a DNA loop and topological isolation of the enhancer from the promoter that prevents enhancer–promoter communication (structure 2). Incorporation of a second insulator between the enhancer and promoter (indicated by arrowhead in structure 2) may result in formation of a competitive DNA loop (structure 3). As a result, the enhancer and promoter would reside in the same topological domain and can efficiently communicate with each other. Many enhancer-blocking properties of insulators and *lac* operators can be rationalized in the framework of the slithering model of enhancer action (see Discussion). Note that only interactions between two eukaryotic insulators are shown (upper panel) while *in vivo* insulators form large 'insulator bodies' (Gerasimova and Corces, 1998; Gerasimova *et al*., 2000; Ishii *et al*., 2002).

Lac operators recapitulate many important properties of eukaryotic insulators observed *in vivo* (Figure 7). Many natural eukaryotic insulators are 'neutral' elements working in a position-dependent way: transcription is inhibited only when an insulator is positioned between an enhancer and promoter (not upstream of the enhancer or downstream of the promoter) and the promoters remain functional (Cai and Levine, 1995; Scott and Geyer, 1995, but see also Wei and Brennan, 2000, 2001). Most likely, insulators prevent enhancer–promoter communication. Two *lac* operators behave similarly: transcription is only inhibited when the promoter is topologically isolated from the enhancer by formation of a DNA loop; and neither enhancer nor promoter is inactivated by loop formation *per se* (Figure 5). *Lac* operators strongly decrease the rate of enhancer–promoter communication (Figure 2). A natural insulator can inhibit transcription independently of its precise location (as long as it is positioned between an enhancer and promoter) and can work over a large distance (Dorsett, 1993; Golovnin *et al*., 1999). Likewise, precise positioning of the two *lac* operators is not essential for their insulator activity, and they can work over a large distance. Finally, in some cases duplication of an insulator positioned between an enhancer and promoter results in loss of insulator activity (Cai and

Table I. Cloning approaches used in this study

Plasmids	Oligonucleotides	Amplified from	Cloned into	Restriction enzymes
pLY10(1-1)S	5′-CTCTAAGCTTAAATTGTGAGCGCTCACAATTCCAC GAAGACCTTTATTCAGAAGGG	pLY10	pLY10	*Hin*dIII
	5′-CCAGGTACCAATTGTGAGCGCTCACAATTAATGC CTTTCCAGCCGCCAATCGAGG			*Kpn*I
pLY10(1-1)2PR	5′-CGCCTTTTCGGCCGAATTTAAAAGTTGGCACAGAT TTCGC	pTH8	pLY10	*Eag*I
	5′-TTTTCCCAGTCCGGACGTTGTAAAACGACGGCCA GTGCC	(1-1)S		*Bsp*EI
pLY10(2-1)S	5′-CGGGTCGCCGGTGAAATTGTGAGCGCTCACAATT CGCTAGCAGCACGCCATAGTGACTGG	pLY10	pLY10	*Sgr*AI
	5′-CCAGGTACCAATTGTGAGCGCTCACAATTAATGC CTTTCCAGCCGCCAATCGAGG	(1-1)S		*Kpn*I
pLY10(2-2)S	5′-GGTGCAAGCTTGCAATTGTGAGCGCTCACAATTA AACTACCGCATTAACGCTTAAATTGTGAGC	pLY10	pLY10	*Hin*dIII
	5′-ATTAGGTACCAAATTGTGAGCGCTCACAATTCAG GTGTTTCCTCCAGCTACCAATTGTGAGC	(1-1)S		*Kpn*I
pLY10(0-1)	5′-CTCTCAGTAAGATCTACAAATATGTTGTGC	pLY10	pLY10	*Bgl*II
	5′-GCATCAATTGCAATTGTGAGCGCTCACAATTCGG CTTTCAGGAACTCGCGGTCCAGATC			*Mfe*I
pLY10(1-1)L	5′-AGATCCGGAAAATTGTGAGCGCTCACAATTACTT CCGCGTTTCCAGACTTTACGAAAC	pLY10	pLY10	*Bsp*EI
	5′-CATGGCGGCCGACGCGCTGGGCTAC		(0-1)	*Eag*I

Shen, 2001; Muravyova *et al*., 2001, but see also Melnikova *et al*., 2002; Kuhn *et al*., 2003). Duplication of similarly positioned *lac* operators also significantly decreases repression of the promoter [Figure 4, construct pLY10(2-1)], presumably because the efficiency of formation of the 'inhibitory' loop is decreased. Taken together, the data suggest that topological isolation could be sufficient for insulation *in vivo*.

Eukaryotic insulators are likely to have other activities in addition to the enhancer-blocking activity. Thus, it is clear that boundary activity of insulators is mechanistically different from the enhancer-blocking activity (Mutskov *et al*., 2002; Recillas-Targa *et al*., 2002); only the enhancer-blocking activity was recapitulated in this study. Moreover, some insulators can functionally interact with promoters (Wei and Brennan, 2000, 2001) suggesting that this activity is part of the mechanism of insulator action. Finally, the ability of some insulators to block enhancer action *in trans* (Krebs and Dunaway, 1998) cannot be immediately explained by the slithering barrier model of insulator action. However, the model could provide a useful framework for further analysis of the mechanism of insulator action.

Materials and methods

Plasmid construction

All templates are derivatives of the pLY10 plasmid containing the enhancer and the *glnAp2* promoter (Liu *et al*., 2001). The plasmids were constructed using PCR amplification of desired fragments, followed by digestion of the fragment and the target plasmids with restriction enzymes and ligation. The pTH8 plasmid was described by Hunt and Magasanik (1985). The cloning approaches outlined in Table I were used.

In vitro transcription assay

All protein components of transcription machinery were purified as described previously (Bondarenko *et al*., 2002; Liu *et al*., 2001). LacI and the R3 mutant repressor were purified as described by Barry and Matthews (1997). Their purity was >95% according to silver staining. *In vitro* transcription reactions were optimized for maximal utilization of promoter on supercoiled templates. Single round transcription assay was carried out in 50 µl aliquots containing 3 nM DNA template, 130 nM core RNA polymerase, 200 nM σ^{54}, 150 nM NtrC, 50 nM NtrB in buffer [50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0)]. The reaction mixture was incubated at 37°C for 10 min. LacI or its mutant R3 were added to the reaction (final concentration 30 nM) and incubated at 37°C for an additional 10 min. To release LacI from DNA, IPTG was added to 1 mM final concentration. ATP was added to the reaction to 0.5 mM final concentration at 37°C for 15 min or for variable time. To start transcription, NTP (final concentration 80 µM), 2.5 µCi [α-^{32}P]GTP, RNase inhibitor (final concentration 0.2 U/µl) and heparin (final concentration 80 µg/ml) were added to the reaction. After incubation at 37°C for 15 min transcription was terminated by adding 100 µl of phenol/chloroform (1:1). End-labeled 227 bp DNA fragment (loading control) was added to the mixture in 50 µl of H_2O. The samples were extracted with phenol/chloroform, precipitated with ethanol, washed with 70% ethanol and dissolved in formamide. The samples were separated on 8% denaturing PAGE, dried and analyzed on a phosphoimager.

Gel-shift assay for DNA loop detection

Supercoiled pLY10(1-1)S template at concentration 15 nM was incubated with or without 50 nM LacI in the binding buffer [50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0)] at 37°C for 20 min in 20 µl aliquots. Then 10 U of *Hin*dIII and *Kpn*I only or together with *Eco*RI were added and incubated at 37°C for 30 min. The reaction was terminated by adding 4 µl of 5× loading buffer containing 50 mM EDTA, 5× TAE buffer and 50% glycerol. The samples were immediately analyzed in 1% agarose gel in TAE buffer. The gel was stained with EtBr and DNA fragments of interest were extracted from the gel by using QIAGEN gel-extraction kit followed by phenol/chloroform extraction and ethanol precipitation. The purified DNA fragments were separated in 6% PAG in TAE buffer and detected by EtBr staining.

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